

## **Rossi's Principles of Transfusion Medicine**

# **Rossi's Principles of Transfusion Medicine**

## **Sixth Edition**

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# Contents

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List of contributors, vii

Preface, x

List of abbreviations, xi

About the companion website, xxii

## Section I: Transfusion medicine from ancient times to the current pandemic

- 1** Transfusion in the new millennium, 3  
*Ennio C. Rossi and Toby L. Simon*

- 2** Disasters and the blood community (including COVID-19), 14  
*Ruth Sylvester and Louis M. Katz*

- 3** Responding to regulatory challenges during public health emergencies, 21  
*Peter W. Marks*

## Section II: Blood donation

- 4** Recruitment and screening of donors and the collection of blood, 27  
*Susan N. Rossmann, Mary Townsend, and Toby L. Simon*

- 5** Blood donor testing, 37  
*Nancy Van Buren and Jed B. Gorlin*

- 6** Acute adverse reactions after blood donation, 49  
*Anne F. Eder*

- 7** Chronic effects of blood and plasma donation, 56  
*Bryan R. Spencer and Toby L. Simon*

- 8** Global perspective: ensuring blood and blood product safety and availability through regulation and certification, 65  
*Eva D. Quinley*

## Section III: Blood groups and pretransfusion testing

- 9** Carbohydrate blood groups, 81  
*Laura Cooling*

- 10** Rh and LW blood group antigens, 100  
*Aline Flock, Don L. Siegel, and Connie M. Westhoff*

- 11** Other protein blood groups, 109  
*Jennifer Ricci Hagman and Jill R. Storry*

- 12** Immunohematology and compatibility testing, 118  
*Connie M. Westhoff and Lynsi Rahorst*

## Section IV: Blood components

### Part I: Red cells

- 13** Red blood cell production and kinetics, 133  
*Mark J. Koury and Lionel Blanc*

- 14** Red blood cell metabolism and preservation, 143  
*John R. Hess and Angelo D'Alessandro*

### Part II: Platelets

- 15** Platelet production and kinetics, 158  
*Thomas C. Binns, Christopher A. Tormey and Henry M. Rinder*
- 16** Platelet immunology and alloimmunization, 168  
*Cheryl L. Maier, Seema R. Patel, and H. Clifford Sullivan*
- 17** Preparation, preservation, and storage of platelet concentrates, 179  
*Moritz Stolla, Valery J. Li, and Johnathan P. Mack*

### Part III: White cells

- 18** Neutrophil production and kinetics: neutropenia and neutrophilia, 188  
*Lawrence Rice and Eric Salazar*
- 19** Granulocyte collection and transfusion, 194  
*Jenny Petkova, Corinne Goldberg, and Jeffrey McCullough*

### Part IV: Plasma

- 20** Composition of plasma, 200  
*Peter Hellstern*
- 21** Plasma and cryoprecipitate for transfusion, 209  
*Torunn Oveland Apelseth, Simon J. Stanworth, and Laura Green*
- 22** The purification of plasma proteins for therapeutic use, 216  
*Nathan Brinkman, Karl McCann, and Barry Gooch*
- 23** Immunoglobulin products, 236  
*Melvin Berger*

## Section V: Apheresis

- 24** Basic principles of apheresis and the collection of blood components by apheresis, 251  
*Edwin A. Burgstaler and Jeffrey L. Winters*
- 25** Therapeutic apheresis: plasma processing, 259  
*Patricia A. R. Brunner and Monica B. Pagano*
- 26** Therapeutic phlebotomy and cellular hemapheresis, 278  
*Patricia A. R. Brunner and Jeffrey A. Bailey*

## Section VI: Blood transfusion

- 27** Patient blood management, 293  
*Darrell J. Triulzi, Jansen N. Seheult, Mark H. Yazer, and Jonathan H. Waters*
- 28** Clinical and technical aspects of blood administration, 306  
*Edward S. Lee, Debra L. Mraz, and Edward L. Snyder*
- 29** Anemia and red blood cell transfusion, 314  
*Jeffrey L. Carson, Asher A. Mendelson, and Paul C. Hébert*

- 30** Sickle cell disease, thalassemia, and hereditary hemolytic anemias, 326  
*Ross M. Fasano, Emily Riehm Meier, and Satheesh Chonat*
- 31** Autoimmune hemolytic anemias and paroxysmal nocturnal hemoglobinuria, 346  
*Christopher A. Tormey and Alexa J. Siddon*
- 32** Hemolytic disease of the fetus and newborn, 364  
*Jennifer Webb, Wen Lu, and Meghan Delaney*
- 33** Obstetric transfusion practice, 373  
*James Sikora and John D. Roback*
- 34** Transfusion in infants and children, 381  
*Jeanne E. Hendrickson and Cassandra D. Josephson*
- 35** Thrombocytopenia and platelet transfusion, 392  
*Michael F. Murphy, Simon J. Stanworth, and Lise J Estcourt*
- 36** Management of immune-mediated thrombocytopenia, 402  
*Caroline Gabe, Donald M. Arnold, James W. Smith, and Theodore E. Warkentin*
- 37** Coagulation concentrates for inherited bleeding disorders, 424  
*Gary M. Woods and Robert F. Sidonio, Jr.*
- 38** Coagulation factor concentrates and pharmacologic therapies for acquired bleeding disorders, 443  
*Natalie Bayli and Ravi Sarode*
- 39** Perioperative transfusion practice, 453  
*Leanne Thalji, Allan M. Klompaas, Matthew A. Warner, Daryl J. Kor, and James R. Stubbs*
- 40** Transfusion therapy in the care of trauma and burn patients, 471  
*John R. Hess, Benjamin R. Huebner, Samuel P. Mandell and Eileen M. Bulger*
- 41** Transfusion support for the oncology patient, 482  
*Kristin M. Stendahl, Wade L. Schulz, and Edward L. Snyder*
- 42** Pathogen-reduced blood components and derivatives, 489  
*Edward S. Lee, Edward L. Snyder, and Jeffrey McCullough*
- 45** Transfusion transmission of parasites and prions, 523  
*Bryan R. Spencer and Paula P. Saá*
- 46** Bacterial contamination of blood components, 533  
*Evan M. Bloch, Richard J. Benjamin, and Sandra Ramirez-Arcos*
- 47** Hemolytic transfusion reactions, 543  
*Sandhya R. Panch and Celina Montemayor*
- 48** Nonhemolytic transfusion reactions, 553  
*Emmanuel A. Fadeyi and Gregory J. Pomper*
- 49** Transfusion-related acute lung injury and other respiratory-related transfusion reactions, 569  
*E. Alexander Dent and H. Clifford Sullivan*
- 50** Transfusion-associated graft-versus-host disease, 582  
*Eric A. Gehrie and Courtney E. Lawrence*
- 51** Transfusional iron overload, 587  
*Sujit Sheth*
- Section VIII: Cellular and tissue transplant technologies**
- 52** Hematopoietic growth factors, 601  
*David J. Kuter*
- 53** Hematopoietic stems cells and transplantation, 617  
*Garrett S. Booth*
- 54** HLA antigens, alleles, and antibodies, 624  
*Scott M. Krummey, Robert A. Bray, Howard M. Gebel, and H. Clifford Sullivan*
- 55** Chimeric antigen receptor T cells and other cellular immunotherapies, 633  
*Andrew D. Fesnak and Don L. Siegel*
- 56** Gene therapy applications to transfusion medicine, 642  
*Seena Tabibi, Eric A. Gehrie, Emanuela M. Bruscia, and Diane S. Krause*
- 57** Tissue engineering and regenerative medicine, 648  
*Cecilia Motta, Qi Xing, Cynthia Wilkins, and Julie Allickson*
- 58** Human tissue allografts: responsibilities in understanding the path from donor to recipient, 660  
*Cassandra D. Josephson and Matthew J. Kuehnert*
- Index, 674

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# Preface

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In 1983, the National Heart, Lung and Blood Institute (NHLBI) awarded five medical school faculty the first of what were to be a host of Transfusion Medicine Academic Awards. The purpose of the program was to enhance instruction in and exposure to the essential principles related to transfusion of blood into patients. This was considered a neglected area in medical education. Embedded in that decision was the idea that blood banking was part of a broader medical field now termed transfusion medicine.

Dr. Ennio C. Rossi was one of these first five awardees. At that time, he was a professor at Northwestern University School of Medicine in Chicago and director of its apheresis unit. Dr. Rossi was approached by Williams and Wilkins to put together a major textbook in this newly identified field of transfusion medicine. Dr. Rossi subsequently recruited two coeditors: Dr. Toby Simon, a board certified transfusion medicine physician who was also one of the first five NHLBI awardees, and Dr. Gerald Moss, a prominent surgeon who had notable research achievements in oxygen transport. Thus, the first edition of *Principles of Transfusion Medicine* was launched by two hematologists and a surgeon. After the second edition, Dr. Rossi retired and Dr. Toby Simon assumed the senior editor role. It was decided to add Dr. Rossi's name to the title in recognition of his conception of the role of the book and to establish continuity for subsequent editions. Sadly, Dr. Rossi passed away on September 3, 2021. We dedicate this book to his memory and quote as follows from the first two paragraphs of the Preface to the first edition published in 1991:

Blood transfusion is an essential part of medical care and indispensable for the support of increasingly more sophisticated surgery. In the past, transfusion decisions were simple because therapeutic options were few. Now, decisions are more complicated. Transplantation biology and immunohematology are tightly intertwined, and transplantation surgery is frequently contingent upon special transfusion support. Advances in the technology of plasma fractionation and apheresis now provide a broad array of services for a large variety of clinical problems. Balanced against these benefits are the risks of blood-transmitted diseases, which have been underscored in the public consciousness by the emergence of acquired immunodeficiency syndrome (AIDS). Autologous transfusion and products of genetic engineering, such as hematopoietic growth factors, are being made available to diminish the risk, albeit small, of transfusion-transmitted disease by homologous blood. As these and other innovations render transfusion therapy more complex, blood banking has developed a clinical arm, transfusion medicine, to deal with these complexities.

*Principles of Transfusion Medicine* will attempt to define the proper use of blood in clinical care. It is intended for the clinicians who prescribe blood, for the students who expect to enter clinical practice, for the scientists, physicians, nurses, technologists, and others who ensure the quality of our blood services. Many diverse sciences are applied to the preparation of blood for transfusion, and virtually all medical and surgical specialties must employ transfusion, from time to time, in care of their patients. For this reason, transfusion medicine is, of necessity, multidisciplinary.

In preparation of this sixth edition, we have also been challenged by a pandemic. In response to this pandemic, two chapters in the

early part of the book have been added, detailing how our specialty responded to the emergency and the lessons learned. In addition, we have chapters focusing on other "megatrends": the application of molecular biology to the basics of matching donor and recipient, the use of apheresis to support new cellular approaches to cancer, the application of pathogen reduction for blood safety, the growth in plasma fractionation to meet the growing use of immune globulin preparations and other plasma-derived derivatives, as well as new approaches to support patients with massive bleeding, coagulopathy, and malignancy.

When the first edition was published, the transition away from whole blood to components and from cold-stored platelets to room-temperature stored platelets was nearly complete. Now we are seeing a reverse trend with recognition of potential benefits for the bleeding patient when treated with whole blood and cold-stored platelets. In the period since 1991, hemophilia care has gone from blood components such as cryoprecipitate, to plasma-derived factor concentrates, to recombinant products, to nonreplacement recombinant treatments, and finally to gene therapy to correct the defect that causes the disease. This is but one example of the evolution of the broader field of transfusion medicine we are capturing in this new sixth edition. We have assembled chapters and authors to guide the reader in understanding the changes that are occurring. At the same time, we have retained a significant amount of still-relevant material from earlier editions.

Contributors for this edition have once again been drawn from various scientific, medical, and surgical disciplines. Thus, this book encompasses topics including encouraging and managing donors, collecting and preserving donated blood, and matching each component to the appropriate recipient, based on the patient's clinical needs. The text also extends these concepts to tissue and goes beyond the field's basic tenets to address new applications.

We can think of no better way to honor Dr. Rossi's legacy than to present a sixth edition that blends transfusion science with clinical medicine, thus facilitating the thoughtful and measured prescription of blood, blood components, and their alternatives. Both the laboratory practice of blood banking and the clinical practice of transfusion medicine remain as important as ever. We proudly attribute the long-term influence of this field to its early leaders, who organized the discipline of transfusion medicine for success by anticipating future practice. We thank our new and returning contributors and the editorial staff at Wiley Blackwell for making possible a sixth edition of *Rossi's Principles of Transfusion Medicine* in this new pandemic-influenced world of transfusion practice.

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# List of abbreviations

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2RBC	double red cell collection	AHG	antihuman globulin
3-PCC / 3F-PCC	three-factor nonactivated prothrombin complex concentrate	AHPI	antihuman polyclonal immunoglobulin
4-PCC / 4F-PCC	four-factor nonactivated prothrombin complex concentrate	AHSP	alpha-hemoglobin stabilizing protein
A3GALT2	isogloboside synthase	AHTR	acute hemophilic transfusion reaction
A4GALT1	PIP <sub>K</sub> synthase	aHUS	atypical hemolytic-uremic syndrome
AA	aplastic anemia	AIDS	acquired immune deficiency syndrome
AABB	Association for the Advancement of Blood and Biotherapies (previously: American Association of Blood Banks)	AIHA	autoimmune hemolytic anemia
AAP	American Academy of Pediatrics	AIS	absent iron stores
aAPC	artificial antigen presenting cell	AKI	acute kidney injury
AATB	American Association of Tissue Banks	ALAS2	5-aminolevulinic acid synthase
AAV	adeno-associated virus	ALI	acute lung injury
AAV	ANCA-associated vasculitis	ALL	acute lymphoblastic leukemia
Ab	antibody	ALT	alanine transferase
ABC	America's Blood Centers	AML	acute myelogenous leukemia
ABC/EBA	America's Blood Centers/European Blood Alliance	AMP	adenosine monophosphate
ABE	acute bilirubin encephalopathy	AMPD	adenosine monophosphate deaminase
ACCP	American College of Chest Physicians	AMR	antibody-mediated rejection
ACD	acid citrate dextrose solution	AMR	Ashwell-Morell receptor
ACD-A	acid-citrate-dextrose formula A	aMSCs	adipose tissue-derived mesenchymal stem cells
ACE	angiotensin-converting enzyme	ANC	absolute neutrophil count
ACEI	angiotensin-converting enzyme inhibitor	ANCA	antineutrophil cytoplasmic antibodies
ACh	acetylcholine	ANG1	angiopoietin 1
AChE	acetylcholinesterase	ANH	acute nonvolemic hemodilution
AChR	acetylcholine receptor	ANK1	ankyrin
ACI	anemia of chronic inflammation	anti-GBM	anti-glomerular basement membrane antibody
ACKR1	atypical chemokine receptor 1	anti-Gov	anti-HPA-15 antibody
ACOG	American College of Obstetricians and Gynecologists	anti-TPO	antithyroid peroxidase
ACS	acute chest syndrome	ANXA2	annexin 2
ACT	activated clotting time	APC	antigen-presenting cell
ADA	adenosine deaminase	aPCC	activated prothrombin complex concentrate
ADCC	antibody-dependent cellular cytotoxicity	APCs	antigen-presenting cells
ADEM	acute disseminated encephalomyelitis	API	alpha <sub>1</sub> -proteinase inhibitor
ADF	actin depolymerizing factor	APS	antiphospholipid antibody syndrome
aDHQ	Abbreviated Donor History Questionnaire	aPTT	activated partial thromboplastin time
ADORA2b	Adenosine interactions with the receptor A2B	AQP1	aquaporin-1
ADP	adenosine diphosphate	AQP3	aquaporin-3
ADSC	adipose-derived stem cell	AQP4-IgG	aquaporin-4 immunoglobulin G antibodies
AECII	alveolar epithelial type cells of the upper airway II	ARC	absolute reticulocyte count
AF	atrial fibrillation	ARDS	acute respiratory distress syndrome
AFSC	amniotic fluid-derived stem cells	ARDS	adult respiratory distress syndrome
AGM	aortogonadomesonephros	ARIPI	Age of Red Blood Cells in Premature Infants Study
AHF	antihemophilic factor	ART	antiretroviral therapy
		AS	additive solution
		ASA	American Society of Anesthesiologists
		ASFA	American Society for Apheresis
		ASH	American society of Hematology
		ASO	antisense oligonucleotides

ASP	antibody-specific prediction	BPAC	FDA Blood Products Advisory Committee
ASPEN	association of sickle cell priapism, exchange transfusion and neurological events	BPD	bronchopulmonary dysplasia
ASRI	American Society for Reproductive Immunology	BRN	World Health Organization Blood Regulators Network
ASSC	acute splenic sequestration crisis	BSA	body surface area
ASTCT	American Society for Transplantation and Cellular Therapy	BSE	bovine spongiform encephalopathy
AT	antithrombin	BSS	Bernard Soulier syndrome
ATF4	activating transcription factor 4	BT	bleeding time
ATG	antithymocyte globulin	BTHC	butyryl-tri-hexyl citrate
ATIII	antithrombin III	BVDV	bovine viral diarrhea virus
ATL	adult T-cell leukemia and lymphoma	C/EBP $\alpha$	CCAAT/enhancer binding protein $\alpha$
ATP	adenosine 5prime*-triphosphate	CAAR	chimeric auto antigen receptor
ATRs	allergic transfusion reactions	CABG	coronary artery bypass graft
ATS	American Thoracic Society	CAD	cold agglutinin disease
AUC	area under the ROC curve	CAEV	arthritis-encephalitis virus of goats
AUG	Augustine blood group	CAFC	cobblestone area-forming cell
AvWWS / AVWS	acquired von Willebrand syndromex	CALR	calreticulin
B-ALL	B-cell acute lymphoblastic leukemia	cAMP	cyclic adenosine monophosphate
B-CAM	basal cell adhesion molecule	CAP	College of American Pathologists
B-CAM/LU	Basal Cell Adhesion Molecule-Lutheran antigen	CAPS	catastrophic antiphospholipid syndrome
B3GALNT1	P synthase	CAR	chimeric antigen receptor
B19V	parvovirus B19	CAR-T cell	CXCL12 abundant reticular (cell)
BAGP	bicarbonate, adenine, glucose, and phosphate	CARS	T cell expressing a chimeric antigen receptor
BART	Blood Conservation Using Antifibrinolytics in a Randomized Trial	CASI	compensatory anti-inflammatory response syndrome
BasoEB	basophilic erythroblast	CASPR2	computer-assisted self-interview
BB/TS	Blood Bank/Transfusion Medicine standards	CBC	contactin-associated protein-2
BC method	buffy-coat method	CBER	complete blood count
BCEs	blood collection establishments	CBF	Center for Biologics Evaluation and Research
BCMA	B cell maturation antigen	CBS	cerebral blood flow
BCSH	British Committee for Standards in Hematology	CCAD	Canadian Blood Services
BCT	blood component therapy	ccc-DNA	Central Cardiac Audit Database
BDD	B-domain-deleted	CCI	covalently closed circular DNA
BECS	blood establishment computer software	CCP	corrected count increment
BELIEVE	An Efficacy and Safety Study of Luspatercept Versus Placebo in Adults Who Require Regular Red Blood Cell Transfusions Due to Beta Thalassemia	CCPD	convalescent Covid plasma
BEN	benign ethnic neutropenia	CDA	complement control protein domain
BFU-Es	burst-forming units-erythroid	CCAD	congenital dyserythropoietic anemia
BFU-MK	burst-forming units-megakaryocyte	CDR	Centers for Disease Control and Prevention
BiKE	bispecific killer engager	CDER	complement-dependent cytotoxicity
BIND	bilirubin-induced neurotoxicity	CD-P-TS	Center for Drug Evaluation and Research
BloodNet	Pediatric Critical Care Blood Research Network	CDR	European Committee on Blood Transfusion
BM-MSCs	bone marrow-derived mesenchymal stem cells	CDRH	complementarity-determining region
BMD	Becker muscular dystrophy	CDSS	Center for Devices and Radiologic Health
BMI	body mass index	CERA	clinical decision support systems
BMP	bone morphogenetic protein	CFB	polyethylene glycol-conjugated recombinant
BMSC	bone marrow stem cell	cffDNA	human erythropoietin
BMT	bone marrow transplantation	CFH	complement factor B
BNP	B-type natriuretic peptide	CFI	cell-free fetal DNA
BOS	bronchiolitis obliterans syndrome	CFR	complement factor H
BP	blood pressure	CFU-Es	complement factor I
		CFU-GM	US Code of Federal Regulations
		CFU-MK	colony-forming units-erythroid
		CGD	progenitor cells with the capacity to generate neutrophils in vitro
		cGMP	colony-forming units-megakaryocyte
		cGMP	chronic granulomatous disease
		CH2-THF	current good manufacturing practice
			cyclic guanosine monophosphate
			methylenetetrahydrofolate

CH3-THF	methyltetrahydrofolate	CREG	cross-reactive group
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate	CRISPR	clustered regularly interspaced short palindromic repeat
	hydrate	CRM	cross-reactive material
CHCM	cell hemoglobin concentration mean	CRPS	chronic regional pain syndrome
CHIKV	chikungunya virus	CRPS II	chronic regional pain syndrome type 2
CHILL REDS-III	Comparison of Donation History and Iron Levels in Teenage Blood Donors	CRRT	continuous renal replacement therapy
ChLIA	chemiluminescent immunoassays	CRS	cytokine release syndrome
CHMP	Committee for Medicinal Products for Human Use	CS	caesarean section
CHO-THF	formyltetrahydrofolate	CSF	cyclosporine
CHOP	Study of Cyclophosphamide, Hydroxydaunorubicin, Oncovin, Prednisone	CT	circulating steel factor
CHR	cellular hemoglobin in reticulocytes	CTA	computerized tomography
CI	confidence interval	CTCL	cancer-testis antigen
CIBMTR	Center for International Blood and Marrow Transplant Research	CTL	cutaneous T-cell lymphoma
CIDP	chronic inflammatory demyelinating polyradiculoneuropathy	CTL2	cytotoxic T-cell
CIT	chemotherapy-induced thrombocytopenia	CTLA-4	choline transporter-like 2 protein
CJD	Creutzfeldt–Jakob disease	CTT	cytotoxic T lymphocyte-associated protein 4
CKD	chronic kidney disease	cTTP	chronic transfusion therapy
CLET	cultured limbal epithelial transplantation	CVAD	congenital thrombotic thrombocytopenic purpura
CLIA	chemiluminescent immunoassay	CVCs	central venous access device
CLIA	Clinical Laboratory Improvement Act	CWD	central venous catheters
CLL	chronic lymphoid leukemia	CXCL12	chronic wasting disease
CM	carboxymethyl	CY	stromal-cell derived factor 1
CM-HUS	complement-mediated hemolytic-uremic syndrome	DAF	cyclophosphamide
CM-TMA	complement-mediated thrombotic microangiopathy	DAH	decay accelerating factor
CMIAs	chemiluminescent microparticle immunoassays	DAMPs	diffuse alveolar hemorrhage
CML	chronic myelogenous leukemia	DARC	damage-associated pattern molecules
CMP	common myeloid precursor	DART	Duffy antigen receptor for chemokines
CMQCC	California maternal quality care collaboration	DAT	Danish Registration of Transfusion Accidents
CMS	Centers for Medicare and Medicaid Services	dATP	direct antiglobulin test
CMV	cytomegalovirus	DBA	deoxy adenosine triphosphate
CNS	central nervous system	DBCD	Diamond–Blackfan anemia
CNSHA	chronic nonspherocytic hemolytic anemia	DBM	Division of Blood Components and Devices
COBLT	Cord Blood Transplant (study)	DC	demineralized bone matrix
CoE	Council of Europe	DCASGPR	dendritic cell
COM	All Common Checklist	DCM	dendritic cell asialoglycoprotein receptor
COOP	continuity of operations plans	DCs	dilated cardiomyopathy
COX2	cyclooxygenase 2	DD	dendritic cells
CP2D	citrate phosphate double dextrose	DDAVP	D-dimers
CPB	cardiopulmonary bypass	DEA	desmopressin
CPD	citrate–phosphate–dextrose	DEAE	diethyleneamine
CPDA	citrate–phosphate–dextrose–adenine	DEC	diethylaminoethyl
CPDA-1	citrate phosphate dextrose adenine	dECM	diethylcarbamazine
CPOE	computerized physician order entry systems	DEHP	decellularised extracellular matrix
CPRA	calculated panel-reactive antibody tests	DEM	diethylhexyl phthalate
CPSI	Canadian Patient Safety Institute	DETTD	Donor Educational Materials
CQ	clindamycin and quinine	DF	Division of Emerging and Transfusion Transmitted Diseases
CR	complete response	DFO	dengue fever
CR1	complement receptor 1	DFP	deferoxamine B mesylate
CRASH-2	Clinical Randomization of an Antifibrinolytic in Significant Hemorrhage trial	DFPP	deferiprone
		DFSD	double-membrane filtration plasmapheresis
		DFX	dry fibrin sealant dressing
		dGTP	deferasirox
		DHF	deoxy guanine triphosphate
		DHFR	dihydrofolate
		DHQ	dihydrofolate reductase
		DHS	Donor History Questionnaire
			Department of Homeland Security

DHSt	dehydrated stomatocytosis	EPO-a	erythropoietin alpha
DHTF	Donor History Task Force	EPO-R	erythropoietin receptor
DHTRs	delayed hemolytic transfusion reactions	ePTFE	expanded polytetrafluoroethylene
DIC	disseminated intravascular coagulation	ERFE	erythroferrone
DIIHA	drug-induced immune hemolytic anemia	ERMAP	erythrocyte membrane-associated protein
DITP	drug-induced immune thrombocytopenic purpura	ESAs	erythropoietin-stimulating agents
DLIs	donor lymphocyte infusions	ESC	embryonic stem cell
DMD	Duchenne muscular dystrophy	ESF	Emergency Support Functions (NRF)
DMH/DHA	dorsomedial nucleus/dorsal area	ESRD	end-stage renal disease
DMS	demarcation membrane system	ET	essential thrombocythemia
DMSO	dimethyl sulfoxide	ETTNO	Effect of Transfusion Thresholds on Neurocognitive Outcomes of extremely low birth weight infants Trial
DOACs	direct oral anticoagulants	EU	European Union
DOT	Department of Transportation	EUHASS	European Hemophilia Safety Surveillance
2,3-DPG	2,3-diphosphoglycerate	EV	extracellular vesicles
DSAs	donor-specific antibodies	EVA	ethylene vinyl acetate
DSBs	double-stranded breaks	EXM	electronic crossmatch
dsDNA	double-stranded DNA	EXT	extreme thrombocytosis
DSEK	Descemet's stripping endothelial keratoplasty	FACT	Foundation for the Accreditation of Cellular Therapy
DSGG	disialogalactosylgloboside	FADH	reduced flavin adenine dinucleotide
dsRNA	double-stranded RNA	FAST	focused ultrasonographic survey for trauma
DSS	decision support system	FBS	fetal blood sampling
DSTR	delayed serologic transfusion reaction	FC	fibrinogen concentrates
DTT	dithiothreitol	fCJD	familial Creutzfeldt–Jacob disease
DVT	deep vein thrombosis	FCR	fraction of cells remaining
EACA	$\epsilon$ -aminocaproic acid	FcRn	neonatal Fc receptor
EBA	European Blood Alliance	FDA	United States Food and Drug Administration
EBI	erythroblastic island	FDAAAA	Food and Drug Administration Amendments Act
EBV	Epstein–Barr virus	FDAMA	Food and Drug Administration Modernization Act
EC	endothelial cells	FDASIA	Food and Drug Administration Safety and Innovation Act
ECBS	Expert Committee on Biological Standardization	FDC	follicular dendritic cell
ECG	electrocardiogram	FDCA	Food, Drug, and Cosmetic Act
ECM	extracellular matrix	FDCs	follicular dendritic cells
ECMO	extracorporeal membrane oxygenation	FDP	fibrin degradation product
ECP	extracorporeal photopheresis	FEIBA	factor VIII inhibitory bypass activity
ECV	extracorporeal volume	FEMA	Federal Emergency Management Agency
EDQM	European Directorate for the Quality of Medicines	FEP	free erythrocyte protoporphyrin
EDTA	ethylenediaminetetraacetic acid	FFI	fatal familial insomnia
EEA	European Economic Area	FFP	fresh frozen plasma
EFIC	exception from informed consent	FGS	focal glomerulosclerosis
EGC	endothelial glycocalyx	FH	familial hypercholesterolemia
EGPA	eosinophilic granulomatosis with polyangiitis	FL	Flt3-ligand
EIA	enzyme immuno(sorbent) assay	FLAER	fluorescent aerolysin
EIAV	infectious anemia virus of horses	FLIPID	Ferritin Levels in Plasma Donors study
ELBW	extremely low birthweight	FMH	fetal–maternal hemorrhage
ELISA	enzyme-linked immunosorbent assay	FNAIT	fetal/neonatal alloimmune thrombocytopenia
EMA	European Medicines Agency	FNHTR	febrile nonhemolytic transfusion reaction
EMAs	emergency management agencies	FOCUS	Functional Outcomes in Cardiovascular Patients Undergoing Surgical Hip Fracture Repair study
EMCV	encephalomyocarditis virus	FSFI	Female Sexual Function Index
EMP3	epithelial membrane protein 3	FSGS	focal segmental glomerulosclerosis
EMP	Embden–Meyerhof–Parnas pathway	FSPT	filtered sunlight phototherapy
EMP	erythroblast–macrophage protein		
ENT1	equilibrative nucleoside transporter 1		
EP	European Pharmacopoeia		
EP3Rs	prostaglandin EP3 receptors		
EPO	erythropoietin		

FT/RA	first-time and reactivated (donors)	HbC	hemoglobin C
FTA-ABS	fluorescent <i>Treponema pallidum</i> antibody absorption	HBc	hepatitis B core
G-CSF	granulocyte colony-stimulating factor	HBcAg	hepatitis B core antigen
G6PD	glucose-6-phosphate dehydrogenase	HbD	hemoglobin D
GABA	$\gamma$ -amino butyric acid	HbE	hemoglobin E
GAD-65	65-kD isoform of glutamic acid decarboxylase	HBeAg	hepatitis B e antigen
GAG	glycoslyaminoglycan	HbF	fetal hemoglobin
Gal	$\beta$ -galactose	HBOCs	hemoglobin-based oxygen carriers
GalNAc	<i>n</i> -acetylgalactosamine	HbS	hemoglobin S
GATA1	GATA-binding factor 1	HBSAg	hepatitis B virus surface antigen
GBM	glomerular basement membrane	HBV	hepatitis B virus
GBS	Guillain–Barré syndrome	HCEC	human corneal endothelial cell
GDF	growth differential factor	HCT	hematopoietic cell transplant
GDP	guanosine diphosphate	HCT/P	human cells, tissues, and cellular and tissue-based product
GEF-H1	guanine nucleotide exchange factor H1	HCV	hepatitis C virus
GEMM-CFC	granulocyte-erythroid-macrophage-megakaryocyte colony-forming cells	HDFN	hemolytic disease of the fetus and newborn
GEN	Laboratory General Checklist	HDI	human development index
GFI1	growth factor interdependent 1	HDIVIG	high-dose intravenous immunoglobulin
GI	gastrointestinal	HDL	high-density lipoprotein
GLUT1	glucose transporter 1	HDN	hemolytic disease of the fetus and newborn
GM-CSF	granulocyte macrophage colony-stimulating factor	HDR	homology-directed repair
GMP	good manufacturing practice	HDV	hepatitis D virus
GP	glycoprotein	HE	hereditary elliptocytosis
GPA	glycophorin A	HEIRS	REDS-III Hemoglobin and Iron Recovery Study
GPA	granulomatosis with polyangiitis	HELLP (syndrome)	hemolysis, elevated liver enzymes, and low platelets
GPB	glycophorin B	HEMPAS	Hereditary erythroblastic multinuclearity with positive acidified serum lysis test
GPC	glycophorin C	HES	hydroxyethyl starch human
GPCR	guanine nucleotide-binding protein-coupled receptor	hESC	human embryonic stem cell
GPD	glycophorin D	HEV	hepatitis E virus
GPI	glycosylphosphatidylinositol	HFMEA	Healthcare Failure Mode and Effect Analysis
GPS	Goodpasture syndrome	Hgb	hemoglobin
GPVI	Glycoprotein VI (platelet)	hGH	human growth hormone
GPX4	glutathione peroxidase 4	HH	hereditary hemochromatosis
GRADE	Grading of Recommendations Assessment, Development and Evaluation	HHV	human herpesvirus
GSH	glutathione	HIC	hydrophobic interaction chromatography
GSL	glycospingolipid	HIF	hypoxia-inducible transcription factor
GSS	Gerstmann–Straussler–Scheinker disease	HIF-PHDs	hypoxia-inducible transcription factor
GT	gestational thrombocytopenia	HIPA	prolyl hydroxylases
GT6	glycosyltransferase family 6	HIT	heparin-induced platelet activation assay
GTA	A-transferase	HIV	heparin-induced thrombocytopenia
GTB	B-transferase	HLA	human immunodeficiency virus
GTP	guanosine triphosphate	HLH	human leukocyte antigen
GTX	granulocyte transfusions	HMW	hemophagocytic lymphohistiocytosis
GVHD	graft-versus-host disease	HMWK	high molecular weight
GVL	graft-versus-leukemia	HNA	high-molecular-weight kininogen
GWA	genome-wide association	HNA-3	human neutrophil antigen
HA	hyaluronic acid	HO1	human neutrophil antigen 3
HA	hydroxyapatite	HPAs	heme oxygenase-1
HAA	hospital-acquired anemia	HPC	human platelet antigens
HAART	highly active antiretroviral therapy	HPCT	hematopoietic progenitor cell
HAV	hepatitis A virus	HPP	hematopoietic progenitor cell
HB-PAN	hepatitis B-associated polyarteritis nodosa	HPV	transplantation
HbAA	normal hemoglobin A	HR	hereditary pyropoikilocytosis
HbAS	hemoglobin A sickle	HRI	human papilloma virus
			hazard ratio
			heme-regulated inhibitor

HRP	histidine-rich protein 2	IPF	immature platelet fraction
HSC	hematopoietic stem cells	IPFA	International Plasma Fractionation Association
HSCT	hematopoietic stem cell transplantation	iPSC	induced pluripotent stem cell
HSV	herpes simplex virus	IPSS	International Prognostic Scoring System
HTA	health technology assessment	IQPP	International Quality Plasma Program
HTLV	human T-cell lymphotropic virus	IR	interventional radiologists
HTR	hemolytic transfusion reaction	IRE	iron-responsive element
hUCMScs	human umbilical cord mesenchymal stem cells	IRP	iron regulatory protein
HUS	hemolytic-uremic syndrome	ISBT	International Society of Blood Transfusion
HVM	handheld vital microscopy	ISTARE	International Surveillance Database for Transfusion Adverse Reactions and Events
hWJCs	Wharton's jelly-derived mesenchymal stem cells	ISTH	International Society on Thrombosis and Hemostasis
HX	hereditary xerocytosis	IT	information technology
%HYPOm	percentage of hypochromic mature red blood cells	ITAC	Inpatient Treatment With Anti-Coronavirus
%HYPOR	percentage of hypochromic red blood cells	ITI	Immunoglobulin Trial
IA-HUS	infection-associated hemolytic-uremic syndrome	ITP	immune tolerance induction
IAP	integrin-associated protein	iTTP	immune thrombocytopenic purpura
IAT	indirect antiglobulin test	IV	immune thrombotic thrombocytopenic purpura
IBCT	incorrect blood component transfused	IVC	intravenous
IBR	intraoperative blood recovery	IVD	inferior vena cava
IC	informed consent	IVIG / IVIg	in vitro diagnostic devices
ICAM4	interstitial cell adhesion molecule-4	JAK2	intravenous immunoglobulin
ICCBBA	International Council for Commonality in Blood Banking Automation	KIR	Janus kinase 2
ICH	International Conference on Harmonization (of Technical Requirements)	KLF-1	killer immunoglobulin-like receptor
ICH	intracranial hemorrhage	LacCer	Krppel-like factor-1
iCJD	iatrogenic Creutzfeldt-Jakob disease	LAD	lactosylceramide
ICU	intensive care unit	LAG3	leukocyte adhesion deficiency
ID NAT	individual nucleic acid test	LAK	lymphocyte-activation gene 3
IDA	iron-deficiency anemia	LCL	lymphokine-activated killer
IDE	iron-deficient erythropoiesis	LCMV	lymphoblastoid line
IDH1	isocitrate dehydrogenase 1	LCR	lymphatic choriomeningitis
IDSA	Infectious Disease Society of America	LCT	locus control region
IDT	individual testing	LDH	lymphocytotoxicity
IE	ineffective erythropoiesis	LDL	lactate dehydrogenase
IFA	immunofluorescence assay	LEMS	low-density lipoprotein
IFAT	immunofluorescent antibody test	LESC	Lambert-Eaton myasthenic syndrome
IFN	interferon	LF	limbal epithelial stem cell
IG/ Ig	immunoglobulin	LFI	low ferritin
IgA	immunoglobulin A	LGI1	lateral flow immunoassay
IGF-1-R	insulin-like growth factor 1 receptor	LGL	leucine-rich glioma inactivated 1
IGF1	insulin-like growth factor-1	LHDAg	large granular lymphocyte
IgG	immunoglobulin G	LHR	long hepatitis D antigen
IgM	immunoglobulin M	LIA	long homologous repeat
IgSF	immunoglobulin superfamily	LIC	latex-enhanced immunoturbidimetric assay
IHD	incorporating isovolemic hemodilution (red cell exchange)	LIF	liver iron concentration
IHN	International Hemovigilance Network	LISS	leukemia inhibitory factor
IL	interleukin	LKE	low ionic strength solution
IM	intramuscular	LMAN	luke antigen on erythrocytes
IMP	inosine monophosphate	LMO2	lectin mannose binding
IND	individual donor	LMW	Lim domain partner of TAL1
IND	investigational new drug	LMWH	low molecular weight
iNKT	invariant natural killer T cell	lncRNAs	low molecular weight heparin
INR	international normalized ratio	LP	long noncoding RNAs
IPC	immature platelet count	LPI	liquid plasma
IPD	individual-patient data	LPS	labile plasma iron
		LR	lipopolysaccharide
		LRP4	leukocyte reduction / leukoreduced
			lipoprotein receptor-related protein 4

LSC	limbal stem cell	MOG	myelin oligodendrocyte glycoprotein
LTA	lipoteichoic acid	8-MOP	8-methyoxyxpsoralen
LTOWB	low-titer group O whole blood	MPA	microscopic polyangiitis
LVDS	large volume delayed sampling	MPO	myeloperoxidase
LVEF	left ventricular ejection fraction	MPP	multipotent progenitor
Mab	monoclonal antibody	MPV	mean platelet volume
MAC	membrane attack complex	MR	magnetic resonance
McC	McCoy antigen	MRI	magnetic resonance imaging
MACE	modified capture enzyme-linked immuno-sorbent assay	mRNA	messenger ribonucleic acid
MAG	myelin-associated glycoprotein	MS	multiple sclerosis
MAHA	microangiopathic hemolytic anemia	MSC	mesenchymal stem (stromal) cell
MAIPA	monoclonal antibody-specific immobilization of platelet antigens	MSM	men who have sex with men
MAP	mean arterial pressure	MTP	massive transfusion protocol
MAPK	mitogen-activated protein kinase	MTX	methotrexate
MART	melanoma antigen recognized by T cells	MuSK	muscle-specific kinase
MATTERs	Military Application of Tranexamic acid in Trauma Emergency Resuscitation study	MVM	minute virus of mice
MB	methylene blue	NAAT	nucleic acid amplification testing
MBFs	microaggregate blood filters	NACSSG	National Acute Chest Syndrome Study Group
MBG	Marburg virus	NAD	nicotinamide adenine dinucleotide
MBP	myelin basic protein	NADH	reduced nicotinamide adenine dinucleotide
MCA	middle cerebral arteries	NADP	nicotinamide adenine dinucleotide
MCFD	multiple coagulation factor deficiency gene	NADPH	phosphate
MCH	mean cell hemoglobin	NAIT	reduced nicotinamide adenine dinucleotide
MCHC	mean corpuscular hemoglobin concentration	NAITP	phosphate
MCP	macrophage chemoattractant protein	NANB	neonatal alloimmune thrombocytopenia
MCV	mean corpuscular volume	NAPTT	neonatal alloimmune thrombocytopenic purpura
MDDS	Medical Device Data Systems	NAT	non-A, non-B hepatitis
MDH1	malate dehydrogenase 1	NATA	non-activated partial thromboplastin time
MDL	Medication Deferral List	NBCUS	nucleic acid testing
MDS	myelodysplastic syndrome	NCAAs	Network for Advancement of Transfusion Alternatives
MECOM	MDS1 and EV11 complex locus protein	NCI	National Blood Collection and Utilization Survey
MEDALIST	A Study of Luspatercept to Treat Anemia Due to Very Low, Low, or Intermediate Risk Myelodysplastic Syndromes	NDDR	national competent authorities
MEHP	mono(2-ethylhexyl) phthalate	NDI	National Cancer Institute
MEP	megakaryocytic-erythroid progenitor	NDMA	National Donor Deferral Registry
MET	mesenchymal–epithelial transition	NEC	neurodevelopmental impairment
MFI	mean fluorescence intensity	NETs	nitrosodimethylamine
MGSA	melanocyte growth-stimulating activity	NF- $\kappa$ B	necrotizing enterocolitis
MGUS	monoclonal gammopathy of undetermined significance	NFE2	neutrophil extracellular traps
MHA-TP	Microhemagglutination Assay for <i>Treponema pallidum</i>	NGC	nuclear factor $\kappa$ B
MHC	major histocompatibility complex	NGS	nuclear factor, erythroid 2
MIRL	membrane inhibitor of reactive lysis	NHLBI	nerve guidance conduits
miRNA	micro RNA	NHS	next-generation sequencing
MK	megakaryocyte	NHSBT	National Heart, Lung, and Blood Institute
MKL	myocardin-like transcription factors	NHSN	National Health Service (UK)
MLR	mixed lymphocyte reaction	NIBSC	National Health System Blood and Transplant Service
MM	multiple myeloma	NICU	National Healthcare Safety Network
MMN	multifocal motor neuropathy	NIH	National Institute of Biological Standards and Control
MMP	matrix metalloproteinase	NIRS	neonatal intensive care unit
MMR	Measles, mumps, and rubella vaccination	NK	National Institutes of Health
MnPO	median preoptic area	NMDAR	near-infrared spectroscopy
MoAbs	monoclonal antibodies	NMDP	natural killer
MODS	multiple-organ dysfunction syndrome	NMOSD	<i>N</i> -methyl-D-aspartate receptor
MOF	multiple-organ failure	NNNI	National Marrow Donor Program

NO	nitric oxide	PCH	paroxysmal cold hemoglobinuria
NOD	non-obese diabetic	PCL	polycaprolactone
Nplate	Romiplostim	PCP	<i>pneumocystis pneumonia carii</i>
NPO	nil per os	PCR	polymerase chain reaction
NRAs	National Regulatory Authorities	PCSK9	proprotein convertase subtilisin-kexin type 9
NRC	Nuclear Regulatory Commission	PD-1	programmed cell death protein 1
NRF	National Response Framework	PDE	phosphodiesterase
NSAID	nonsteroidal anti-inflammatory drug	pdFVII	plasma-derived factor VII
NTBI	non-transferrin-bound iron	pdFX	plasma-derived factor X
NTDT	nontransfusion-dependent thalassemia	pdFXIII	plasma-derived factor XIII
NTT	number needed to treat	PDGF-B	Platelet-derived growth factor subunit B
NYHA	New York Heart Association	PDLLA	poly-D,L-lactide
OBI	occult hepatitis B infection	PDMP	plasma-derived medicinal product
OBRR	Office of Blood Research and Review	PEA	P-selectin expression assay
OCS	open canalicular system	PEG	polyethylene glycol
OEF	oxygen extraction fraction	PEG-rHuMGDF	pegylated recombinant human megakaryocyte growth and development factor
OGP	osteogenic growth peptide	PEI	Paul Ehrlich Institute
OHI	occult hepatitis infection	PENUT	Preterm Erythropoietin Neuroprotection Trial
OHSt	overhydrated hereditary stomatocytosis	PF	platelet factor
OMCL	Official Medicines Control Laboratory	PF4	platelet factor 4
OPN	osteopontin	PF24	24-hour frozen plasma
OR	odds ratio	PFA-100	platelet function analyzer 100
ORC	oxidized regenerated cellulose	PfEMP(-1)	<i>Plasmodium falciparum</i> erythrocyte membrane protein(-1)
OrthoEB	orthochromatic erythroblast	PGA	poly(glycolic acid)
OSHA	Occupational Safety and Health Administration	PGE2	prostaglandin E2
OTAT	Office of Tissues and Advanced Therapies	PhEur	European Pharmacopeia
OthoEBs	orthochromatic erythroblasts	PHS	Public Health Service
P-OH	prolyl hydroxylation	PHSA	Public Health Service Act
PAB	pseudoautosomal boundary	PI	platelet increment
PAF	platelet-activating factor	PI3K	phosphatidylinositol-3-kinase
PAGGGSM	phosphate-adenine-glucose-guanosine-glucuronate-saline-mannitol	PIC/S	Pharmaceutical Inspection Co-operation Scheme and Pharmaceutical Inspection Convention
PAGGSM	phosphate-adenine-glucose-guanosine-saline-mannitol	PICC	peripherally inserted central catheter
PaGIA	particle gel immunoassay	PIG-A	phosphatidylinositol glycan class A
PAH	pulmonary arterial hypertension	PINT	Premature Infants in Need of Transfusion Study
PAI-1	plasminogen activator inhibitor type 1	PIV	peripheral intravenous
PAIgG	platelet-associated IgG	PIVKAs	proteins induced in vitamin K absence
PALISI	Pediatric Acute Lung Injury and Investigators Network	PK	penetrating keratoplasty
PAN	polyarteritis nodosa	PKA	protein kinase A
PANDAS	pediatric autoimmune neuropsychiatric disorders associated with streptococcal infections	PKD	pyruvate kinase deficiency
PAR1	pseudoautosomal region 1	PLA	poly(lacticacid)
PAS	platelet additive solution	PLADO	Optimal Platelet Dose Strategy to Prevent Bleeding in Thrombocytopenia Patients
PASSPORT	Post Approval Surveillance Study of Platelet Outcomes, Release Tested (protocol)	PLC	poly(caprolactone)
PAT	passive alloimmune thrombocytopenia	PLGA	poly(lactic-co-glycolic acid)
PBM	patient blood management	PLS	passenger lymphocyte syndrome
PBMC	peripheral blood mononuclear cell	PME	partial mutual exchange
PBPC	peripheral blood progenitor cell	PMMA	polymethylmethacrylate
PBR	postoperative blood recovery	PMN	polymorphonuclear neutrophil
PBSC	peripheral blood stem cell	PNH	paroxysmal nocturnal hemoglobinuria
PC	platelet concentrate	PNI	peripheral nerve injury
PCAM	platelet-endothelial cell adhesion molecule-1	PNM	neutrophil
PCC	prothrombin complex concentrates	POEMS	polyneuropathy, organomegaly, endocrinopathy, monoclonal protein, and skin changes (syndrome)

POISE / POISE-2	Perioperative Ischemic Evaluation trial	RECESS	Red Cell Duration Study
PolyEB	polychromatophilic erythroblast	REDS-III	Recipient Epidemiology and Donor
PPH	postpartum hemorrhage		Evaluation Study-III
PPi	pyrophosphate	REF	febrile nonhemolytic transfusion reaction
PPP	pentose phosphate pathway	rFIX	recombinant factor IX
PPR	percent platelet recovery	RFLP	restriction fragment length polymorphism
PPTA	Plasma Protein Therapeutics Association	rFVIIa	recombinant activated factor VII
PR	pathogen reduction	rFVIII	recombinant factor VIII
PR3	proteinase 3	RhAG	Rh-associated glycoprotein
PRA	panel-reactive antibody tests	RhD	rhesus D protein
PRAC	Pharmacovigilance Risk Assessment Committee	rhEPO	recombinant human erythropoietin
PRBCs	packed red blood cells	RhIG	Rh immune globulin
PRCA	pure red blood cell aplasia	RhoA	Ras homolog family member A
PRES	posterior leukoencephalopathy	rhTPO	recombinant human thrombopoietin
ProEB	proerythroblast	rHuEPO	Recombinant human erythropoietin
PROMMTT	Prospective Observational Multicenter Massive Transfusion Trial	RING	Safety and Effectiveness of Granulocyte Transfusion in Resolving Infection in People with Neutropenia study
PROPPR	Pragmatic Randomized Optimal Plasma and Platelet Ratios trial	RIPA	radioimmunoprecipitation assay
PRP	platelet-rich plasma	RIR	replication-incompetent retrovirus
PrP <sup>C</sup>	membrane-bound prion protein	RISE study	Retrovirus Epidemiology and Donor Study
PRPP	phosphoribosyl pyrophosphate	RLS	reporting and learning systems
PRT	Pathogen Reduction Technology	ROC	receiver operating characteristic
PRV	pseudorabies virus	ROS	reactive oxygen species
PS	phosphatidylserine	ROTEM	rotational thromboelastometry
PSA	prostate-specific antigen	RP	reticulated platelet
PSGL1	platelet sialoglycoprotein ligand-1	RPa	raphe pallidus nucleus in the medulla
PSOs	patient safety organizations	RPR	rapid plasma reagent
PSV	peak systolic velocity	RPs	reticulated platelets
PT	prothrombin time	RR	repeat reactive
PTFE	polytetrafluoroethylene	RSV	respiratory syncytial virus
PTLD	posttransplant lymphoproliferative disease	RT	room temperature
PTP	post-transfusion purpura	RTTIs	relevant transfusion-transmitted infections
PTR	platelet transfusion refractoriness	rVIIa	recombinant activated factor VII
PTT	partial thromboplastin time	rVWF	recombinant von Willebrand factor
PUP	previously untreated patient	S/D	solvent and detergent
PVC	polyvinyl chloride	S1P	sphingosine-1-phosphate
PvDBP	P. vivax Duffy binding protein	SAA	severe aplastic anemia
PVH	hypothalamic paraventricular nucleus	SABM	Society for the Advancement of Blood Management
pVHL	von Hippel-Lindau protein	SAG	saline, adenine, and glucose
PVR	poliovirus receptor	SAG-M	saline, adenine, and glucose with mannitol
QA	quality assurance	SAL	sterility assurance level
QAE	quaternary amino ethyl	SAO	Southeast Asian Ovalocytosis
QALY	quality-adjusted life years	SBDS	Shwachman–Bodin–Diamond syndrome
QC	quality control	SC	subcutaneous
RA	rheumatoid arthritis	sc-TPA	single-chain tissue plasminogen activator
RANTES	regulated on activation, normal T-cell expressed and secreted	sc-UPA	single-chain urokinase plasminogen activator
RBCCs	red blood cell concentrates	SCD	sickle cell disease
RBC(s)	red blood cells	SCF	stem cell factor
RBDM	risk-based decision-making	scFv	single-chain variable fragment
RBM15	RNA binding motif protein 15	SCI	silent cerebral infarcts
RCAS1	receptor-binding cancer antigen expressed on SiSo cells	SCID	severe combined immunodeficiency
RCDADs	relevant communicable disease agents or diseases	SCIG	subcutaneous IgG
RCE	red cell exchange	sCJD	sporadic Creutzfeldt-Jakob disease
RE-LY	Randomized Evaluation of Long-Term Anticoagulant Therapy trial	SCL	stem cell leukemia
REACT	Renal Autologous Cell Therapy	SCN	severe congenital neutropenia
		SCs	Schwann cells
		SDF-1	stromal-cell-derived factor 1
		SDS	sodium dodecyl sulfate

SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis	TACO	transfusion-associated circulatory overload
Se	secretor-positive	TAD	transfusion-associated dyspnea
SHDAg	short Hepatitis D antigen	TAFI	thrombin-activatable fibrinolysis inhibitor
SHOT	Serious Hazards of Transfusion program (UK)	TALENs	transcription activator-like effector nucleases
shRNA	short hairpin RNA	TAMMv	timed average mean maximum velocity
SID	Sd <sup>a</sup> antigen	TAPS	Transfusion Alternatives Preoperatively in Sickle cell disease trial
SID	secondary immune deficiencies	TAPS	twin anemia-polycythemia sequence
SINV	Sindbis virus	TAXI	Pediatric Critical Care Transfusion and Anemia EXPertise Initiative
siRNA	small interfering RNA	TBSA	total body surface area
SIRS	systemic inflammatory response syndrome	tc-TPA	two-chain tissue plasminogen activator
SIS	small intestinal submucosa	tc-UPA	two-chain urokinase plasminogen activator
SIT trial	Silent Infarct Transfusion trial	TCD	transcranial Doppler ultrasound
SI	Swain-Langley antigen	TCP	tricalcium phosphate
SLE	systemic lupus erythematosus	TCR	T-cell receptor
SMC	smooth muscle cell	TDT	Transfusion-dependent thalassemia
SNO-Hb	S-nitrosohemoglobin	TEE	thromboembolic event
SNP	single nucleotide polymorphism	TEG	thromboelastography
SNV	single nucleotide variation	TEVG	tissue-engineered vascular graft
SoGAT	International Working Group on the Standardization of Genomic Amplification Techniques for the Virological Safety Testing of Blood and Blood Products	TF	tissue factor
SOP	standard operating procedure	TFPI	tissue factor pathway inhibitor
SP	source plasma	TGA	thrombin generation assay
SP	sulfopropyl	TGF(-β)	transforming growth factor(-β)
SPRCA	solid-phase red cell adherence	Th	T helper (cell)
SPS	stiff-person syndrome	THA	total hip arthroplasty
SQUID	superconducting quantum interference device	THBD	Thrombomodulin
SRA	serotonin-release assay	THF	tetrahydrofolate
SRF	serum response factor	TI	tincture of iodine
SSCs	spermatogonial stem cells	TIL	tumor-infiltrating lymphocyte
ssDNA	single-stranded DNA	TIM3	T-cell immunoglobulin and mucin domain containing-3
SSOP(H)	sequence-specific oligonucleotide probe	TITRe2	The Transfusion Indication Threshold Reduction trial
SSP(-PCR)	hybridization	TJC	The Joint Commission
	sequence-specific primer polymerase chain reaction	TLR	toll-like receptor
ssRNA		TM	thalassemia major
STAT(5)	single-stranded RNA	TMAA	thrombotic microangiopathic anemia
	signal transduction and activator of transcription(-5)	TMA	thrombotic microangiopathy
sTfR	soluble transferrin receptor	TMA	transcription-mediated amplification
STOP	Stroke Prevention Trial in Sickle Cell Anemia Trial	TMER	Transfusion Medicine Epidemiology Review study
STOP 2	Optimizing Primary Stroke Prevention in Sickle Cell Anemia Trial	Tmod1	modulatory T cell 1
STS/SCA	Society of Thoracic Surgeons and Society of Cardiovascular Anesthesiologists	TNC	total nucleated cell count
Stx	shiga toxins	TNF	tumor necrosis factor
suPAR	soluble urokinase plasminogen activator receptor	TNFα	tumor necrosis factor-α
SVC	superior vena cava	TOP	transfusion of prematures study
SWiTCPH	Stroke With Transfusions Changing to Hydroxyurea trial	TOPIC	Transfusion of Fresh Frozen Plasma in Nonbleeding ICU Patients trial
Syk	spleen tyrosine kinase	TOTM	triethyl hexyl trimellitate
t-PA	tissue-type plasminogen-activator	TP	<i>Treponema pallidum</i>
TA-GVHD	transfusion-associated graft-versus-host disease	tPA	tissue plasminogen activator
TA-MC	transfusion-associated microchimerism	TPB	theory of planned behavior
		TPE	therapeutic plasma exchange
		TPMT	thiopurine methyltransferase
		TPO	thrombopoietin
		TPO-RAs	thrombopoietin receptor agonists
		TRAIL	tumor necrosis factor-related apoptosis-inducing ligand

TRALI	transfusion-related acute lung injury	vCJD	variant Creutzfeld–Jakob disease
TRAP	Trial to Reduce Alloimmunization to Platelets	VECs	vascular endothelial cells
Treg	regulatory T cell	VEGF	vascular endothelial growth factor
TRICC	Transfusion Requirements in Critical Care	VEGFR	vascular endothelial growth factor receptor
TRICK	transfusion-related inhibition of cytokines	VGCC	voltage-gated calcium channel
TRIM	transfusion-related immunomodulation	VGKC	voltage-gated potassium channel
TRIPICU	Transfusion Strategies for Patients in Pediatric Intensive Care Units study	VIP	von Willebrand Disease International Prophylaxis study
TRS	Technical Report Series (WHO)	VITT	vaccine-induced immune thrombotic thrombocytopenia
TSEs	transmissible spongiform encephalopathies	VKA	vitamin K antagonists
TSO	Transfusion Safety Office	VKDB	vitamin K deficiency bleeding
TSOs	transfusion safety officers	VKDFs	vitamin K-dependant coagulation factors
TSP	tropical spastic paraparesis	VKOR	vitamin K epoxide reductase
TT	thrombin time	VLBW	very-low-birthweight
TT-CMV	transfusion-transmitted cytomegalovirus infection	VLDL	very-low-density lipoprotein
TTB	transfusion-transmitted babesiosis	VML	volumetric muscle loss
TTD	transfusion transmitted disease	VMV	visna-maedi virus of sheep
TTI	transfusion-transmissible infection	VOC	vaso-occlusive crisis
TTISS	Transfusion Transmitted Injuries Surveillance System	VP	viral structure protein
TTM	transfusion-transmitted malaria	VPS	vascular positioning system
TTP	thrombotic thrombocytopenic purpura	VSMCs	vascular smooth muscle cells
TTTS	twin-to-twin transfusion syndrome	VWD / VWD	von Willebrand disease
TTV	TT virus	vWF	von Willebrand factor
TTViS	transfusion-transmitted viral infections	VXM	virtual crossmatch
TWEAK	TNF-like weak inducer of apoptosis	WAIIA	warm autoimmune hemolytic anemia
TWiTCH	transcranial Doppler ultrasound With Transfusions Changing to Hydroxyurea trial	WAS	Wiskott–Aldrich syndrome
TXA	tranexamic acid	WB	Western blot
UBC / UCB	umbilical cord blood	WBC	whole blood
UDHQ	Uniform Donor History Questionnaire	WBD	white blood cell
UEA	<i>Ulex europeaus</i>	WBDPs	whole blood derived
UFH	unfractionated heparin	WBIT	whole blood derived platelets
ULR	universal leukocyte reduction	WCC	wrong blood in tube
UNOS	United Network for Organ Sharing	WFH	WHO Collaborating Center
USP	US Pharmacopoeia and National Formulary	WHIM (syndrome)	World Federation of Hemophilia
UTR	untranslated region	WHO	warts, hypogammaglobulinemia, infections, and myelokathexis
UV-A	ultraviolet A	WNV	World Health Organization
UV-B	ultraviolet B	WOMAN	West Nile virus
UV-C	ultraviolet C	ZFN	World Maternal Antifibrinolytic trial
VATS	Viral Activation by Transfusion Study	ZIKV	zinc finger nuclease
VCAM1	vascular cell adhesion molecule 1	ZnPP	Zika virus
			zinc protoporphyrin

## About the companion website

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This book is accompanied by a companion website.

[www.wiley.com/go/simon/Rossi6](http://www.wiley.com/go/simon/Rossi6)



The website features:

- The figures from the book in downloadable PowerPoint slides.
- Downloadable PDFs of all chapters.
- Downloadable PDFs of the complete reference lists from the book.

The password for the website is the first word of Chapter 1. Please use all lowercase.

## **SECTION I**

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# **Transfusion medicine from ancient times to the current pandemic**

## CHAPTER 1

# Transfusion in the new millennium

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Prehistoric man left drawings of himself pierced by arrows.<sup>1</sup> This means he was as aware of blood as he was of his own limbs. The flint implements he used as tools and weapons distinguished him from other creatures and contributed to the violence of his era. As he hunted food and fought enemies, he observed bleeding and the properties of blood. A cut, received or inflicted, yielded a vivid red color. If the cut was shallow, there was little blood. But if the cut was deep, a red torrent flowing from the stricken victim quickly led to death, with shed blood congealed and darkening in the sun. Fatal hemorrhage was commonplace. Nonetheless, the sight must have been fearful and possibly existential as life flowed red out of the body of an enemy or a wounded animal.<sup>2</sup> It is no wonder, then, that at the dawn of recorded history, blood was already celebrated in religious rites and rituals as a life-giving force.

The cultural expressions of primitive and ancient societies, although separated by time or space, can be strikingly similar. Whether these expressions emerged independently or were diffused about the world by unknown voyagers will probably always remain clouded in mystery.<sup>2</sup> Nonetheless, there is a common thread in the ancient rituals that celebrate blood as a mystical vital principle. In Leviticus 17:11, “the life of the flesh is in the blood,” and the Chinese Neiching (circa 1000 BCE) claims the blood contains the soul.<sup>2</sup> Pre-Columbian North American Indians bled their bodies “of its greatest power” as self-punishment,<sup>3</sup> Egyptians took blood baths as a recuperative measure, and Romans drank the blood of fallen gladiators in an effort to cure epilepsy.<sup>4</sup>

The Romans also practiced a ceremony called taurobolium—a blood bath for spiritual restoration. A citizen seeking spiritual rebirth descended into a pit or *fossa sanguinis*. Above him on a platform, a priest sacrificed a bull, and the animal’s blood cascaded down in a shower upon the beneficiary. Then, in a powerful visual image, the subject emerged up from the other end of the pit, covered with blood and reborn.<sup>1</sup>

The legend of Medea and Aeson taken from Ovid’s *Metamorphoses* and quoted in Bulfinch’s *Mythology*<sup>5</sup> also ascribed rejuvenating powers to blood. Jason asked Medea to “take some years off his life and add them to those of his father Aeson.” Medea, however, pursued an alternative course. She prepared a cauldron with the blood of a sacrificed black sheep. To this, she added magic herbs, hoarfrost gathered by moonlight, the entrails of a wolf, and many other things “without a name.” The boiling cauldron was stirred

with a withered olive branch, which became green and full of leaves and young olives when it was withdrawn. Seeing that all was ready,

Medea cut the throat of the old man and let out all his blood, and poured into his mouth and into his wound the juices of her cauldron. As soon as he had imbibed them, his hair and beard laid by their whiteness and assumed the blackness of youth; his paleness and emaciation were gone; his veins were full of blood, his limbs of vigour and robustness. Aeson is amazed at himself and remembers that such as he now is, he was in his youthful days, 40 years before.

This legend seems to echo the apocryphal story of Pope Innocent VIII, who is said to have received the blood of three young boys in 1492 while on his deathbed. As the story goes, a physician attempted to save the pope’s life by using blood drawn from three boys 10 years of age, all of whom died soon thereafter. Some nineteenth-century versions of this tale suggest the blood was transfused. However, earlier renditions more plausibly suggest that the blood was intended for a potion to be taken by mouth. In any event, there is no evidence the pope actually received any blood in any form.<sup>6,7</sup>

The folklore that flowed with blood was not accompanied by a great deal of accurate information. The ancient Greeks believed that blood formed in the heart and passed through the veins to the rest of the body, where it was consumed. Arteries were part of an independent system transporting air from the lungs. Although Erasistratos (circa 270 BCE) had imagined the heart as a pump, his idea was ahead of its time. As long as veins and arteries were dead-end channels transporting blood and air, there was little need for a pump in the system. Although Galen (131–201 CE) finally proved that arteries contain blood, communication with the venous system was not suspected. Blood, formed in the liver, merely passed through the blood vessels and heart on its way to the periphery.<sup>1</sup> These teachings remained in place for 1400 years until they were swept away in 1628 by Harvey’s discovery of the circulation.

The realization that blood moved in a circulating stream opened the way to experiments on vascular infusion. In 1642, George von Wahnrendorff injected wine<sup>8</sup>—and, in 1656, Christopher Wren and Robert Boyle injected opium and other drugs<sup>9</sup>—intravenously into dogs. The latter studies, performed at Oxford, were the inspiration for Richard Lower’s experiments in animal transfusion.

## The first animal transfusion

Richard Lower (1631–1691) was a student at Oxford when Christopher Wren and Robert Boyle began their experiments on infusion. In due course, Lower joined their scientific group and studied the intravenous injection of opiates, emetics, and other substances into living animals.<sup>10</sup> In time, the transfusion of blood itself became the objective. The announcement of the first successful transfusion, performed by Richard Lower at Oxford in February 1665, was published on November 19, 1666, in the *Philosophical Transactions of the Royal Society* in a short notation titled, “The Success of the Experiment of Transfusing the Blood of One Animal into Another.”<sup>11</sup> The entire notation is as follows:<sup>11</sup>

This experiment, hitherto look'd upon to be of an almost insurmountable difficulty, hath been of late very successfully perform'd not only at Oxford, by the directions of that expert anatomist Dr. Lower, but also in London, by order of the R. Society, at their publick meeting in Gresham Colledge: the Description of the particulars whereof, and the Method of Operation is referred to the next opportunity.

The December 17, 1666, issue of the *Transactions* contained the full description as promised.<sup>12</sup> It was taken from a letter<sup>13</sup> written by Lower to Robert Boyle on July 6, 1666, in which Lower described direct transfusion from the carotid artery of one dog to the jugular vein of another. After describing the insertion of quills into the blood vessels of the donor and recipient dogs, Lower wrote:<sup>13</sup>

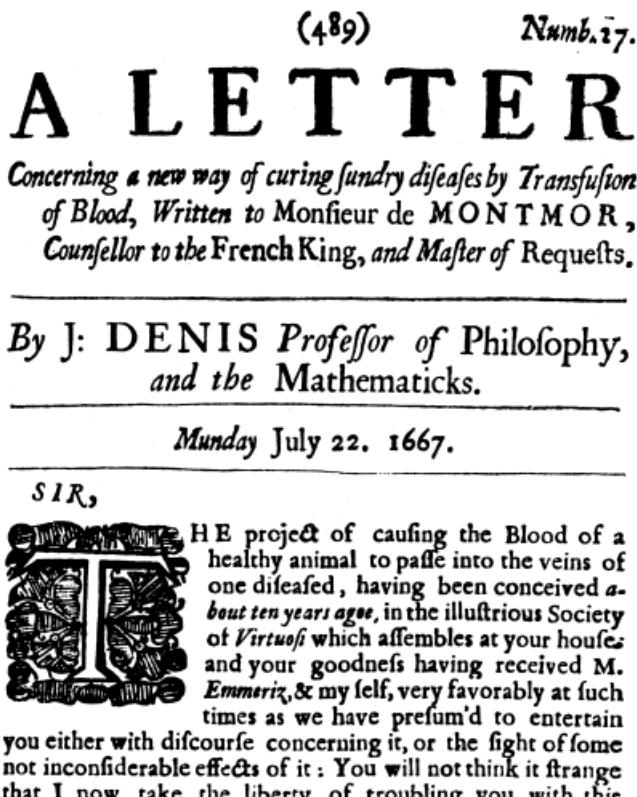
When you have done this you may lay the dogs on their side and fasten them densely together as best you may to insure the connection of the two quills. Quickly tighten the noose around the neck of the receiving animal as in venesection, or at all events compress the vein on the opposite side of the neck with your finger, then take out the stopper and open the upper jugular quill so that while the foreign blood is flowing into the lower quill, the animal's own blood flows out from the upper into suitable receptacles—until at last the second animal, amid howls, faintings, and spasms, finally loses its life together with its vital fluid.

When the tragedy is over, take both quills out of the jugular vein of the surviving animal, tie tightly with the former slipknots, and divide the vein. After the vessel has been divided, sew up the skin, slacken the cords binding the dog, and let it jump down from the table. It shakes itself a little, as though aroused from sleep, and runs away lively and strong, more active and vigorous perhaps, with the blood of its fellow than its own.

These studies inevitably led to the transfusion of animal blood to humans. In England, this occurred on November 23, 1667, when Lower and Edmund King transfused sheep blood into a man named Arthur Coga.<sup>14</sup> Described by Samuel Pepys as “a little frantic,” Coga was paid 20 shillings to accept this transfusion, with the expectation that it might have a beneficial “cooling” effect. One week later, Coga appeared before the Society and claimed to be a new man, although Pepys concluded he was “cracked a little in the head.”<sup>15</sup> However, this was not the first transfusion performed in a human. The credit for that accomplishment belongs to Jean-Baptiste Denis (1635–1704), who had performed the first human transfusion several months earlier in Paris.

## The first animal-to-human transfusion

The founding of the Royal Society in London in 1662 was followed in 1666 by the establishment of the Academie des Sciences in Paris under the patronage of King Louis XIV. The new Academie reviewed the English reports on transfusion with great interest. Denis probably read of Lower’s experiments in the *Journal des Savants* on January 31, 1667, and he began his own studies approximately one month later.<sup>15,16</sup> The first human transfusion was



**Figure 1.1** The first human transfusion. Source: Denis (1967).<sup>17</sup> Figure 01, p 01 / With permission of The Royal Society.

then performed on June 15, 1667, when Denis administered the blood of a lamb to a 15-year-old boy (Figure 1.1).

Although discovery of the circulation had suggested the idea of transfusion, indications for the procedure remained uninformed. Transfusion was still thought to alter behavior and possibly achieve rejuvenation. The blood of young dogs made old dogs seem frisky; the blood of lions was proposed as a cure for cowardice; and, five months later, Arthur Coga would receive a transfusion of sheep blood because of its presumed “cooling” effect. Denis used animal blood for transfusion because he thought it was “less full of impurities”<sup>17</sup>:

Sadness, Envy, Anger, Melancholy, Disquiet and generally all the Passions, are as so many causes which trouble the life of man, and corrupt the whole substance of the blood: Whereas the life of Brutes is much more regular, and less subject to all these miseries.

It is thus ironic that the symptoms of the first transfusion recipient may have been explained in part by profound anemia; the single transfusion of lamb blood may have produced temporary amelioration owing to increased oxygen transport. Denis described the case as follows:<sup>17</sup>

On the 15 of this Moneth, we hapned upon a Youth aged between 15 and 16 years, who had for above two moneths bin tormented with a contumacious and violent fever, which obliged his Physitians to bleed him 20 times, in order to asswage the excessive heat.

Before this disease, he was not observed to be of a lumpish dull spirit, his memory was happy enough, and he seem'd cheerful and nimble enough in body; but since the violence of this fever, his wit seem'd wholly sunk, his memory perfectly lost, and his body so heavy and drowsie that he was not fit for anything. I beheld him fall asleep as he sate at dinner, as he was eating his Breakfast, and in all occurrences where men seem most unlikely to sleep. If he went to bed at nine of the clock in the Evening, he needed to be wakened several times before he could be got to rise by nine the next morning, and he pass'd the rest of the day in an incredible stupidity.

I attributed all these changes to the great evacuations of blood, the Physitians had been oblig'd to make for saving his life.

Three ounces of the boy's blood were exchanged for 9 ounces of lamb arterial blood. Several hours later the boy arose, and "for the rest of the day, he spent it with much more liveliness than ordinary." Thus, the first human transfusion, which was heterologous, was accomplished without any evident unfavorable effect.

This report stimulated a firestorm of controversy over priority of discovery.<sup>18,19</sup> The letter by Denis was published in the *Transactions* on July 22, 1667, while the editor, Henry Oldenburg, was imprisoned in the Tower of London. Oldenburg, following some critical comments concerning the Anglo-Dutch War then in progress (1665–1667), had been arrested under a warrant issued on June 20, 1667. After his release two months later, Oldenburg returned to his editorial post and found the letter published in his absence. He took offense at Denis's opening statement, which claimed that the French had conceived of transfusion "about ten years ago, in the illustrious Society of Virtuosi" (Figure 1.1). This seemed to deny the English contributions to the field. Oldenburg cited these omissions in an issue of the *Transactions* published September 23, 1667, "for the Months of July, August, and September." By numbering this issue 27 and beginning pagination with 489, Oldenburg attempted to suppress the letter by Denis.<sup>18</sup> However, as is evident, this did not ultimately succeed. Nonetheless, subsequent events created even greater difficulties for Denis.

Although the first two subjects who underwent transfusion by Denis were not adversely affected, the third and fourth recipients died. The death of the third subject was easily attributable to other causes. However, the fourth case initiated a sequence of events that put an end to transfusion for 150 years.

Anthony du Mauroy was a 34-year-old man who suffered from intermittent bouts of maniacal behavior. On December 19, 1667, Denis and his assistant Paul Emmerez removed 10 ounces of the man's blood and replaced it with 5 or 6 ounces of blood from the femoral artery of a calf. Failing to note any apparent improvement, they repeated the transfusion 2 days later. After the second transfusion, du Mauroy experienced a classic transfusion reaction:<sup>20</sup>

His pulse rose presently, and soon after we observ'd a plentiful sweat over all his face. His pulse varied extremely at this instant, and he complain'd of great pains in his kidneys and that he was not well in his stomach.

Du Mauroy fell asleep at about 10 o'clock in the evening. He awoke the following morning and "made a great glass full of urine, of a color as black, as if it had been mixed with the soot of chimneys."<sup>20</sup> Two months later, the patient again became maniacal, and his wife again sought transfusion therapy. Denis was reluctant but finally gave in to her urgings. However, the transfusion could not be accomplished, and du Mauroy died the next evening.

The physicians of Paris strongly disapproved of the experiments in transfusion. Three of them approached du Mauroy's widow and encouraged her to lodge a malpractice complaint against Denis. She instead went to Denis and attempted to extort money from him in return for her silence. Denis refused and filed a complaint before

the Lieutenant in Criminal Causes. During the subsequent hearing, evidence was introduced to indicate that Madame du Mauroy had poisoned her husband with arsenic. In a judgment handed down at the Chatelet in Paris on April 17, 1668, Denis was exonerated, and the woman was held for trial. The court also stipulated "that for the future no transfusion should be made upon any human body but by the approbation of the Physicians of the Parisian Faculty."<sup>21</sup> At this point, transfusion research went into decline, and within 10 years it was prohibited in both France and England.

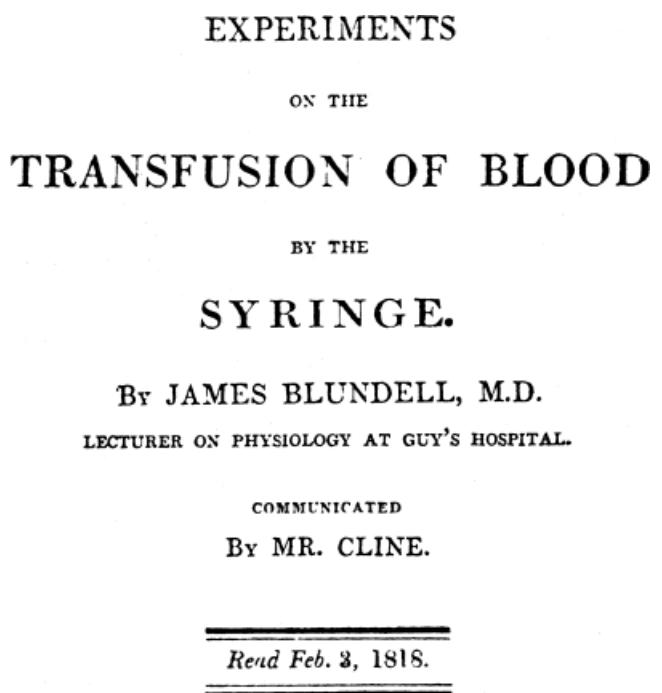
## The beginnings of modern transfusion

After the edict that ended transfusion in the seventeenth century, the technique lay dormant for 150 years. Stimulated by earlier experiments by Leacock, transfusion was "resuscitated" and placed on a rational basis by James Blundell (1790–1877), a London obstetrician who had received his medical degree from the University of Edinburgh.<sup>22</sup> Soon after graduation, Blundell accepted a post in physiology and midwifery at Guy's Hospital. It was there that he began the experiments on transfusion that led to its rebirth. The frequency of postpartum hemorrhage and death troubled Blundell. In 1818, he wrote:<sup>23</sup>

A few months ago I was requested to visit a woman who was sinking under uterine hemorrhage. . . . Her fate was decided, and notwithstanding every exertion of the medical attendants, she died in the course of two hours.

Reflecting afterwards on this melancholy scene . . . I could not forbear considering, that the patient might very probably have been saved by transfusion; and that . . . the vessels might have been replenished by means of the syringe with facility and promptitude.

This opening statement introduced Blundell's epoch-making study titled "Experiments on the Transfusion of Blood by the Syringe"<sup>23</sup> (see Figure 1.2). Blundell described in detail a series



**Figure 1.2** The beginnings of modern transfusion. Source: Blundell (1818).<sup>23</sup> Figure 01, p 01 / With permission of The Royal Society of Medicine.

of animal experiments. He demonstrated that a syringe could be used effectively to perform transfusion, that the lethal effects of arterial exsanguination could be reversed by the transfusion of either venous or arterial blood, and that the injection of 5 drams (20 cc) of air into the veins of a small dog was not fatal but transfusion across species ultimately was lethal to the recipient.<sup>23</sup> Thus, Blundell was the first to clearly state that only human blood should be used for human transfusion. The latter conclusion was confirmed in France by Dumas and Prevost, who demonstrated that the infusion of heterologous blood into an exsanguinated animal produced only temporary improvement and was followed by death within six days.<sup>24</sup> These scientific studies provided the basis for Blundell's subsequent efforts in clinical transfusion.

The first well-documented transfusion with human blood took place on September 26, 1818.<sup>25</sup> The patient was an extremely emaciated man in his mid-thirties who had pyloric obstruction caused by carcinoma. He received 12 to 14 ounces of blood in the course of 30 or 40 minutes. Despite initial apparent improvement, the patient died two days later. Transfusion in the treatment of women with postpartum hemorrhage was more successful. In all, Blundell performed 10 transfusions, of which 5 were successful. Three of the unsuccessful transfusions were performed on moribund patients, the fourth was performed on a patient with puerperal sepsis, and the fifth was performed on the aforementioned patient with terminal carcinoma. Four of the successful transfusions were given for postpartum hemorrhage, and the fifth was administered to a boy who bled after amputation.<sup>22</sup> Blundell also devised various instruments for the performance of transfusion. They included an "impellor," which collected blood in a warmed cup and "impelled" the blood into the recipient via an attached syringe, and a "gravitator"<sup>26</sup> (Figure 1.3), which received blood and delivered it by gravity through a long vertical cannula.

The writings of Blundell provided evidence against the use of animal blood in humans and established rational indications for transfusion. However, the gravitator (Figure 1.3) graphically demonstrated the technical problems that remained to be solved. Blood from the donor, typically the patient's husband, flowed into a funnel-like device and down a flexible cannula into the patient's vein "with as little exposure as possible to air, cold, and inanimate surface."<sup>25</sup> The amount of blood transfused was estimated from the amount spilled into the apparatus by the donor. In this clinical atmosphere, charged with apprehension and anxiety, the amount of blood issuing from a donor easily could be overstated. Clotting within the apparatus then ensured that only a portion of that blood actually reached the patient. Thus, the amount of blood actually transfused may have been seriously overestimated. This may explain the apparent absence of transfusion reactions. Alternatively, reactions may have been unrecognized. Patients who underwent transfusion frequently were agonal. As Blundell stated, "It seems right, as the operation now stands, to confine transfusion to the first class of cases only, namely, those in which there seems to be no hope for the patient, unless blood can be thrown into the veins."<sup>26</sup> Under these circumstances, "symptoms" associated with an "unsuccessful" transfusion might be ascribed to the agonal state rather than the transfusion itself. For a time, the problem of coagulation during transfusion was circumvented by the use of defibrinated blood. This undoubtedly increased the amount of blood actually transfused. However, there were numerous deaths. Interestingly, these deaths were attributed to intravascular coagulation when in actuality they

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### OBSERVATIONS

ON

### TRANSFUSION OF BLOOD.

BY DR. BLUNDELL.

*With a Description of his Gravitator.\**

STATES of the body really requiring the infusion of blood into the veins are probably rare; yet we sometimes meet with cases in which the patient must die unless such operation can be performed; and still more frequently with cases which seem to require a supply of blood, in order to prevent the ill health which usually arises from large losses of the vital fluid, even when they do not prove fatal.

\* The instrument is manufactured by Messrs. Maw, 55, Aldermanbury.

In the present state of our knowledge respecting the operation, although it has not been clearly shown to have proved fatal in any one instance, yet not to mention possible, though unknown risks, inflammation of the arm has certainly been produced by it on one or two occasions; and therefore it seems right, as the operation now stands, to confine transfusion to the first class of cases only, namely, those in which there seems to be no hope for the patient, unless blood can be thrown into the veins.

The object of the Gravitator is, to give help in this last extremity, by transmitting the blood in a regulated stream from one individual to another, with as little exposure as may be to air, cold, and inanimate surface; ordinary venesection being the only operation performed on the person who emits the blood; and the insertion of a small tube into the vein usually laid open in bleeding, being all the operation which it is necessary to execute on the person who receives it.

The following plate represents the whole apparatus connected for use and in action:—

Tab. I.



No. 302.

Y

Figure 1.3 Blundell's gravitator. Source: Blundell (1828).<sup>26</sup> With permission of Jeremy Norman & Co., Inc.

were probably fatal hemolytic reactions caused by the infusion of incompatible blood.<sup>27</sup>

Transfusion at the end of the nineteenth century, therefore, was neither safe nor efficient. The following description, written in 1884, illustrates this point:<sup>28</sup>

Students, with smiling faces, are rapidly leaving the theatre of one of our metropolitan hospitals. The most brilliant operator of the day has just performed immediate transfusion with the greatest success. By means of a very beautiful instrument, the most complex and ingenious that modern science has yet produced, a skilful surgeon has transfused half a pint, or perhaps a pint, of blood from a healthy individual to a fellow creature profoundly collapsed from the effects of severe hemorrhage. Some little difficulty was experienced prior to the operation, as one of the many stop-cocks of the transfusion apparatus was found to work stiffly; but this error was quickly rectified by a mechanic in attendance. Towards the close of the operation the blood-donor, a powerful and heavy young man, swooned. Two porters carried him on a stretcher into an adjoining room.

In the latter half of the nineteenth century, there were many attempts to render transfusion a more predictable and less arduous procedure. In 1869, Braxton-Hicks,<sup>29</sup> using blood anticoagulated with phosphate solutions, performed a number of transfusions on women with obstetric bleeding. Many of the

patients were in extremis and ultimately all died. Unfortunately, a detailed description of terminal symptoms was not provided.<sup>29</sup> Some investigators attempted to rejuvenate animal-to-human transfusion, and Oscar Hasse persisted in this approach despite disastrous results. Studies by Emil Ponfick and by Leonard Landois finally put an end to this practice. Ponfick, in carefully controlled studies, confirmed the lethality of heterologous transfusion and identified the resulting hemoglobinuria along with its donor erythrocyte source. Landois documented the poor results of animal-to-human transfusion and demonstrated the lysis of sheep erythrocytes by human serum in vitro.<sup>8</sup>

Frustration with blood as a transfusion product led to even more bizarre innovations. From 1873 to 1880, cow, goat, and even human milk was transfused as a blood substitute.<sup>30</sup> The rationale derived from an earlier suggestion that the fat particles of milk could be converted into blood cells. Milk transfusion was particularly popular in the United States,<sup>30</sup> where the practice of animal-to-human transfusion was recorded as late as 1890.<sup>31</sup> Fortunately, these astonishing practices were discontinued when saline solutions were introduced as "a life-saving measure" and "a substitute for the transfusion of blood."<sup>32</sup> A passage from an article written by Bull in 1884<sup>32</sup> is particularly instructive:

The danger from loss of blood, even to two-thirds of its whole volume, lies in the disturbed relationship between the calibre of the vessels and the quantity of the blood contained therein, and not in the diminished number of red blood-corpuscles; and . . . This danger concerns the volume of the injected fluids also, it being a matter of indifference whether they be albuminous or containing blood corpuscles or not.

Mercifully, volume replacement with saline solutions deflected attention from the unpredictable and still dangerous practice of blood transfusion. Accordingly, transfusions were abandoned until interest was rekindled by the scientific and technical advances of the early twentieth century.

### The twentieth century

The twentieth century was ushered in by a truly monumental discovery. In 1900, Karl Landsteiner (1868–1943) observed that the sera of some persons agglutinated the red blood cells of others. This study, published in 1901 in the *Wiener Klinische Wochenschrift*<sup>33</sup> (Figure 1.4), showed for the first time the cellular differences in individuals from the same species. In his article, Landsteiner wrote:<sup>34</sup>

In a number of cases (Group A) the serum reacts on the corpuscles of another group (B), but not, however, on those of group A, while, again, the corpuscles of A will be influenced likewise by serum B. The serum of the third group (C) agglutinates the corpuscles of A and B, while the corpuscles of C will not be influenced by the sera of A and B. The corpuscles are naturally apparently insensitive to the agglutinins which exist in the same serum.

Aus dem pathologisch-anatomischen Institute in Wien.  
Ueber Agglutinationserscheinungen normalen  
menschlichen Blutes.

Von Dr. Karl Landsteiner, Assistenten am pathologisch-anatomischen  
Institute.

Figure 1.4 Landsteiner's description of blood groups. Source: Landsteiner (1901).<sup>33</sup> With permission of ScienceOpen.

With the identification of blood groups A, B, and C (subsequently renamed group O) by Landsteiner and of group AB by Decastello and Sturli,<sup>35</sup> the stage was set for the performance of safe transfusion. For this work, Landsteiner somewhat belatedly received the Nobel Prize in 1930. But even that high recognition does not adequately express the true magnitude of Landsteiner's discovery. His work was like a burst of light in a darkened room. He gave us our first glimpse of immunohematology and transplantation biology and provided the tools for important discoveries in genetics, anthropology, and forensic medicine. Viewed from this perspective, the identification of human blood groups is one of only a few scientific discoveries of the twentieth century that changed all of our lives.<sup>34</sup> Yet the translation of Landsteiner's discovery into transfusion practice took many years.

At the turn of the twentieth century, the effective transfer of blood from one person to another remained a formidable task. Clotting, still uncontrolled, quickly occluded transfusion devices and frustrated most efforts. In 1901, the methods used in transfusion were too primitive to demonstrate the importance of Landsteiner's discovery. Indeed, the study of in vitro red cell agglutination may have seemed rather remote from the technical problems that demanded attention. An intermediate step was needed before the importance of Landsteiner's breakthrough could be perceived and the appropriate changes could be incorporated into practice. This process was initiated by Alexis Carrel (1873–1944), another Nobel laureate, who developed a surgical procedure that allowed direct transfusion through an arteriovenous anastomosis. Carrel<sup>36</sup> introduced the technique of end-to-end vascular anastomosis with triple-threaded suture material. This procedure brought the ends of vessels in close apposition and preserved luminal continuity, thus avoiding leakage or thrombosis. This technique paved the way for successful organ transplantation and brought Carrel the Nobel Prize in 1912. It was also adapted by Carrel<sup>37</sup> and others<sup>38,39</sup> to the performance of transfusion. Crile<sup>38</sup> introduced the use of a metal tube to facilitate placement of sutures, and Bernheim<sup>39</sup> used a two-piece cannula to unite the artery to the vein (Figure 1.5). Because all of these procedures usually culminated in the sacrifice of the two vessels, they were not performed frequently. Direct transfusion was also fraught with danger. In a passage written two decades later, the procedure was recalled in the following manner:<sup>40</sup>

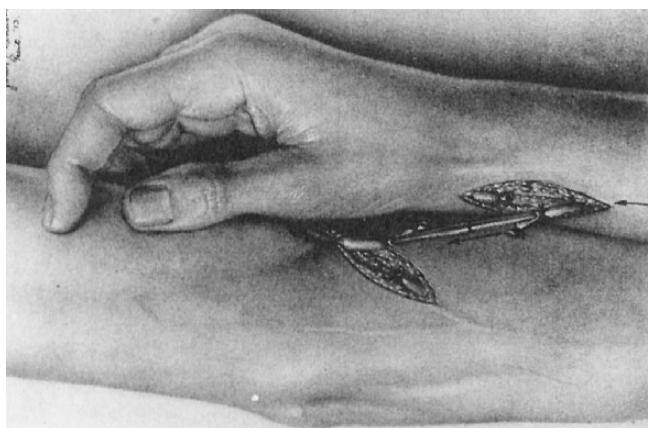


Figure 1.5 Direct transfusion by means of arteriovenous anastomosis through the two-pieced cannula of Bernheim. Source: Bernheim (1917).<sup>39</sup> With permission of J. B. Lippincott & Company.

## A CASE OF FATAL HEMOLYSIS FOLLOWING DIRECT TRANSFUSION OF BLOOD BY ARTERIOVENOUS ANASTOMOSIS.\*

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AND

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**Figure 1.6** Report of a fatal transfusion reaction. Source: Pepper and Nisbet (1907).<sup>41</sup> With permission of American Medical Association.

The direct artery to vein anastomosis was the best method available but was often very difficult or even unsuccessful. And, what was almost as bad, one never knew how much blood one had transfused at any moment or when to stop (unless the donor collapsed). (I remember one such collapse in which the donor almost died—and the surgeon needed to be revived.)

Despite these many difficulties, direct transfusion through an arteriovenous anastomosis for the first time efficiently transferred blood from one person to another. The process also disclosed fatal hemolytic reactions that were undeniably caused by transfusion<sup>41</sup> (Figure 1.6). However, the relation of these fatal reactions to Landsteiner's discovery was not recognized until Reuben Ottenberg (1882–1959) demonstrated the importance of compatibility testing.

Ottenberg's interest in transfusion began in 1906 while he was an intern at German (now Lenox Hill) Hospital in New York. There, Ottenberg learned of Landsteiner's discovery and in 1907 began pretransfusion compatibility testing.<sup>42</sup> Ottenberg accepted an appointment at Mount Sinai Hospital the next year and continued his studies on transfusion. In 1913, Ottenberg published the report that conclusively demonstrated the importance of preliminary blood testing for the prevention of transfusion "accidents"<sup>43</sup> (Figure 1.7). This was not Ottenberg's only contribution. He observed the Mendelian inheritance of blood groups,<sup>44</sup> and he was the first to recognize the relative unimportance of donor

## ACCIDENTS IN TRANSFUSION

THEIR PREVENTION BY PRELIMINARY BLOOD EXAMINATION: BASED ON AN EXPERIENCE OF ONE HUNDRED TWENTY-EIGHT TRANSFUSIONS \*

REUBEN OTTENBERG, M.D.

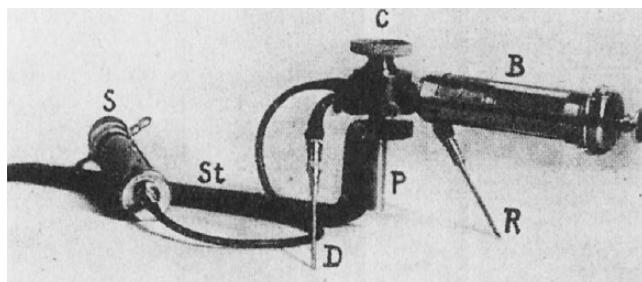
AND

DAVID J. KALISKI, M.D.

NEW YORK

Accidents following transfusion have been sufficiently frequent to make many medical men hesitate to advise transfusion, except in desperate cases. It has been our opinion since we began making observations on this question in 1908 that such accidents could be prevented by careful preliminary tests, leading to the exclusion of agglutinative or hemolytic donors. Our observations on over 125 cases have confirmed this view and we believe that untoward symptoms can be prevented with absolute certainty.

**Figure 1.7** Report of the importance of testing before transfusion. Source: Ottenberg and Kaliski (1913).<sup>43</sup> Figure 01, p 01 / With permission of American Medical Association.



**Figure 1.8** Apparatus for Unger's two-syringe, four-way stopcock method of indirect transfusion. Source: Unger (1915).<sup>49</sup> Figure 01, p 02 / With permission of American Medical Association.

antibodies and consequently the "universal" utility of type O blood donors.<sup>45</sup>

Further advances in immunohematology were to occur in succeeding decades. The M, N, and P systems were described in the period between 1927 and 1947.<sup>46</sup> The Rh system was discovered in connection with an unusual transfusion reaction. In 1939, Levine and Stetson<sup>47</sup> described an immediate reaction in a group O woman who had received her husband's group O blood soon after delivery of a stillborn fetus with erythroblastosis. This sequence of events suggested that the infant had inherited a red cell agglutininogen from the father that was foreign to the mother. At about the same time, Landsteiner and Wiener<sup>48</sup> harvested a rhesus monkey red cell antibody from immunized guinea pigs and rabbits. This antibody agglutinated 85% of human red cell samples (Rh-positive) and left 15% (Rh-negative) unaffected. When the experimentally induced antibody was tested in parallel with the serum from Levine's patient, a similar positive and negative distribution was observed, and the Rh system had been discovered. Other red cell antigen systems were subsequently described, but when Rh immunoglobulin was introduced as a preventive measure for hemolytic disease of the newborn, it became one of the major public health advances of the century.

Despite the introduction of compatibility testing by Ottenberg, transfusion could not be performed frequently as long as arteriovenous anastomosis remained the procedure of choice. Using this method, Ottenberg needed five years (Figure 1.7) to accumulate the 128 transfusions he reported in his study on pretransfusion testing.<sup>43</sup> New techniques, such as Unger's two-syringe method introduced in 1915<sup>49</sup> (Figure 1.8), eventually put an end to transfusion by means of arteriovenous anastomosis. However, transfusions did not become commonplace until anticoagulants were developed and direct methods of transfusion were rendered obsolete.

## Anticoagulants, the blood bank, and component therapy

The anticoagulant action of sodium citrate completely transformed the practice of transfusion. Early reports from Belgium<sup>50</sup> and Argentina<sup>51</sup> were followed by the work of Lewisohn<sup>52</sup> that established the optimal citrate concentration for anticoagulation. The work of Weil<sup>53</sup> then demonstrated the feasibility of refrigerated storage. Subsequently, Rous and Turner<sup>54</sup> developed the anticoagulant solution that was used during World War I.<sup>55</sup> Despite its very large volume, this solution remained the anticoagulant of choice until World War II, when Loutit and Mollison<sup>56</sup> developed an acid-citrate-dextrose (ACD) solution. Used in a ratio of 70 mL ACD to 450 mL blood, ACD provided 3–4 weeks of preservation of a more concentrated red

cell infusion. Thus, the two world wars were the stimuli for the development of citrate anticoagulants and the introduction of indirect transfusion.<sup>46</sup> For the first time, the donation process could be separated, in time and place, from the actual transfusion. Blood drawn and set aside now awaited the emergence of systems of storage and distribution. Again, it was the provision of medical support during armed conflict that stimulated these developments.

A blood transfusion service, organized by the Republican Army during the Spanish Civil War (1936–1939), collected 9000 L of blood in citrate-dextrose anticoagulant for the treatment of battle casualties.<sup>57</sup> At about that same time, Fantus<sup>58</sup> began operation of the first hospital blood bank at Cook County Hospital in Chicago. His interest had been stimulated by Yudin's report<sup>59</sup> on the use of cadaveric blood in Russia. Apart from certain scruples attached to the use of cadaveric blood, Fantus reasoned that a transfusion service based on such a limited source of supply would be impractical. Accordingly, he established the principle of a "blood bank" from which blood could be withdrawn, provided it had previously been deposited. As Fantus<sup>58</sup> himself stated, "[J]ust as one cannot draw money from a bank unless one has deposited some, so the blood preservation department cannot supply blood unless as much comes in as goes out. The term 'blood bank' is not a mere metaphor." The development of anticoagulants and the concept of blood banks provided an infrastructure upon which a more elaborate blood services organization could be built. World War II was the catalyst for these further developments.

At the beginning of World War II, blood procurement programs were greatly expanded.<sup>46</sup> In Great Britain, an efficient system had been developed through the organization of regional centers. When the war started, these centers, already in place, were able to increase their level of operation. In the United States, the use of plasma in the management of shock had led to the development of plasma collection facilities.<sup>60</sup> The efficient long-term storage of plasma had been further facilitated by the process of lyophilization developed by Flosdorf and Mudd and the introduction of ABO-independent "universal" plasma produced by pooling several thousand units of plasma.<sup>61</sup> In 1940, the United States organized a program for the collection of blood and the shipment of plasma to Europe. The American Red Cross, through its local chapters, participated in the project, which collected 13 million units by the end of the war.<sup>46</sup>

The national program of the American Red Cross ceased at the end of the war. However, many of the local chapters continued to help recruit donors for local blood banks, and in 1948, the first regional Red Cross blood center was begun in Rochester, New York. By 1949–1950 in the United States, the blood procurement system included 1500 hospital blood banks, 1100 of which performed all blood bank functions. There were 46 nonhospital blood banks and 31 Red Cross regional blood centers. By 1962, these numbers had grown to 4400 hospital blood banks, 123 nonhospital blood banks, and 55 American Red Cross regional blood centers, and the number of units collected had grown to between 5 and 6 million per year.<sup>62</sup>

During this time, blood was collected through steel needles and rubber tubing into rubber-stoppered bottles. After washing and resterilization, the materials were reused. On occasion, "vacuum bottles" were used to speed up the collection. However, the high incidence of pyrogenic reactions soon led to the development of disposable plastic blood collection equipment.

In a classic article written in 1952, Walter and Murphy<sup>63</sup> described a closed, gravity technique for whole blood preservation. They used a laminar flow phlebotomy needle, an interval donor tube, and a collapsible bag of polyvinyl resin designed so that the unit could be

assembled and ready for use after sterilization with steam. The polyvinyl resin was chemically inert to biologic fluids and nonirritating to tissue. Soon thereafter, Gibson *et al.*<sup>64</sup> demonstrated that plastic systems were more flexible and allowed removal of plasma after sedimentation or centrifugation. In time, glass was replaced with plastic, and component therapy began to emerge. This development was enhanced by the US military's need to reduce the weight and breakage of blood bottles during shipment in the Korean War.

Component and derivative therapy began during World War II, when Edwin J. Cohn and his collaborators developed the cold ethanol method of plasma fractionation.<sup>65</sup> As a result of their work, albumin, globulin, and fibrinogen became available for clinical use. As plastic equipment replaced glass, component separation became a more widespread practice, and the introduction of automated cell separators provided even greater capabilities in this area.

Clotting factor concentrates for the treatment of patients with hemophilia, and other hemorrhagic disorders were also developed during the postwar era. Although antihemophilic globulin had been described in 1937,<sup>66</sup> unconcentrated plasma was the only therapeutic material until Pool discovered that Factor VIII could be harvested in the cryoprecipitable fraction of blood.<sup>67</sup> This resulted in the development of cryoprecipitate, which was introduced in 1965 for the management of hemophilia. Pool showed that cryoprecipitate could be made in a closed-bag system and urged its harvest from as many donations as possible. The development of cryoprecipitate and other concentrates was the dawn of a golden age in the care of patients with hemophilia. Self-infusion programs, made possible by technologic advances in plasma fractionation, allowed early therapy and greatly reduced disability and unemployment. This golden age came abruptly to an end with the appearance of the AIDS virus.

### Transfusion in the age of technology

In contrast to the long ledger of lives lost in previous centuries because of the lack of blood, transfusion in the twentieth century saved countless lives. In 1937, during those early halcyon days of transfusion, Ottenberg wrote:<sup>40</sup>

Today transfusion has become so safe and so easy to do that it is seldom omitted in any case in which it may be of benefit. Indeed the chief problem it presents is the finding of the large sums of money needed for the professional donors who now provide most of the blood.

It is ironic that Ottenberg's statement should refer to paid donors and foreshadow difficulties yet to come. However, experience to that point had not revealed the problem of viral disease transmission. More transfusions would have to be administered before that problem would be perceived.

After the introduction of anticoagulants, blood transfusions were given in progressively increasing numbers. At Mount Sinai Hospital in New York, the number of blood transfusions administered between 1923 and 1953 increased 20-fold<sup>68,69</sup> (Table 1.1). This increase was particularly notable after the establishment of blood banks. It was during this period that Beeson wrote his classic description of transfusion-transmitted hepatitis.<sup>70</sup> He had been alerted to the problem by the outbreaks of jaundice that followed inoculation programs with human serum during World War II. Thus, blood transfusion entered a new era. Blood components not only saved lives but also transmitted disease. The discovery of the Australian antigen<sup>71</sup> and the subsequent definition of hepatitis A virus and B virus (HBV) still left residual non-A and non-B diseases,<sup>72</sup> a gap that has been largely filled by the discovery of the

**Table 1.1** Increase in the Number of Blood Transfusions at Mount Sinai Hospital, New York, 1923–1953

Year	Number of Transfusions
1923	143
1932	477
1935	794
1938	(Blood bank started)
1941	2097
1952	2874
1953	3179

Source: Lewisohn (1955).<sup>68</sup> Reprinted with permission of Elsevier.

hepatitis C virus (HCV).<sup>73</sup> However, it was the outbreak of AIDS that galvanized public attention to blood transfusion.

The AIDS epidemic was first recognized in the United States, and the first case of AIDS associated with transfusion was observed in a 20-month-old infant.<sup>74</sup> Subsequently, the suspicion that AIDS could be transmitted by means of transfusion was confirmed.<sup>75</sup>

The human immunodeficiency virus (HIV) was identified,<sup>76</sup> and an effective test to detect the HIV antibody was developed.<sup>76</sup>

## Concern for blood safety

Since 1943, transfusion therapy has been shadowed by the specter of disease transmission. In that year, Beeson described post-transfusion hepatitis and unveiled a problem that has grown with time. As transfusion increased, so did disease transmission. In 1962, the connection between paid donations and post-transfusion hepatitis was made.<sup>77</sup> A decade later, the National Blood Policy mandated a voluntary donation system in the United States. And yet, blood usage continued to increase.

Concern about post-transfusion hepatitis was not sufficient to decrease the number of transfusions. Although the use of whole blood declined as blood components became more popular, total blood use in the United States doubled between 1971 and 1980

(Table 1.2).<sup>78–85</sup> This pattern changed as the emergence of AIDS exposed all segments of society to a revealing light.

AIDS probably arose in Africa in the 1960s and spread quietly for years before it was detected. By 1980, an estimated 100,000 persons were infected, and by 1981, when the first cases were reported, a worldwide pandemic lay just beneath the surface. The initial response of the public and officials seemed trifling and insufficient as the outbreak grew to proportions few had foreseen. Criticism was levied against the news media for initially ignoring the story, the government for delay in acknowledging the problem, gay civil rights groups for resistance to epidemiologic measures, research scientists for unseemly competition, blood services for delayed response in a time of crisis, and the US Food and Drug Administration (FDA) for inadequate regulatory activity. Historians with the perspective of time will determine whether there really were more villains than the virus itself.<sup>89</sup>

The realization that transfusion can transmit an almost invariably fatal disease had a chilling effect on the public. Two major changes in blood services have occurred in the aftermath of the AIDS epidemic. The FDA, using pharmaceutical manufacturing criteria not “tailored to . . . blood banks,” became more aggressive in regulatory actions against blood collection establishments.<sup>90</sup> And, finally, blood use moderated for approximately 10 years. Through the 1980s and early 1990s, red cell and plasma transfusion peaked and began to stabilize (Table 1.2). Only platelet use and human progenitor cell transplantation, driven by the demands of cancer chemotherapy, continued to increase.<sup>78–85</sup> Educational programs to encourage judicious use of blood have been initiated, and they have been favorably received by practicing physicians. Use of red cells and plasma fell from 2008 to 2011. This was a combined impact of the great recession reducing healthcare utilization and the widespread use of patient blood management programs intended to reduce blood transfusion. This represents the first time since the end of the Second World War that the growth in transfusion of blood products in the United States and other developed countries stopped and was reversed for a sustained period.

Relentless public pressure for a “zero-risk” blood supply resulted in dividends through continued scientific and technologic improvements. Enhanced sensitivity and better use of serologic testing, along with improved scrutiny of donors, resulted in major reductions in risk of transmitted disease by the mid-1990s.<sup>91</sup> Discovery that pools of units subjected to nucleic acid testing almost closed the window for HIV and HCV virus resulted in the application of this testing for both whole blood and plasma donations beginning between 1998 and 2000.<sup>91,92</sup> This, combined with virus reduction and inactivation of the final product, resulted in plasma derivatives that have not transmitted AIDS or hepatitis since 1994.<sup>93</sup> For whole blood and platelet components, risks have become low. Pathogen reduction has driven the risks lower for plasma and platelets, and very low for plasma-derived therapies such as immune globulin.

With the reduction in the risk of viral transmission, the focus in the developed world has shifted to transfusion-related acute lung injury (TRALI)—possibly from recipient-directed leukocyte antibodies and lipid mediators in transfused plasma—and bacterial infection primarily occurring in room-temperature stored platelets. In order to make incremental gains against these risks, the use of male plasma only and the culture of platelets before they are released have been implemented. The 2011 blood utilization survey indicated a 28.8% reduction in TRALI, suggesting a significant breakthrough with regard to this risk.<sup>85</sup> Geographic exclusions have been aimed at reducing the potential for variant

**Table 1.2** Transfusions in the United States (in Millions of Units)<sup>78–88</sup>

Year	Whole Blood and Red Blood Cells	Platelets	Plasma
1971	6.32	0.41	0.18
1979	9.47	2.22	1.29
1980	9.99	3.19	1.54
1982	11.47	4.18	1.95
1984	11.98	5.53	2.26
1986	12.16	6.30	2.18
1987	11.61	6.38	2.06
1989	12.06	7.26	2.16
1992	11.31	8.33	2.26
1994	11.11	7.87	2.62
1997	11.52	9.04	3.32
1999	12.39	9.05	3.32
2001	13.90	10.20	3.93
2004	14.18	9.88	4.09
2006	14.65	10.39	4.01
2008	15.02	2.28*	4.48
2011	13.79	2.17	3.88
2013	13.40	2.17	3.62
2015	11.35	1.98	2.72
2017	10.65	1.94	2.37

\* Platelets reported in apheresis units in this and subsequent years.

Source: Adapted from Surgenor and Schnitzer (1985)<sup>78</sup>; Surgenor et al. (1998)<sup>79</sup>; Wallace et al. (1989)<sup>80</sup>; Wallace et al. (1990)<sup>81</sup>; Sullivan et al. (2002)<sup>82</sup>; Sullivan et al. (2005)<sup>83</sup>; US Department of Health and Human Services (2008)<sup>84</sup>; AABB (2012)<sup>85</sup>; Chung et al. (2016)<sup>86</sup>; Ellingson et al. (2017)<sup>87</sup>; Jones et al. (2020)<sup>88</sup>.

Creutzfeldt–Jakob disease (vCJD) transmission by transfusion, although in the United States such occurrence seems highly unlikely. In many countries, universal leukocyte reduction has been a response to the vCJD risk. Ironically, universal application of leukocyte reduction is probably ineffective for vCJD but has stimulated controversy over its use for preventing other problems.<sup>94</sup>

Finally, focus on the understanding, management, and prevention of medical errors in general might lead to progress against remaining nemesis hemolytic transfusion reactions caused by mis-transfusion. Bar code technology at the bedside, commonly applied to prevent errors in medication administration, has shown efficacy in reducing transfusion errors.<sup>95</sup> Radiofrequency identification shows further promise in error-prone situations such as operating rooms.<sup>96</sup> Transfusion safety officers and hemovigilance systems are other initiatives that have been instituted.

“Zero risk” has still not been achieved. Emerging global infections such as West Nile virus, Chagas disease, and Chikungunya virus remain future potential threats and have encouraged further test development and implementation of strategies for pathogen reduction.

## Current status

As shown in Figure 1.9, there is a complex sequence of events that occurs today between recruitment of the donor and the final hemovigilance and quality reviews. In the developed world, increasing sophistication of methods to ensure safety and availability increased the cost of blood components to hospitals up to 2013 when a leukocyte-reduced red cell in the United States cost hospitals a mean of \$228, an apheresis platelet unit \$557, a unit of fresh frozen plasma \$63.<sup>98</sup> Blood usage, as measured by a national utilization study, peaked in 2008 before the financial crisis. It then began a steady, slow decline. The latest survey available of the 2017 utilization suggested the decline had slowed.<sup>88</sup> During this time, pathogen reduction began to be introduced. In addition, new data suggested cold-stored platelets might be superior to room-temperature stored platelets in some situations because of better immediate function, and whole blood became preferred by some centers for trauma and other massively bleeding patients. This was a “back to the 1970s” scenario. Thus, new complexities were introduced into the blood operations which had simplified in the 1980s and 1990s to component preparation to red cells and plasma for either transfusion or further manufacture into derivatives with platelets predominantly



**Figure 1.9** The blood donation and transfusion chain: from donor arm to patient vein. The bold arrows outline nine important stages in the system that ensures safe and effective patient blood transfusions. Graphic design by Kimberly E. Crookston. Source: Crookston et al. (2015).<sup>97</sup> Reproduced with permission of Lippincott, Williams & Wilkins.

from apheresis. It is not clear where blood utilization is headed in a postpandemic world.

In the developed world, no cost has usually been spared in meeting public demands for blood safety. In the underdeveloped world, the picture is quite different. The greatest blood need is for women hemorrhaging during childbirth, infants and children with anemia caused by malaria, and victims of trauma. In 80 of 172 countries responding to a World Health Organization (WHO) survey, less than 1% of the population donate blood. In sub-Saharan Africa, fewer than 3 million units of blood are collected annually for a population of more than 700 million. Of the 148 countries reporting data to WHO, 41 are unable to screen for minimum safety (HIV, HBV, HCV, and syphilis). WHO estimates that unsafe blood in these countries results in 16 million new infections with HBV, 5 million with HCV, and 160,000 with HIV each year (accounting for 5–10% of the world's HIV infections). Fortunately, there is progress in some nations in achieving an all-volunteer supply and minimum screening. Thus, there are two drastically different pictures of blood safety and availability worldwide.<sup>99</sup>

## Organization of blood services in the United States

Blood collection for transfusion in the United States is accomplished by an eclectic system that has evolved since World War II. Close to half of the blood for transfusion in the United States has historically been collected by the American Red Cross ([www.redcross.org](http://www.redcross.org)). Community blood centers were established nationwide in areas where the Red Cross did not operate. Most of these non-profit collection agencies are now loosely affiliated with a trade organization known as America's Blood Centers. In recent years, consolidation among regional blood centers has been driven by the impact of reduced transfusions on the blood center business model. This has added several larger national blood programs independent of the American Red Cross such as Vitalant, Versiti, New York Blood Center Enterprises, and OneBlood.

Many blood centers are affiliated with national blood banking organizations, including AABB (founded in 1947—see [www.aabb.org](http://www.aabb.org)). The “voluntary” standards set by the AABB are so well regarded that these often become the standard of care in North America and in other areas of the world, even without government regulatory intervention.

Many hospitals have found it impractical to maintain hospital-based collections. Although hospital-based collection still exists in the United States, the increasing cost, external regulation, shortage of medical technologists, and variable supply and demand have progressively shifted collection activities to blood centers. However, the proportion of blood collected by hospitals in 2011 remained relatively constant when compared to 2004 (6.6 vs 6.4%, respectively).<sup>100,101</sup> Even hospitals that collect their own blood usually do not perform donor testing, and out of necessity they maintain a supplemental blood contract with the local blood supplier. The US Department of Defense continues to operate an independent blood services organization for its hospitals as well as its military mission.

Blood components collected for transfusion in the United States are from volunteer donors; in contrast, most plasma for further manufacture into plasma-derived therapies also known as plasma-derived medicinal products (PDMPs) is collected from paid donors in commercial centers.

## Organization of blood services outside the United States

Most high-income countries have adequate blood services, maintaining a full range of donor screening and quality assurance procedures, while producing most of the components needed in the region. However, there exist a wide variety of dissimilar organizational structures that are successful in meeting the transfusion needs of areas served. These models can be centralized or decentralized, governmental, military, private, hospital based, or mixed. For the most part, the local transfusion system reflects the administrative system of each country. For instance, in countries that have strong governmental control, there is often a centralized transfusion system operated by government-run institutions. The World Health Organization (WHO) encourages strong governmental leadership in establishing national transfusion networks.<sup>102</sup> Many countries outside the United States have instituted national blood programs. Overall, the provision of blood services worldwide is heterogeneous and varies with historical development, the socioeconomic development of the region, and the influence of national scientific and political factors.<sup>102</sup>

Donor recruitment is sometimes performed externally to the blood collection agencies. Recruitment is sometimes carried out by independent organizations that have a special relationship with the procurement agency, such as a country's national Red Cross. The International Federation of Blood Donor Organizations was established in 1955 as a support network for donor recruitment ([www.fiods-ifbdo.org](http://www.fiods-ifbdo.org)). Seventy-two nations participate, although there is a notable absence of many English-speaking countries. The goal of the organization is member state self-sufficiency in blood from voluntary, unpaid blood donors, while also improving safety and, in turn, confidence in the national blood supplies by developing minimum standards for donations, inspection, and quality assurance.

In 1975, the 28th World Health Assembly passed a resolution recognizing the value of voluntary blood donation and called on member states to promote national blood transfusion services based on voluntary unpaid donations.<sup>102</sup> Voluntary donation has been a goal for some time as it is perceived as a safer alternative. In 2002, the European Union approved legislation that established comprehensive standards for blood products that included a requirement for voluntary donation.<sup>102</sup> The WHO Global Database on Blood Safety 2011 report presents data from 164 countries representing 92% of the global population.<sup>103</sup> It shows that half of the global blood collection occurs in high-income countries, home to only 15% of the world population. Global blood donations from voluntary unpaid donors increased from 2004 to 2012. Seventy-three countries collect over 90% of their blood supply from voluntary unpaid blood donors. However, 72 countries collect more than 50% of their blood supply from family/replacement or paid donors. Around 1.5 million donations intended for transfusion were collected from paid donors.<sup>104</sup> Nevertheless, countries are increasingly moving toward voluntary blood donation.

WHO recommends that, at minimum, all blood for transfusion should be screened for HIV, hepatitis B, hepatitis C, and syphilis. Twenty-five countries are not able to screen all donated blood for one or more of these infections. Irregular supply of test kits is one of the most commonly reported barriers to screening.<sup>100</sup> Yet, even in countries that are able to screen all of their blood, the risk of transfusion-transmitted infections (TTIs) varies. The prevalence of TTIs in blood donations in high-income countries is considerably lower than in low- and middle-income countries.<sup>4</sup> External quality control assessment is often lacking: whereas 97% of blood-screening

laboratories in high-income countries are monitored, only 33% of middle-income and 16% of low-income countries have external quality assessment.<sup>104</sup>

There is still heterogeneity in the practices that are taken for granted as mandatory in North America, even among the most effective blood services. For instance, in Norway, nucleic acid testing for HIV is not performed on repeat blood donors due to the low incidence in the recruited donor population and the effectiveness of the serological screening.

Low- and middle-income countries often struggle to provide the same level of safety as high-income countries with established blood procurement systems. The costs of procurement and testing of the blood are often prohibitive. For instance, in sub-Saharan Africa, although blood transfusion has a long history,<sup>105</sup> collection services are fragmented. Blood is sometimes in short supply, and safety seldom can be guaranteed.<sup>4</sup>

Some high-income countries such as the United States have implemented a “precautionary principle” that has meant extreme costs for marginal added safety when measured by standards such as quality-adjusted life-years.<sup>106</sup> Many countries simply do not have resources to implement this strategy, even if it were medically justified. In stark contrast to the precautionary principle, blood in some parts of the world is still transfused without testing. In addition, when testing can be done, there may not be resources to notify and counsel donors about positive test results, such as HIV. This provides an opportunity for the world community to make a great impact on global health through collaboration and collegiality. In response, WHO established the Global Collaboration for Blood Safety in 1994, a voluntary partnership of organizations, institutions, associations, agencies, and experts that are concerned with the safety and availability of blood. Many of these functions are now carried out by the WHO Global Blood Safety Network and the WHO Global Forum for Blood Safety (<http://www.who.int/bloodsafety/collaboration/en/>).

Even in the developed world, availability has been a challenge in the past.<sup>107</sup> A clear need is for more group O red cells; populations of non-European ethnicity generally have an increased proportion of group O.<sup>108</sup>

Another approach to maintain adequate availability is to control usage by ensuring that blood is used appropriately. Some US blood centers have been successful in bringing their transfusion medicine expertise into the patient care setting by providing transfusion services to hospitals. The model in Seattle, Washington, has operated for decades.<sup>109,110</sup> In the United Kingdom, liaison systems for blood centers to hospitals employing web-based technology for supply chain management have been introduced.<sup>111,112</sup> In Denmark, success has been reported using the Thromboelastograph (Haemoscope, Niles, IL) hemostatic system to manage coagulopathy in conjunction with treating physicians—something also done in many US hospitals.<sup>113</sup> Other point-of-care tests to assess the state of the coagulation system and tissue oxygenation could also result in more accurately targeted component transfusion. Transfusion medicine specialists in hospitals—whether from pathology groups, blood center staff, or other areas such as anesthesiology or hematology—are critical to the successful use of blood transfusion in patient care. Today, patient blood management has become a new standard for assuring transfusion as indicated based on best evidence. This has significantly reduced blood utilization in developed countries and has been supported by indications that restrictive blood transfusion strategy does not impair outcomes in most cases, although certain subsets of patients such as cancer patients might require a higher

hemoglobin threshold.<sup>114,115</sup> At the same time, the treatment of trauma is returning to what is in effect whole blood for massive bleeding by administering plasma and platelets in proportion to red cells early in the care of victims.<sup>116</sup>

Although some advances in transfusion medicine at the end of the last millennium have been difficult to implement, such as the use of hemoglobin solutions, the field has continued to advance into new areas of stem cell biology, regenerative medicine, and cord blood banking. In addition, transfusion medicine specialists increasingly function in collaboration with surgeons, oncologists, and hematologists in treating the acutely ill patient with complex medical problems. With all the added sophistication, the optimal hemoglobin and platelet triggers and endpoints for transfusion remain unsettled. Clinicians are less likely to use oxygenation transport endpoints to determine the need for red cell transfusion but are beginning to look for other means to assess tissue oxygenation. If a patient's hemoglobin is too high (even when below normal), complications such as thromboembolism can result. Too low an endpoint exposes some patients to the risk of tissue hypoxia. The clearest trend has been away from autologous transfusion, although some medical centers seek bloodless medicine and surgery combining pharmacotherapy (mainly erythropoietin and iron), blood recovery and reinfusion, and conservative triggers and endpoints.<sup>117</sup> More conservative triggers and endpoints for platelet transfusion are becoming accepted, but approaches to alloimmunized patients and bacterial contamination are still in question.<sup>118</sup> Also debatable is whether transfusion, through some poorly quantifiable mechanism such as immunomodulation, confers a poorer prognosis on acutely ill patients.<sup>114</sup>

The COVID-19 pandemic challenged the blood supply in the United States and throughout the world. Blood centers adjusted by increasing collection of convalescent plasma and decreasing other collections to match reduced demand for routine transfusions. As the developed world began to exit the pandemic, demand for convalescent plasma was replaced by demand for routine transfusions leading to shortages. The emergence from the pandemic thus revealed issues with blood supply chain, as it did for other needs. An analysis in the United States suggests the need for stronger national policies, attention to structural issues in the supply chain, and better integration with national and global emergency preparedness efforts.<sup>119</sup>

From ancient times into the new millennium, blood has been a substance that fascinates mankind. Despite unresolved controversies, blood transfusion remains of critical importance in the care of sick patients throughout the world.

## Disclaimer

The authors have disclosed no conflicts of interest.

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## CHAPTER 2

# Disasters and the blood community (including COVID-19)

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This chapter is an attempt to explain and link the reader to the resources available to guide the development of an organization wide disaster preparedness process. As such, it is not all inclusive. Given the recent history of abundant catastrophes ranging from earthquakes and tsunamis, extreme weather events and most recently the COVID-19 pandemic, most facilities should already have undertaken this activity, and this information may function as a checklist to be sure that critical elements have all been addressed. Issues presented by the COVID-19 pandemic will be discussed as well.

Disaster preparedness, emergency response, and disaster risk reduction are all terms referring to the process of preparing for and responding to a disaster. These are defined by the World Health Organization as “an occurrence that disrupts the normal functioning of a community or society and causes a level of suffering (human, material, economic, or environmental) that exceeds the capacity of adjustment of the affected community.”<sup>1</sup> Disasters vary in size, and what is a disaster in one area may not be in another. A blood collection facility in Iowa’s tornado alley will have a different list of priorities to confront than one in the hurricane prone Gulf Coast locale or in California near the San Andreas fault. Disasters can be natural events such as earthquakes, weather related like hurricanes or tornadoes or man-made, for example, bombs, industrial accidents, or mass shootings. They can be internal to an organization, the crash of critical IT infrastructure, or external, responding to a mass shooting incident in a center’s or hospital’s service region. Anything that overwhelms local capacity can be considered a disaster.<sup>2</sup>

Management focus has grown from only responding when an event happens to reducing the potential for and impact of disasters. Using a process approach, disaster management addresses the entire cycle from planning, response, and recovery followed by learning from the experience and modifying plans accordingly. We will discuss strategies herein, but tactical and procedural resources relevant to the blood community can be found in the AABB Disaster Operations Handbook.<sup>3</sup>

Transfusion services rarely stand-alone but are generally a component of a hospital or medical complex. As such, transfusion services do not generally develop disaster plans in isolation, rather they contribute to the overall facility’s planning activities. Regardless, it is critical to ensure blood needs, and support are included in all disaster-planning activities.

## Background

Disaster management encompasses pre-event planning, response, and recovery phases. Planning is by nature iterative with improvement dependent on simulations and especially on learning from prior events.<sup>4</sup> The cycle of disaster management includes four functional areas: mitigation, preparedness, response, and recovery. Each should be included in an organization’s disaster planning activities.

*Mitigation* efforts are those that prospectively limit the damage an unpredictable event can cause. Accordingly, preparation for disasters begins with a thorough risk assessment.<sup>5</sup> Risk is the potential for losses (physical, economic, and social) associated with a hazard and can be defined in terms of estimated probability and frequency, causative factors, and locations or areas affected. Such a risk assessment serves to identify the known risks to and vulnerabilities of one’s physical plant, personnel, and operations, and evaluates the likelihood and damage potentials for each threat. Potential internal disasters include fires, flooding from burst water pipes, workplace violence, and hazardous spills. External includes earthquakes, wild-fires, pandemics, hurricanes, and mass casualty incidents. Resources are available to assist in identifying known risks and threats in various countries around the world and can be used in this endeavor.<sup>3,6</sup>

Risks should be eliminated or controlled where possible. This may include adjustments to plant and property design. These initiatives range from site locations to building or retrofitting physical structures to protect employees and assets with redesigns to evolving building codes, flood plain analyses, storm shelters, redundant data pathways, and communications systems. Smaller actions can reduce loss or injury (e.g., securing bookshelves or file cabinets to a wall). All such interventions can contribute to the protection of both physical resources and human capital.

*Preparedness* addresses risks that cannot be minimized by mitigation alone through planning and testing those plans. These efforts should focus on people, assets, and organizational systems that can be affected by different threats. Plans should include actions required if an event directly affects the organization as well as responses needed in support of disasters in the local/regional area. How an organization responds to a mass shooting within the facility versus one in the local area are different and plans must account for both. Finally, plans are words on paper, and staff must be trained on them with simulations and drills. It is the reinforcement from

repetition that hones leader's and staff's ability to think and react quickly during unexpected occurrences during a disaster.

*Response* occurs during the event(s) and is essentially putting predeveloped plans into effect and adjusting the plan to facts on the ground. These are time-sensitive action steps taken by the staff to protect life and property, ensure ongoing safety, and to stabilize operations. These will always involve communication with leadership, staff, "customers" (broadly defined) and often the media, and operational adjustments to maintain the ability to collect process, distribute, and transfuse blood products.

Effective response strategies center on a formal incident command process<sup>7</sup> using defined processes and procedures under explicitly assigned scopes of responsibility based on subject matter expertise. Clear lines of authority are obviously needed. These should be practiced during drills and suitable for immediate implementation any time of the day or night. Written checklists drafted using the outputs of the risk assessment process are an example of "job aids" that can be useful for an incident command group to avoid missing important tasks during simulations or actual disaster responses when time may be of the essence. Such documents were prepared at AABB (formerly the American Association of Blood Banks) starting in 2005 for an anticipated influenza pandemic and are available for modification.<sup>8</sup>

Recovery refers to efforts to return toward normal operations and to evaluate the response for lessons learned and begins after the initial response has been initiated. Restoring and preserving critical systems to maintain business operations (e.g., communications, water, power, sewage, and data) must be prioritized. Once systems and normal operations are restored, the final step of the disaster management cycle begins with an honest, thorough analysis of the event, actions taken, and challenges encountered. Such critical self-review is imperative to learn lessons that

will allow organizations to prevent recurrences and improve future disaster responses.

An important detail in disaster response is the contemporaneous recording of all actions and decisions made as events unfold to enable accurate reconstruction of the real-time evolution of a disaster in the future. It is virtually impossible to remember after the fact all details that occur in the rapidly changing environment of an unfolding catastrophe. While everyone should be encouraged to keep notes and all notes made should be retained and used in the reconstruction of the event, organizations should assign recording responsibility to ensure completion of the task. This will assist in after action reporting, analysis, and identifying lessons learned.

## Organization for emergency operations

National governmental authorities generally have agencies responsible for the coordination of disaster planning and response in their respective countries. Important exemplars of international sources of information and training on disaster planning and emergency operations are provided in Table 2.1.

It is important for incident command staff to understand where their organization fits in the disaster management framework among the multiple levels of governmental and nongovernmental organizations (NGOs) to ensure effective coordination during an event or drill.

In the United States, the Department of Homeland Security (DHS) and its Federal Emergency Management Agency (FEMA) are responsible for developing rules and guidelines for responding to emergencies and coordinating any national response when necessary. DHS and FEMA get their statutory authority to respond to disasters from the Robert T. Stafford Disaster Relief and Emergency Assistance Act (the "Stafford Act") signed into law in 1988.<sup>9</sup>

**Table 2.1** Resources to Assist in Development and Improvement of Local Plans

Organization	Website
European Union (EU)—Disaster Preparedness	<a href="https://ec.europa.eu/echo/what/humanitarian-aid/disaster_preparedness_en">https://ec.europa.eu/echo/what/humanitarian-aid/disaster_preparedness_en</a>
United Nations (UN) Office for Disaster Risk Reduction—Sendai Framework for Disaster Risk Reduction	<a href="https://www.undrr.org/implementing-sendai-framework/what-sendai-framework">https://www.undrr.org/implementing-sendai-framework/what-sendai-framework</a>
International Federation of the Red Cross and Red Crescent Societies (IFRC)	<a href="https://www.ifrc.org/en/what-we-do/disaster-management/about-disasters/what-is-a-disaster/">https://www.ifrc.org/en/what-we-do/disaster-management/about-disasters/what-is-a-disaster/</a>
Asian Preparedness Partnership (APP)	<a href="https://www.adpc.net/igo/Default.asp#">https://www.adpc.net/igo/Default.asp#</a>
Australia Queensland Government	<a href="https://www.disaster.qld.gov.au/Pages/default.aspx">https://www.disaster.qld.gov.au/Pages/default.aspx</a>
United Kingdom Guidance: Emergency response and recovery	<a href="https://www.gov.uk/guidance/emergency-response-and-recovery">https://www.gov.uk/guidance/emergency-response-and-recovery</a>
National Institute of Disaster Management—Ministry of Home Affairs, Government of India	<a href="https://nidm.gov.in">https://nidm.gov.in</a>
United States—Department of Homeland Security's Federal Emergency Management Agency (FEMA)	<a href="https://www.fema.gov/">https://www.fema.gov/</a>
United Kingdom National Health Service—Clinical guidelines for major incidents and mass casualty events	<a href="https://www.england.nhs.uk/publication/clinical-guidelines-for-major-incidents-and-mass-casualty-events/">https://www.england.nhs.uk/publication/clinical-guidelines-for-major-incidents-and-mass-casualty-events/</a> <a href="https://www.premiersafetyinstitute.org/safety-topics-az/emergency-preparedness/emergency-preparedness/">https://www.premiersafetyinstitute.org/safety-topics-az/emergency-preparedness/emergency-preparedness/</a>
Premier Safety Institute—Emergency Preparedness for Healthcare	<a href="https://www.who.int/hac/techguidance/preparedness/health-emergency-and-disaster-risk-management-framework-eng.pdf?ua=1">https://www.who.int/hac/techguidance/preparedness/health-emergency-and-disaster-risk-management-framework-eng.pdf?ua=1</a> <a href="https://www.calhospitalprepare.org/">https://www.calhospitalprepare.org/</a>
World Health Organization (WHO) Health Emergency and Disaster Risk Management Framework	<a href="https://www.cdc.gov/cpr/index.htm">https://www.cdc.gov/cpr/index.htm</a>
California Hospital Association—Emergency Preparedness Preparing Hospitals for Disasters	<a href="https://www.cdc.gov/niosh/topics/healthcare/emergency.html">https://www.cdc.gov/niosh/topics/healthcare/emergency.html</a>
U.S. Centers for Disease Control and Prevention (CDC) Public Health Center for Preparedness and Response	<a href="https://www.cdc.gov/nchb/hsb/disaster/Facilitator_Guide.pdf">https://www.cdc.gov/nchb/hsb/disaster/Facilitator_Guide.pdf</a> <a href="https://emedicine.medscape.com/article/765495-overview#a1">https://emedicine.medscape.com/article/765495-overview#a1</a>
CDC National Institute for Occupational Safety and Health (NIOSH) Emergency Preparedness and Response	<a href="https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5300000/">https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5300000/</a>
CDC Disaster Preparedness and Response Training—Complete Course	<a href="https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5300000/">https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5300000/</a>
Medscape—Disaster Planning	<a href="https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5300000/">https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5300000/</a>
FEMA Ready Planning Resources	<a href="https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5300000/">https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5300000/</a>
Center for Risk Communication	<a href="https://centerforriskcommunication.org/">http://centerforriskcommunication.org/</a>
CDC Crisis and Emergency Risk Communication	<a href="https://emergency.cdc.gov/cerc/">https://emergency.cdc.gov/cerc/</a>
American Red Cross How to Prepare for Emergencies	<a href="https://www.redcross.org/get-help/how-to-prepare-for-emergencies.html">https://www.redcross.org/get-help/how-to-prepare-for-emergencies.html</a>

The National Response Framework (NRF) “is a guide to how the nation responds to all types of disasters and emergencies. It is built on scalable, flexible, and adaptable concepts that align key roles and responsibilities.<sup>10</sup> The NRF is composed of 15 Emergency Support Functions (ESF) that group resources into functional areas. Blood is specifically covered under the medical and public health section.<sup>11</sup> Blood collection and hospital facilities must be familiar with the language contained in the NRF in order to request and receive assistance during major disaster declarations.

The AABB Interorganizational Task Force on Domestic Disasters and Acts of Terrorism (Task Force) was formed in 2002 to provide specific support to blood collection and transfusion facilities during major emergencies integrating with the NRF and other cross-disciplinary systems. Blood centers and hospital facilities should review the AABB Disaster Operations Handbook and be familiar with how to contact the Task Force during emergencies.<sup>3</sup>

Disasters are inherently *local* events requiring individuals, local organizations, NGOs, and authorities to work together. The lynchpin to any disaster response will be local emergency management agencies (EMAs) that include public health, police, fire, and ambulance services that will coordinate the response. State, territorial, and national support systems are in place to support local plans, with development and training and with response when localities are overwhelmed. It is imperative that blood centers and transfusion services be familiar with and participate with local EMAs as blood support is key to minimizing morbidity and mortality of injuries.

## Planning for blood and transfusion in disasters

Blood collection and distribution systems vary by country, some centralized, others dispersed. In the United States, blood is collected in regional blood centers where it is tested, processed, labeled, and stored. Other countries centralize different aspects of the process. The process can take anywhere from 24 to 72 hours to complete depending on national regulations and local operations. Blood is then distributed to intermediate hubs or directly to hospitals for use in patient care. Complex distribution systems heavily rely on robust communication and information systems, transportation and logistics support, and critical utilities such as fuel and electricity. EMA personnel are generally not familiar with how blood collection, processing, and distribution work, and often make assumptions that blood is always available. It is incumbent upon blood center and hospital transfusion service personnel to continually educate EMAs on how the local blood supply works, the average general inventory on hand, and what is required for resupply.

Most mass casualty events and disasters do not seriously stress blood supplies or distribution systems.<sup>12</sup> Needs are met via established inventories and relationships with regional and national colleagues. Those relationships need to be established before an event occurs, but organizations like the AABB task force can facilitate the availability of blood products needed, including urgent transportation requirements where disruptions to usual routes have occurred.

The public/donor response to disasters can be overwhelming and if not controlled can lead to overcollection, the eventual outdate of some blood products, and medium-term donor unavailability while they are deferred awaiting the passage of minimum interdonation intervals.<sup>13</sup> Controlling this generous and understandable donor response is difficult but can benefit from having template messages ready in advance and disseminated early via all

available routes, including and especially social media. Securing the cooperation of EMAs and the media in their dissemination is pivotal. Unified messages about need, or lack thereof, at all levels are critical (local, regional, and national) and should address some minimum elements including a situation report regarding supply and demand with a clear “ask”—e.g., delay donation to the recovery period or a “donate now” ask that specifies what is needed, where and when.

Disasters from the use of biologic, chemical, or nuclear agents may result in widespread donor deferrals and in the potential quarantine of blood components already in the manufacturing process or storage until the nature and effect of the agent can be determined. Radiologic attacks may dramatically increase the demand for platelets, other blood products, or stem cells for weeks or months following an event.

The COVID pandemic saw immediate decreases in blood use as elective medical care was delayed, followed by excess demand when care returned suddenly toward normal at the end of the first wave in the US (Personal communication from Basavaraju S, CDC Office of Blood Organs and Other tissue Safety, March 8, 2021).

Staff absenteeism and donor shortages develop as individuals are unable to travel to work, become ill, need to stay home to take care of loved ones, face quarantine, or fear exposure to a pathogen in public places. Careful consideration of who can and how they will work from home is critical. Those who must be physically present to collect, process, and distribute blood must be made to feel secure at work. At collection facilities during the SARS-CoV-2 pandemic, many clerical and administrative tasks were transitioned to remote systems. A variety of physical interventions recommended by public health, including physical and social distancing, and installing or making available physical barriers and personal protective equipment both for staff and donors, were adapted to collection and manufacturing operations, and to transfusion service environments.

These same considerations may disrupt just-in-time supply chains and the availability of critical supplies as manufacturers face the same difficulties as collection facilities and hospitals.<sup>14</sup> Due diligence during the selection and qualification of vendors of critical supplies requires careful consideration of their disaster planning in support of an uninterrupted flow of goods. This includes, importantly, the companies that develop, install, maintain, and upgrade the information technology resources the blood community relies upon in the highly regulated environment of transfusion medicine, up to and including complete duplication of all applications and archives at a “hardened” remote site.

Hospitals and collection facilities need crisis standards of care that include blood triage to optimize blood use during a severe shortage.<sup>15</sup> It is critical that these be sufficiently detailed to apply both conservative transfusion thresholds for multiple products and clinical scenarios and decisions to curtail blood-intensive nonurgent services and to allocate scarce components at need, be available, and regularly reviewed in advance of a severe disaster. Blood centers should work with their hospital customers to ensure that they have triage plans in place to avoid making collection facility medical personnel, remote from the bedside, responsible for rationing. The Minnesota Department of Health has developed an exemplary menu of materials for hospitals preparing to respond to disasters. They include clinical guidelines that will be useful to assist healthcare facilities in planning for events that would overwhelm the system’s resources, including blood and blood components.<sup>16</sup>

Certain disasters may affect a blood collector's ability to collect and process blood before, during, and after an event. For instance, as a hurricane approaches, a blood collector may suspend collections for a few days before and after the storm has passed, losing expected collections. Elective surgeries typically are postponed during the same period, alleviating the strain on blood availability. However, lessons learned from previous disasters indicate that blood collection and transfusion facilities should prepare for the potential acute shortage of blood components in the days following a hurricane-type event, with special attention given to the supply of platelets. Accordingly, collection facilities should work to augment supplies before a predictable hazard (e.g., hurricane or severe winter storm) and plan for a managed donor surge as the threat recedes. The AABB Disaster Task Force or analogous organizations can provide for support if routine supply channels are inadequate to bolster supplies.

## Continuity of operations plans

Disaster planning is an essential element of a successful business.<sup>17</sup> Accrediting organizations, such as AABB, the College of American Pathologists (CAP), and The Joint Commission (TJC), require participating organizations to have an emergency plan. Planning for responding to a disaster is an iterative process. Plans can have various names, but one commonly accepted term is a continuity of operations plan (COOP). Such a plan is best developed by a cross-functional team representing the major areas within the organization. Coordination with key suppliers and customers is essential to ensure their plans integrate with those of the planning entity. Hospital-based blood bank and transfusion service planning may be subsumed in an overall hospital or department effort but must be reviewed by subject matter experts to be certain that issues specific to blood and transfusion are recognized and attended to.

Once a plan is drafted, it must be validated, the staff trained, and the strategy practiced. It must be reviewed frequently, annually at a minimum, revised, and the process must be repeated. Plans are based on assumptions and are only as good as those assumptions and the experiences of the people writing them. It is the process of testing the plans, either through simulation or during actual events, that provides the necessary challenges to identify shortfalls in the plan. No plan will include all potential scenarios that can occur as each disaster can be unique. Key elements for consideration to include in a COOP are outlined in Table 2.2.

**Table 2.2** Elements Critical for Inclusion in a Continuity of Operations Plan: Minimal Elements of a COOP.

Element	Purpose
Essential Functions	Identify functions needed for business continuity. They are the essential functions that must continue. The most basic functions include: <ul style="list-style-type: none"> <li>• <i>Blood Center:</i> Procurement and storage of blood and blood components, transportation to hospitals. Collection and testing can be interrupted, but the supply of blood to customers is essential.</li> <li>• <i>Hospitals:</i> Receive, store, and transfuse blood to appropriate patients.</li> </ul>
Chain of Command	Leadership is critical to successful control and direction during an emergency. Organizations must have clearly defined and communicated primary and alternative chains of decision-making authority.
COOP Decision Trees	Flow charts and decision trees listing options for various scenarios to guide leaders during emergency operations.

**Table 2.2** (Continued)

Element	Purpose
Emergency Communications	Effective communications are vital during a disaster. Communication failures are the most cited lesson from all disasters. The ability to communicate with staff, EMAs, customers, suppliers, donors, community, and media all must be maintained. Redundant modes of communication should be planned on and tested periodically.
Staffing	Staff are vulnerable to local events and risks. Critical staff/essential personnel responsibilities should be identified, and personnel occupying those positions should be informed. Cross-training to protect essential operations during staff shortages can be considered. Additional contingency staff (volunteers, retirees, etc.) should be identified and trained periodically. Staff should be encouraged to complete family preparedness plans.
Key Contacts	A contact list for both internal and external key personnel should be maintained. It should be available both electronically and as hard copy.
Logistics	Plans are needed to ensure critical supplies, and equipment required to perform essential functions remain available. Identify and validate alternative sources of essential supplies as primary sources may be unavailable as a result of the disaster.
Transportation	Transportation has repeatedly proven vulnerable during past disasters due to a myriad of causes (e.g., flooding, fuel shortages, and road blockages). During the COVID pandemic, routine commercial flights were reduced suddenly and without warning. Coordination with local EMAs or national organizations may be necessary to ensure the delivery of blood, samples, and supplies.
Alternate Facilities	Identify alternative facilities that can be used to continue operations or safeguard equipment pending recovery.
Security and Safety	Plans to secure facilities, fleet, staff, information systems, fuel, and hazardous waste. Additional consideration should be given to the potential for large crowds at collection sites following large mass casualty events. Coordination with local EMAs and insurance companies is suggested.
Vital Records	Protection of essential records is critical in the highly regulated field of blood. Records related to the safety and availability of blood, human resource files, legal records, and financial records are critical to business continuity.
Insurance	Adequate insurance is essential to survive disasters, both property and liability. Business interruption insurance should also be considered.
Cash Reserves	Cash reserves, equal to several months of operating expenses, should be accrued to ensure the survivability of the business until normal operations can be resumed.
Human Resources	Consideration should be given to alternative work options that may be required during a disaster such as shelter in place, work from home, salary, and benefit continuation. Predisaster consideration of such possibilities allows for thorough evaluation.
Utilities	Basic utility services such as water, sewer, power, fuel, telecommunications, and Internet can be vulnerable particularly in weather-related events. Contingency plans for their priority availability based on the nature of the blood community mission should be made with service providers.
Media Communications	Creation and dissemination of clear messaging about the status of local and national blood supplies is critical. Media spokespersons should be preidentified and trained in risk and emergency communication anticipating media interactions. Resources are available on effectively communicating with the media (CDC, 2018) (CFRC, 2020).

## Regulatory considerations in disasters

The blood community is highly regulated worldwide and even slight changes to processes and procedures can have far-reaching effects for facilities and patients, especially during an emergency. Blood collection and transfusion facilities must address regulatory impacts as part of their disaster planning.

## National authority

Most countries have a national authority with overall responsibility for the safety of health care delivery often called the Ministry of Health or Department of Health. Within these organizations will be an agency responsible for the safety of blood and blood components. In the United States, this agency is the Food and Drug Administration (FDA). FDA's Center for Biologics Evaluation and Research (CBER) has the primary responsibility to ensure the safety, purity, and potency of biologic products, which includes blood and blood components.<sup>18</sup> Consideration must be given to the regulations and requirements of the relevant national regulatory agencies when developing emergency response plans. Such regulatory considerations during disasters generally fall into the following three categories.

## Determination of the acceptability of components in available inventory

Written emergency procedures at centers and hospitals should address emergency power and temperature and other environmental monitoring during storage. For example, the impact on acceptability of temperature excursions (either high or low) should be identified as well as any other effects, such as exposure to smoke or water. Consideration should also be given to unprecedented emergencies that may affect blood, such as a radiologic event or exposure to a biologic or chemical agent. Components potentially exposed in an event may require quarantine for a determination of component suitability.<sup>19</sup> When the safety, purity, or potency of a component is in question, the appropriate regulatory agencies should be consulted. An exception or variance may be needed to use such components in certain situations, especially when clinically important shortages are extant. The resources from the AABB Disaster Task Force can be a useful intermediary of such questions.<sup>3</sup>

Blood released for use during a disaster should generally be compliant with applicable regulations and standards, including quality control and testing for infectious diseases. Exceptions may be needed if blood supplies are exhausted, resupply is not possible, and blood is needed immediately to save lives. Appropriate samples should be retained for retrospective testing as soon as conditions permit. Documentation of the circumstances is essential, and hospitals and physicians should be notified about any normal processing steps that have not been completed on the blood.

In extreme circumstances, national regulatory authorities may issue emergency guidance to help ensure an adequate blood supply. For example, on September 11, 2001, following the terrorist attacks on the United States that shut down commercial air travel, the FDA issued "Policy Statement on Urgent Collection, Shipment, and Use of Whole Blood and Blood Components Intended for Transfusion to Address Blood Supply Needs in the Current Disaster Situation" (this document is no longer available on the FDA website). Likewise, some donor eligibility requirements were amended during the recent SARS-CoV-2 pandemic, especially those regarding the deferral of men who have had sex with men and for reducing the risk from malaria and from variant Creutzfeldt-Jakob disease to transfusion recipients. While these changes had long been advocated by blood collectors and were under reconsideration by FDA, the revisions were expedited early in the COVID pandemic to address potential blood shortages.<sup>20</sup>

Disasters can affect donor eligibility, particularly emergencies involving infectious diseases or hazardous chemicals. These agents should be evaluated for (1) their potential to harm to recipients and (2) potential for an asymptomatic interval of infectivity or toxicity

after exposure but before or after any donor illness that would allow collection of an unsafe donation. If an agent is suspected to be transmissible, an appropriate deferral period must be considered, donors must be deferred appropriately, and testing may be considered. For example, in the United States, various precautionary approaches to such issues have recently been applied to the agent of SARS (donor deferrals) and Zika Virus (donor deferrals and donor testing requirements). Again, national regulatory authorities should be consulted for guidance as issues arise during a disaster.

## Potential consequences on operations

Blood component usage during disasters, particularly mass casualty events, may be altered to ensure rapid availability of blood products to those most likely to benefit. It remains essential that all patients admitted who may require transfusion have a baseline sample obtained prior to transfusion. Extreme care for sample-patient identification should be taken given that patient identification is often incomplete and pseudoidentifiers may be used in emergencies. Still, there are acceptable circumstances for interventions like the initial use of Group O red blood cells before blood typing results are available and reservation of Rh-negative blood for females of childbearing age.<sup>15</sup> These are ideally enumerated prospectively in an acceptable disaster plan and not left to on-the-fly decisions.

Adequate staffing can be an issue during any disaster. Key to any and all decisions made during disasters should be the safety and well-being of facility staff. In the days and hours before an anticipated weather event, during and after an emergency, staff members must be able to tend to their families and personal needs. Still, only trained staff should be used for regulated functions. Volunteers can be used to perform tasks for which they have been trained, especially nonregulated functions such as predonation education and maintenance of the canteen. Likewise, appropriate licensure is required to safely operate fleet vehicles. Emergency plans should include maintaining a list of staff trained in multiple functions. For example, staff members who have transferred from one area to another (and have maintained competency in the prior area) can be used in the previous function with appropriate certification and licensing.<sup>21</sup>

Equipment and supplies should also be assessed for exposure to water, humidity, and temperature extremes. FDA has produced useful information guides about the effect of disasters on medical devices.<sup>22</sup>

## Records management

Vital records must be secured and maintained. These documents include records of donors, donations, manufacturing, testing, quality assurance, product disposition, as well as transfusion records. Proactive planning needs to locate storage sites in protected spaces adequate for the kinds of disasters being anticipated (e.g., not locating servers or records in the basements of flood-prone sites). If these records are damaged or lost during a disaster, blood in inventory at that time may require quarantine and recall. Efforts should be made to retrieve and preserve any damaged records.

## The COVID-19 pandemic and transfusion medicine

There is no better example of a catastrophe impacting blood collection facilities and transfusion services than the emergence of SARS-CoV-2 and the resultant pandemic. In the earliest stages of the worldwide spread of this virus, preventive lockdowns to mitigate

transmission were widely implemented. As this occurred, collection organizations expanded stocks where feasible and prepared for an adverse impact on donor availability. The lockdowns eliminated most nonurgent medical care with data from the National Healthcare Safety Network documenting a steep decline in transfusion of red blood cells in the United States during March and April of 2020, followed by a recovery when health care began to reopen in May and June (personal communication from Basavaraju S, CDC, Office of Blood Organs and Other Tissue Safety, March, 8, 2021). Varying degrees of lockdown have been recurrent across varying jurisdictions in subsequent waves. At the time of this writing, the United States is in its fourth wave.

Preliminary publications in the United States and Canada have confirmed these trends and reinforced the utility of collaboration among collectors and providers to prevent and control severe shortages.<sup>23,24</sup>

Generally speaking, transfusion services made no or few changes in their procedures for receipt, testing or storage of pretransfusion samples for infection control purposes, while there was augmentation of personal protective measures for staff in some. On-the-fly staff training regarding proper employment of infection control measures was nearly universal and generally developed in the context of the institutional response to COVID-19. Shortage mitigation strategies, beyond those inherent in the cancellation and delay of nonurgent care, included clinician reminders about appropriate blood use and encouraging alternatives to the use of labile products.<sup>25</sup>

Collection facilities were heavily focused on maintaining adequate collections in an environment that remained safe enough both to attract donors and protect staff, and the blood community developed extensive materials in support of that goal.<sup>26</sup> The effort received major support with the designation of staff in blood collection facilities as essential health care workers.<sup>27</sup> This allowed their priority for receipt of personal protective equipment and vaccines, some increased flexibility in isolation and quarantine and supported business continuity during the implementation and deployment of appropriate nonpharmaceutical interventions to mitigate the risk of introduction and transmission of the virus at collection sites. When lockdowns eliminated the large bulk of mobile blood drives in the United States, collections were pushed into fixed sites and community events were scheduled in areas where fixed sites were not accessible, requiring the rapid implementation of recruitment and access policies to prevent symptomatic or exposed donors (and staff) from presenting. Amendments were made to human resources policies regarding contact, quarantine and isolation, and staff wellness screening. Requirements for physical distancing, masks and shields, and barriers between donors and staff in donor rooms, labs, and processing and distribution areas were imposed. Environmental cleaning procedures were reviewed and revised in compliance with public health recommendations. These policies were rapidly developed, often adapting materials developed more than a decade earlier in anticipation of pandemic flu.<sup>8</sup> The changes all occurred on the background of a move from in-person to remote working for many administrative personnel that both minimized their risks of exposure and the risk that they would introduce the virus into the working environment.

As a result of a very decentralized, some would say dysfunctional, public health response in the United States, collection facilities with operations in multiple states were faced with the additional complication of compliance with variable recommendations in diverse public health jurisdictions.

A frequent early question was whether SARS-CoV-2 was a transfusion transmissible pathogen.<sup>27</sup> This concern was largely based on obvious theoretical concerns raised by the multiorgan clinical expression of COVID-19 and the identification of viral RNA in both patients and healthy blood donors, in the absence of any evidence of such transmission. Subsequently, no report or allegation implicating SARS-CoV-2 as transfusion-transmitted has been published. There is an emerging consensus that if it occurs, it is rare and not a material threat where reasonable care is applied to donor selection.<sup>28,29,30,31</sup>

In the absence of effective therapy for COVID-19, there was great fervor in the blood community and among clinicians and regulators to develop and implement programs to collect, distribute, and evaluate convalescent COVID-19 plasma (CCP) for use as passive immunotherapy providing therapeutic antibodies.<sup>32</sup> Collection organizations, generally in collaboration with their hospital and health system collaborators, and some hospital systems mobilized rapidly in March and April to do so.<sup>33,34</sup> In the United States, an observational protocol, under an expanded access Investigational New Drug exemption, was developed as a collaboration by collectors, the Mayo Clinic, treating clinicians and the FDA, aiming to facilitate access to a promising blood component while gathering information on its potential value and safety when infused into hospitalized high-risk COVID-19 patients with features of severe infection. By August, results were sufficient for the FDA to grant an emergency use authorization for the wide use of CCP, based on evidence of clinical benefit, most especially with use earlier in infection of CCP with higher levels of antibody.<sup>35,36</sup>

Subsequently, randomized controlled trials have not shown consistent benefit in hospitalized patients (early studies were reviewed in ref. [37]). The largest of these trials, RECOVERY in the United Kingdom, enrolled over 11,000 of a planned 20,000 patients from May 2020 until it was suspended for futility in January 2021.<sup>38</sup> By Spring of 2021, collection and use of CCP, an innovative product created in the midst of an unprecedented disaster, was declining rapidly.<sup>39</sup> An additional large, international, randomized controlled trial in hospitalized patients (CONCOR-1) has been published that was also suspended for futility.<sup>40</sup> If any broad benefit is to be realized from CCP, infusing well-characterized plasma with high titers of neutralizing antibody very early in infection, likely prior to hospitalization as was done in an Argentine trial,<sup>41,42</sup> might be required (as has been observed for the use of monoclonal antibodies also available under emergency use authorization in the United States).<sup>43</sup> Unfortunately, the "SIREN-C3PO" study of early treatment was suspended for futility in March 2021.<sup>44</sup> Determining whether there are "niche" indications for prophylactic or therapeutic CCP in patients who have not yet or are unable to mount an adaptive immune response or when monoclonal antibodies cannot be used will require further studies.<sup>45</sup>

Transfusion of convalescent plasma carries with it all the usual concerns about the use of labile blood products including the inherent variability of the product, transfusion reactions (especially TRALI and TACO), and the transmission of infections, e.g., HIV, HCV, and HBV. Human immunoglobulins (intravenous and subcutaneous) and especially hyperimmune globulin from recovered patients have been proposed as a solution that would standardize the product, essentially eliminate the risk of transfusion-transmitted infection, and simplify treatment allowing the infusion of a relatively low volume of an immunoglobulin preparation with high titers of anti-SARS-CoV-2 activity.

High-quality data on COVID-19 clinical outcomes associated with the use of standard intravenous immunoglobulin are not yet available. A randomized trial in 59 patients with severe COVID-19 using “off-the-shelf” IVIg during the Spring of 2020 found a mortality benefit.<sup>46</sup> Given that the preparation used could not have contained significant levels of anti-SARS-CoV-2, the authors speculate that the effect is related to immunomodulation and anti-inflammatory effects. Subsequently, data have demonstrated that levels of SARS-CoV-2 antibodies in standard intravenous immunoglobulin (IVIg) preparations have risen progressively during the pandemic,<sup>47</sup> but clinical data on the effect of these levels are not available.

Multiple fractionators (the CoVIg Plasma Alliance) responded to the pandemic and the lack of highly effective treatment options for COVID-19 by establishing an urgent collaboration with the National Institutes of Health during the Summer and Fall of 2020 to collect plasma from convalescent donors, test their units with standard assays for infectious diseases, titer the plasma for anti-SARS-CoV-2 antibodies using a consensus assay, and pool the collections. The pools were subjected to fractionation with its orthogonal viral inactivation and removal processes and to concentrate the relevant IgG fraction. This hyperimmune globulin preparation was used in a phase three randomized clinical trial versus placebo in seriously ill COVID-19 patients (Inpatient Treatment With Anti-Coronavirus Immunoglobulin (ITAC)). Detailed published or preprint data are not yet available. However, a media announcement on April 2, 2021, stated that the trial had failed to “meet its endpoints.” These were clinical improvement compared to the standard of care in an ordinal outcome based on the patient’s clinical status on Day 7 plus a number of secondary outcomes.<sup>48</sup> Whether trials will be conducted using this preparation for postexposure prophylaxis of high-risk exposed individuals or infected patients very early in clinical illness has not been determined.

Even if effective in clinical trials, passive immunotherapy faces the potential operational barriers raised by the selection and increasingly wide geographical distribution of SARS-CoV-2 mutational variants.<sup>49</sup> Early evidence demonstrates that antibodies and vaccines designed against early progenitor strains of SARS-CoV-2 may lose activity against variant strains evolving locally.<sup>50,51</sup> For CCP, this raises the question of whether a unit collected several months previous to anticipated use or in another locale can be as effective as cotemporary plasma and/or plasma from convalescent donors near to the intended recipient. A post hoc analysis of observational data from the United States expanded access IND suggests that “near sourced” CCP (collected within 150 miles of the recipient) may be clinically superior to distantly sourced plasma.<sup>52</sup> Further, the cycle

time for lots of intravenous immune globulin preparations exceeds six months. If the same holds for hyperimmune globulin preparations, they may be “outrun” by the emergence of variants in diverse locales. Substantial effort will be required to understand the impacts of variant emergence on all these preventive and therapeutic interventions in enough detail to inform our need and ability to continually “redesign” them in response.

It is a truism that “when you have seen one disaster, you have seen one disaster.”<sup>53</sup> The breadth of scope of catastrophes makes it a fool’s task to try to touch every aspect of disaster planning and response in a short chapter. Herein, we try to enumerate the critical elements organizational leaders must consider during these activities and provide references to materials we have found to be of use in our own blood banking careers. It is “US-centric” but should be applicable to the iterative process of developing, exercising, and implementing disaster response capacity independent of where the reader is located. Perhaps the most important take home message is the need to be proactive in planning, aggressive in training and simulation, and blunt during after-action critiques when disasters occur. These must be active processes and the plans that evolve from them must not gather dust between occurrences.

The need and ability to develop entirely new “product lines” during an unprecedented disaster suggest our historical planning processes were adequate to do so while maintaining a sufficient blood supply to meet clinical need. That said, the many lessons learned and being learned pending the subsidence of COVID-19 will need to be incorporated into the plans of individual organizations and up the food chain to the national and international agencies responsible for imagining the future before they can be returned to the bookshelf awaiting the next catastrophe.

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## CHAPTER 3

# Responding to regulatory challenges during public health emergencies

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### Connection between public health emergencies and blood transfusion

The outbreak of Severe Acute Respiratory Distress Syndrome Coronavirus-2 (SARS-CoV-2), which is responsible for the COVID-19 pandemic that started in 2020, has served as a reminder of the importance of emergency response preparedness.<sup>1,2</sup> Particularly, blood and blood components are critical medical products needed for use during natural and man-made disasters as well as during epidemics and pandemics caused by various pathogens. The transfusion of blood and blood components in turn occupies a unique position among medical products, in that in addition to being potentially life-saving medical interventions, for certain infectious diseases, blood transfusion may become a method of infectious disease transmission.<sup>3</sup> This has most notably been the case for the viral pathogens hepatitis B, hepatitis C, and human immunodeficiency virus (HIV), among others (Table 3.1).

As blood transfusion became a more routine practice during the twentieth century, adverse effects outside of immediate and delayed transfusion reactions became more apparent. In particular, the development of hepatitis following the transfusion of blood products was noted in the 1940s to 1950s.<sup>4</sup> During the 1960s and 1970s, it was ultimately realized that about 30% of chronically transfused individuals developed hepatitis not attributable to hepatitis A or B, now known mostly to be attributable to hepatitis C.<sup>5</sup> The rapid development of acquired immunodeficiency syndrome (AIDS) associated with the spread of HIV among the hemophilia community in the early 1980s drove home the critical need for better screening of the blood supply and better manufacturing methods for blood derivatives.<sup>6</sup> Initial measures relying on questionnaire-based donor deferrals gave way to iterative generations of testing for hepatitis viruses and HIV. In addition, pathogen reduction technologies, such as heat inactivation, solvent-detergent treatment, and nanofiltration, were introduced into processes for the manufacture of blood derivatives.<sup>7</sup>

The relatively robust nature of pathogen inactivation technologies, reducing the infectious titers of most pathogens by many logs, with the notable exception of parvoviruses, has transformed the overall safety of blood derivatives prepared from plasma. However, transfusible blood components remain susceptible to emerging pathogens that are not addressed by current deferrals or screening

technologies. During the past few decades, West Nile virus, Zika virus, and babesia are just a few of the pathogens that have had to be addressed in the United States.<sup>8</sup> Although pathogen reduction technologies have now been developed for all blood components and are approved in the United States for use in platelet and plasma preparation, the time and expense associated with their implementation have limited their application to date.<sup>9</sup> Pending the development and introduction of a more streamlined safe and effective pathogen reduction technology, the blood supply will remain particularly vulnerable to the emergence of novel pathogens until a method for their detection can be developed.

Pending introduction of highly efficient and effective pathogen reduction technologies, the ability of the blood supply to become a vector for disease transmission will remain a weak point in our ability to address emerging pathogens. When novel pathogens emerge, a rapid response will continue to be necessary for the identification of the responsible pathogen and implementation of appropriate deferral or screening technologies to maintain a safe blood supply. In the end, therefore, our ability to keep the blood supply safe will ultimately rely on the basic pandemic response strategies described below.

### Approach to pandemic management

At any given time, there are hundreds of pathogens that could potentially be associated with epidemics or pandemics, and a number of these are known to be transmitted by blood transfusion or could potentially be transmitted by blood transfusion.<sup>8</sup> Four key aspects of addressing emerging infectious diseases (Table 3.2) include: (1) identifying the infectious agent and maintaining accurate epidemiologic surveillance, (2) developing and deploying accurate diagnostic tests, (3) instituting appropriate containment or mitigation measures, and (4) developing and deploying effective therapeutics and vaccines. Although SARS-CoV-2 has not been a pathogen of particular concern for blood-borne transmission, vigilance must always be maintained for this possibility in the setting of emerging infectious diseases. This is particularly true given the importance of blood product transfusion to routine medical care, to the management of individuals with immunocompromise and cancer, and due to its importance during natural and man-made disasters.<sup>10</sup>

**Table 3.1** Some Established and Potential Transfusion Transmitted Infections

<b>Established Transfusion Transmissible Pathogens</b>
Human immunodeficiency virus (HIV)
Hepatitis B virus (HBV)
Hepatitis C virus (HCV)
Human lymphotropic virus types 1 and 2 (HTLV-1 and HTLV-2)
Cytomegalovirus (CMV)
West Nile Virus (WNV)
Zika virus (ZIKV)

<b>Emerging Pathogens with Potential for Transfusion Transmission</b>
Ebola virus
Marburg virus
Dengue virus
Chikungunya virus
Lassa virus
Nipah virus
Powassan virus

**Table 3.2** Four Key Aspects of Epidemic and Pandemic Response

<b>Epidemiologic Surveillance</b>	<b>Containment and Mitigation</b>
Identify the infectious agent and maintain accurate epidemiologic surveillance	Institute appropriate containment or mitigation measures
<b>Diagnostic Testing</b>	<b>Therapeutics and Vaccines</b>
Develop and deploy accurate diagnostic tests	Develop and deploy effective therapeutics and vaccines

## Epidemiologic surveillance

Early recognition of infectious disease outbreaks is critical since the implementation of a containment strategy is most effective when the disease is localized. For known pathogens, the clinical identification of an outbreak should lead to the rapid implementation of appropriate diagnostic and treatment modalities. For novel emerging pathogens, the identification of a common symptom complex in a population may initially point to a potential infectious source and lead to isolation of the pathogen. The emergence of nucleic acid testing by polymerase chain reaction (PCR) has been transformational as it allows the rapid development of a diagnostic test.<sup>11</sup> Since such infectious diseases frequently originate in remote locations, and given the importance of PCR to early diagnosis, this technology has been adapted for use in remote settings.

## Diagnostic testing

As noted above, once epidemiologic intelligence suggests a novel outbreak, the rapid development of easily implemented diagnostic tests is needed. Although PCR performed in a laboratory initially may be useful, as an epidemic or pandemic progresses, the deployment of a more convenient testing methodology is desirable. The COVID-19 pandemic was quite instructive regarding the importance of the necessary characteristics of such tests.<sup>12</sup> Diagnostic accuracy coupled with ease of deployment and implementation are critical elements that should be addressed. Such accurate diagnosis is needed to determine disease epidemiology, which is critical for the implementation of a containment strategy, if one is possible. In addition to qualitative testing for nucleic acids, antigens, or antibodies for the determination of the presence or absence of disease, the availability of quantitative testing to determine the extent of the

antibody response to a passive or actively elicited immune response may also be relevant. For example, accurate determination of neutralizing antibody titers, either directly or through a surrogate marker, can be critical both for diagnostic and therapeutic applications. For example, determining the extent of the immune response may be critical for the identification of high titer units of convalescent plasma, which could possibly be used early on in an epidemic or pandemic, prior to the availability of more specific therapies,<sup>13</sup> and it is also very important for assessing the response to prophylactic immunization.

Another recent outbreak that illustrated the importance of the rapid development of testing strategies was the 2016–2017 Zika virus outbreak.<sup>14</sup> In the absence of a diagnostic test, blood donors had to be deferred based on location and travel history due to the possibility of exposure to the virus, which could be transmitted by blood transfusion. The development by manufacturers of screening assays for the blood supply allowed the implementation of a national testing program ensuring the safety of the blood supply.<sup>15</sup> As an aside, the complexity and cost of that implementation were not without controversy; however, early on in the outbreak, there was significant concern that significant morbidity could be associated with extensive spread of the Zika virus as the potential for its spread via multiple modes of transmission, including intimate sexual contact, was not yet fully understood.

## Containment and mitigation

With the identification of an infectious disease outbreak and the development of clinical or laboratory criteria to document infection, the next step is implementing a strategy to minimize morbidity and mortality. Initially, containment may be used to try to halt the spread of illness to new locations; if it is not successful, a transition to mitigation is necessary.<sup>16</sup>

Implementation of a containment strategy within a given location may involve the quarantine of individuals known to be infected with a pathogen as well as those who have had a high risk of having been exposed. On a broader level, containment may involve limiting travel either within a country or across international borders. Such limitations may involve screening of individuals to identify and defer travel by those with signs of illness to blanket deferrals of travel across borders for entire populations. The success of a containment strategy largely depends on how fast an outbreak is detected. Though it may seem paradoxical, infectious diseases that rapidly cause severe morbidity in most or all of individuals infected are generally easier to contain than those that are associated with variable symptoms, including a relatively high number of asymptomatic carriers. The situation with more asymptomatic carriers allows more disease spread prior to the implementation of containment measures. Ebola virus is good example of the former, and SARS-CoV-2 the latter, situation.<sup>17</sup>

A mitigation strategy generally involves the implementation of measures to reduce the effect of widespread disease transmission within a community. Such measures could include everything from cancellation of large-scale gatherings and attendance at schools or offices to wearing masks to prevent the spread of respiratory viruses. The mitigation phase of an epidemic or pandemic also includes the time during which nonspecific supportive care measures may be implemented prior to the development of disease-specific therapeutics and preventative vaccines. The introduction of specific therapeutics and vaccines generally takes place during the mitigation phase of the response to an infectious outbreak.

## Therapeutics and vaccines

Epidemics and pandemics associated with newly emerging pathogens present special challenges as first the pathogen must be identified and characterized in detail and then work must proceed rapidly to identify known or new drugs that can treat disease caused by the pathogen and vaccines that can prevent disease caused by the pathogen.<sup>18</sup> Those epidemics and pandemics associated with known pathogens may allow the deployment of existing antimicrobial agents and the adaptation of existing vaccines to the specific pathogen circulating. Known pathogens for which products have previously been developed, potentially using special pathways, such as the animal rule, can generally be relatively rapidly deployed, if they can be manufactured in sufficient quantity.<sup>19,20</sup>

For newly emerging pathogens, large-scale randomized clinical trials of therapeutics and vaccines may be required to demonstrate safety and effectiveness. These can be quite challenging to conduct during a pandemic for both practical and ethical reasons, and innovative clinical trial designs, such as ring vaccination, cluster randomization, and stepped wedge designs, among others, may need to be deployed.<sup>19</sup> Although the conduct of clinical trials may seem to be a potentially rate-limiting step in this process, prior influenza outbreaks, as well as the COVID-19 pandemic, have illustrated that manufacturing represents a step that is potentially even more limiting in the deployment of effective therapeutics and vaccines. In part, this is simply because of the scale of production that may be required. For example, even in the simplest case of a vaccine that requires only a single dose to provoke protective immunity, several hundred million doses need to be produced in order to protect the population of the United States, let alone allow for global export. Once a therapeutic or vaccine is produced, the challenges of facilitating its effective distribution and administration during a situation with pandemic limitations must be overcome. Preventive vaccines can be given at mass vaccination clinics established for this purpose. However, the administration of prophylactic or therapeutic parenteral agents may prove more challenging than it might first seem. Because health-care facilities may not have sufficient space for the containment of infectious disease in the outpatient setting, the use of special centers or even mobile units may need to be considered.

Ultimately, careful coordination of all of the above individual pandemic response actions is absolutely critical to most effectively controlling a pandemic as quickly as possible.

## Case study of a therapeutic: COVID-19 convalescent plasma

Cases of COVID-19 started to emerge in late 2019. By January 2020, the potential severity of infection with SARS-CoV-2 became apparent, and in China a clear need for potentially effective therapeutic interventions arose. Convalescent plasma obtained from individuals infected with other respiratory viruses had shown some potential benefit in other outbreaks, and the evidence was strongest when

high titer plasma was administered early in the course of infections.<sup>21</sup> Because of the prior experience suggesting its potential benefit, convalescent plasma was employed in this setting. Initial reports from China regarding small numbers of individuals with COVID-19 treated with convalescent plasma were somewhat encouraging.<sup>22,23</sup> Efforts in the United States to implement randomized clinical trials were slowed by challenges developing a reliable assay to assess the neutralizing antibody titers of the convalescent plasma collected from donors as well as challenges in a medical care system that in many places was overwhelmed. Ultimately, an expanded access program was implemented that treated close to 100,000 individuals.<sup>24</sup> Simultaneously and subsequently, several randomized clinical trials reported conflicting results. Ultimately, when confounding factors were deconvoluted, it appears that the infusion of high neutralizing antibody titer COVID-19 convalescent plasma does indeed produce a very modest survival benefit in those individuals who are earlier in the disease course and not intubated, as well as in those individuals who cannot develop an antibody response such as those with hematologic malignancies.<sup>25</sup> In retrospect, had the appropriate assays and clinical trial infrastructure been in place, the answer to the clinical question of whether COVID-19 plasma could provide survival benefit could have been answered much more efficiently.

## Summary

Although the COVID-19 pandemic that has taken millions of lives across the world is not yet under control as of mid-2021, there is hope that global vaccination campaigns will achieve this in the next few years, at most. Even as we come out of this pandemic, we must be prepared for the next infectious disease outbreak, whether in an epidemic or pandemic form. Fundamental to any epidemic or pandemic response plan for those pathogens capable of being transmitted by blood is the development of appropriate measures to protect the blood supply. The application of lessons learned in the current pandemic will hopefully expedite the response to future outbreaks and reduce the loss of life.

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## **SECTION II**

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# **Blood donation**

## CHAPTER 4

# Recruitment and screening of donors and the collection of blood

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The blood transfusion system begins with the donors. The methods and details of blood donor recruitment, assessment, and collection have changed over time and with technology, but the principles remain the same. An adequate number of donors must be available, they must be drawn with consideration for the safety of the donor and the recipient, and the products collected must be tailored to the needs of the patients needing transfusion. In the United States and many countries, routine blood donors are not usually remunerated, but donors of plasma for further manufacture generally donate more frequently and are usually paid. Plasma collections for this purpose are dealt with at the end of this chapter.

### Donor demographics

In 2019, the most recent year for which large-scale US data are available, 9,777,000 allogeneic whole blood (WB) units were collected, based on analysis from the CDC in the National Blood Collection and Utilization Survey, representing a decrease of 6% from the 2017 survey. This includes blood centers and hospital-based blood collectors but excludes about 14,000 autologous and directed donations. Additionally, 1,800,000 red blood cell (RBC) units were collected by apheresis (automated) procedures, an increase of 0.7% from 2017 in apheresis units. After losses due to testing and other reasons, the WB and RBC supply was 10,879,000, a decline of 5.8% from 2017, more than the 3% decrease between 2015 and 2017. Also, 2,359,000 apheresis platelets were collected, an increase of 0.9% since 2017.<sup>1</sup>

In 2019, a total of 10,981,000 successful donations of all types of blood products were made, 1.1% fewer than in 2017. The total number of individual donors was 7,316,000, about 8.5% fewer than in 2017. About 70% were repeat donors, while 30% donated for the first time. Of the total donations, 16.1% were made by individuals 65 years of age or older, an increase of 10.5% from 2017.<sup>2</sup> The age of the US donation population has been a continued concern. The first large-scale blood donation efforts in the US occurred during World War II (WWII), when both red cells and plasma were sought in volume to support the combat soldiers. Perhaps it is not surprising then that, until today, the greatest number of donors have come from the donors who “learned to donate” during that time and their children, the baby boomers. Over time, the demographics of blood

donors have skewed older as the baby boomers have aged.<sup>3,4</sup> Although donation is safe for older donors,<sup>5</sup> this age group cannot support donation indefinitely as their numbers decline and medical conditions render them unable to donate. Concern is particularly strong about apheresis platelet donations,<sup>6</sup> and various strategies have been proposed to ensure supply.<sup>7</sup> High school collections remain an important part of the blood supply in the United States; in one national study, 39% of first-time donors were under 20 years of age.<sup>8</sup>

Young donors contribute very much to the diversity of the donor pool, but in general, donations from racial and ethnic minorities are disproportionately less than whites,<sup>8,9</sup> and this is of concern for several reasons. Diverse ethnicities are a growing part of the US population and so are needed as donors if the country is to have a robust blood supply. Also, since many red cell antigens are associated with particular geographic and/or ethnic groups, donors of particular groups are needed to meet the needs of certain patients. For example, patients with sickle cell disease may need blood with specific types available almost exclusively within the African American community. However, donations from minorities make up less than a quarter of all donations in most studies. Increasing this proportion is a priority for blood collectors throughout the country. Studies of demographic and attitudinal differences in donation from ethnic groups, as well as possible interventions, continue to make contributions to this effort.<sup>10–14</sup>

In 2019, the number of males and females presenting to donate was roughly equal (6,644,000 males and 6,378,000 females), but females had a much higher rate of deferral (particularly due to low hemoglobin), so the number of donations by males was higher.<sup>2</sup> Female deferrals for the presence or risk of HLA antibodies which may contribute to TRALI also contribute to their lower donation numbers and exacerbate the apheresis platelet shortage.

### Donor recruitment and retention

In almost all circumstances, blood donors must be actively recruited from the population. Even in the most trying times, as during WWII when a blood program was being established for combat soldiers, donors must be deliberately sought.<sup>15</sup>

## Theories of donation behavior

In recent years, several groups have worked to establish a theory or framework for blood donor behavior. Ideally, such a theory would explain the observed behavior of donors and would provide information leading to the development of more successful methods of recruiting appropriate donors. It is generally assumed that the motivation of donation is altruism, as it is widely known that blood transfusion can save lives. The *theory of planned behavior* (TPB), proposed by Ajzen,<sup>16</sup> has been most widely used and applied to many health behaviors. *Donation* (the behavior) is preceded by an *intention* to donate. This intention is formed by the interaction of attitudes, subjective norms, and perceived behavioral control. *Attitudes* are the positive or negative evaluation of donating blood, while *subjective norms* are the perceptions of social pressure about donation. *Perceived behavioral control* in this model is the potential donor's own assessment of his or her degree of control over the donation. Emotional aspects of the blood donation process, beyond simple motivation, have recently been emphasized by several workers.<sup>17–19</sup> Adding emphasis to this component, including but extending beyond simple motivation, may improve the utility of the models to suggest areas for study and change. These changes may lead to improvements in the blood donor recruitment and retention process. We need not only more donors in general, but also more specific types of donors.

Ideally, the validation of any theory would occur by measuring actual blood donations. Unfortunately, this is difficult in the short run as for most donation types blood donation cannot occur very often, and it is difficult to follow a large panel of potential donors across a long period. Most blood donor recruitment studies and metrics are cross-sectional or run over only a short interval. Evaluation of the TPB mostly relies upon measurement of *intent* to donate as measured through other values that have been validated to correlate with actual donation. Other metrics have also been suggested and used in studies of TPB.<sup>20</sup>

The initial motivation for donation has long been assumed to be *altruism*, that is, the donation will benefit society. Altruism need not be “pure,” that is, of no benefit to the donor, and other types of altruism have been suggested and measured.<sup>21–23</sup> Notable is “warm glow,” which is a sense of feeling good after a donation (“I would feel good about myself after donating”) which has been shown to be important in some contexts, particularly for repeat donors. A number of workers have shown that motivation can be strengthened by motivational interviewing among nondonors and less motivated donors, leading in some cases to increased intention and retention as donors.<sup>24–26</sup> The effect on the most experienced and committed donors may be less noticeable.<sup>27–29</sup>

Perceived behavioral control is an important part of forming the intention to donate, and in particular the donor's control is threatened by fear of fainting, the risk of which is overestimated by the inexperienced donor.<sup>30–32</sup> The fear itself can increase the risk of fainting. Education and specific steps to reduce this occurrence not only has a direct benefit to the safety of the donor but also can improve the donor's sense of coping, and may lead to improved return and retention rates.<sup>33–37</sup>

Perceived control of donation is also threatened by deferral for any reason, and donors who have been deferred are less likely to return.<sup>38</sup> This is especially true for those who present to donate for the first time and those who have not donated in a long time, with longer deferrals causing more damage to return rates.<sup>39,40</sup> This is an important consideration in the careful crafting of donor deferral policies.

## Donor retention and repeated donations

Donors who repeatedly donate provide most of the blood donations in this country, and are highly sought. The TPB provides clues to a path for developing a first-time donor into a repeat donor, but research has not been particularly successful in achieving behavioral change. Several studies indicate that first-time donors who make a second donation or even several donations within the first year are much more likely to become regular donors,<sup>41</sup> but we do not have a clear idea of why or how that happens. Anxiety may be a part for many who do not return,<sup>42</sup> and even experienced donors may feel anxiety that is a barrier to more donation.<sup>43</sup> Traditional reminders and incentives may not be helpful in this situation. In the absence of a clear and effective intervention, individualized psychological support may be most useful.<sup>44</sup>

The issue of incentives for regular blood donation is not well-settled. Small incentives, such as T-shirts, are entrenched in the US donor system, and the FDA has rules on the amounts and types of incentives that may be offered to the “volunteer” donor.<sup>45</sup> Some donors, especially experienced and committed ones, do not seem to need or even desire additional incentives, at least not monetary ones. However, most studies of monetary rewards have been limited to surveys rather than trials. Within the European Union, policies and attitudes vary, and even those with a positive attitude to incentives did not feel they would change willingness to donate.<sup>46</sup> A study in Germany suggested that monetary incentives could encourage return of first-time donors after the first donation, but would not improve the long-term commitment of donors.<sup>47</sup> In recent years, the subject of remuneration of donors in the United States has repeatedly come up as the system is challenged to collect specific products (such as group O red cells, AB plasma, and apheresis platelets) in sufficient volumes to meet clinical needs. Several authors and authorities have suggested considering a model of remuneration, and trials are ongoing.<sup>7,48</sup>

## Extraordinary donations following disasters

Extraordinary circumstances may bring a sudden influx of donors, particularly when the event involves traumatic injury to a large number of people and thus might require a large number of units of blood for treatment. In fact, the blood needed most immediately must already be available to the hospitals, and so has been collected at least a day before the event. Donors donating shortly after the event do serve to replenish the supplies that have been used and provide robust supplies for coming days, but much of the donated blood may be wasted. In the mass shooting in Las Vegas on October 1, 2017, 58 persons were killed and 600 injured, with 220 hospital admissions. Five hundred blood components were transfused during the first 24 hours, and the public donated almost 800 units. More than 17% of the donated blood went unused.<sup>49</sup> Collections nationally were also increased over the next few days.<sup>49</sup> Following 9/11, about 572,000 units over the normal number were collected in September, October, and November,<sup>50</sup> and collections were increased more than 200% in the days following the San Francisco Bay Area earthquake of October 17, 1989.<sup>51</sup> In a study of some large blood centers following 9/11, there was a fivefold increase in first-time donors, while repeat donors increased 1.5 times.<sup>50</sup> In San Francisco, first-time donors increased from 26 to 45% following the earthquake.<sup>51</sup> Females showed a larger increase than males in both these events. The first-time donors had a return rate over the next months that was similar to their baseline, not increased.<sup>50–52</sup> No exceptional increase in positive test results was seen in either of the sites, considering the usual higher rates in first-time donors.

The SARS-CoV-2 pandemic led to some particular challenges in blood requirements and donor response. The large-scale social isolation that was enforced to stop the spread of the virus slowed blood collections tremendously. Normal medical procedures, notably elective surgeries, were cancelled for fear of infection or lack of hospital capacity. Gradually, severe blood shortages developed, and a public campaign by national leaders such as the Surgeon General led to a tremendous public response. Throughout, the donors and staff had to deal with personal protective issues and fears. The analysis of this disaster-type response in the face of a respiratory pathogen has led to new insights about donor motivation and acceptance of risks, and exemplified aspects that were shown in studies of “intention” to donate during previous avian flu outbreaks.<sup>53–55</sup>

### **Recipient-specific blood donation**

Following the recognition that HIV could be transmitted by transfusion, the notion of giving your own blood for elective surgery (autologous) or asking a friend or relative to donate blood for you (directed) gained attention from physicians and patients alike. A third type of recipient-specific donation (designated) is based less on fear of blood than from an exceptional medical need.

### **Autologous donations**

As might be expected, the collection of autologous RBCs peaked in the mid-1980s and early-1990s when the risk of transfusion-transmitted HIV was high, making autologous blood a safer option. Beginning in the early mid- to late-1990s, as testing technology advanced, autologous donation plummeted. The 2011 NBCUS showed a 59% drop in collections over 2008, with 58% of units ending in discard. 2013 NBCUS<sup>56</sup> showed a decline in autologous collection of 47% from the 2011 survey; by the 2015 NBCUS, autologous collections had dropped an additional 54.2%.<sup>57</sup> 2017 saw an additional drop in the collection of autologous red cells by 61.9%, followed by an additional reduction of 51% by 2019. Factors contributing to this steep decline in collections over time include the recognition of the higher cost of production due to special procuring and labeling requirements, increased waste due to discard, problematic hospital reimbursement, the widespread advent of blood management programs, and dramatic enhancements in the detection of relevant transfusion-transmitted infectious diseases (RTTIs). Etchason *et al.* reported that the additional cost of autologous blood ranged from \$68 to \$4873 per unit of blood and resulted in little benefit based on quality-adjusted years of life (QALY),<sup>58</sup> reaching many millions of dollars cumulatively.<sup>59</sup> Currently reasonable indications include surgeries for which there exists a high likelihood of transfusion such as select orthopedic, vascular, and cardiothoracic surgeries. Autologous blood may also be a rational approach for patients for which finding compatible blood may be challenging or even impossible, such as those with extremely rare blood types, multiple alloantibodies, or antibodies to high-frequency red cell antigens.<sup>60</sup>

While many of the processes used for the collection of whole blood are the same as those used to collect autologous RBCs, there are number of key differences. To collect autologous blood, the donor/patient's physician must write an order or prescription noting the anticipated date of use (surgery date), and the type and number of products needed. Eligibility criteria are less stringent and are targeted at protection of the donor/patient. Finally, the lower acceptable hemoglobin level is 11.0 g/dL for both males and females. Since autologous donors do not generally meet the strict criteria to which allogeneic, voluntary donors are held, if the

donated unit(s) are not transfused, they may not be used for other patients and must be discarded.

### **Directed donations**

The rise of directed donations coincided, as did autologous, with the recognition of HIV as an RTTI. Out of fear, patients anticipating transfusion began asking friends and family to donate blood on their behalf. Unlike autologous donations, directed donors must meet all criteria for volunteer nonremunerated donors. If the directed unit(s) are not transfused into the intended recipient, the units can be placed in general inventory for other patients. Contrary to popular belief, directed donations are not safer than those donated by volunteer donors. Often such donors are first-time donors, known to be less safe than regular, repeat donors. Further, because the donor is known to the recipient, the donor may be reluctant to divulge high-risk behaviors, and there is a risk of loss of confidentiality for the donor. In common with autologous units, directed units are more expensive due to costs associated with procuring, labeling, and ensuring they are reserved for a specific recipient. Since 2013, both the collection and transfusion of directed donations have declined; the decline from 2015 to 2017 was 25.7% with an additional 46.6% from 2017 to 2019. The rate of transfusion dropped 14.7% from 2015 to 2017 and another 83.9% from 2017 to 2019.

### **Exceptional medical need**

Collection of components for an exceptional medical need is infrequent and limited to very few indications. Donors must meet the same criteria as volunteer donors, with some exceptions. Such collections may occur once or regularly depending on the need. Examples include HLA-matched platelets collected from family members or volunteers, or donors with unusual blood types or red cell antigen types that may be used for patients with rare blood types, multiple alloantibodies, or alloantibodies to high-frequency red cell antigens. Such donors may give at regular intervals, with their components used fresh or frozen in rare donor registries. Ill neonates with neonatal alloimmune thrombocytopenia (NAIT) may require urgent transfusion of antigen-negative platelet products. Large BCEs and rare blood registries may keep a list of suitable donors who can be called on in an emergency to donate platelets. In areas where such services do not exist, platelets (washed) may be collected from the antigen-negative mother; this is considered an exceptional need due to waiver of the postpartum restriction (Chapter 34). Finally, granulocytes for patients with select infections or neutrophil function defects comprise a special product that must be collected and infused quickly. Donors are usually treated with steroids or filgrastim, and they may donate several times over a brief period of time as needed. Such donors are often called “designated” donors. Since granulocytes may need to be infused prior to completion of infectious disease testing, predonation testing is common (Chapter 19).

## **The collection process for blood components for transfusion: screening, informed consent, phlebotomy, collection, and postdonation**

### **Donor screening**

The basis of donor screening is twofold: to minimize risks both to the recipient and to the donor. The Food and Drug Administration (FDA) has long considered donor screening as the first layer of defense in a long line of mitigation steps to protect the blood supply.

Although all blood donations undergo testing for RTTIs, among many other tests, not all RTTIs can be detected readily by testing, and for some, approved tests may not exist. Even for RTTIs for which reliable tests exist, such as HIV and hepatitis B and C, there is a period between the time of infection and the time of detection, known as the infectious window period. If a donor donates during this infectious window, the test result may be (falsely) negative. In order to interdict RTTIs for which no test exists or for donors in the infectious window, donors are screened to identify risk factors that could indicate a potentially infected donor. For RTTIs for which there is no test, donor screening may be the sole method to eliminate infected donors. Questioning may include direct questions ("Have you ever had HIV?" or "Have you ever had malaria?"), indirect questions to identify risk factors ("Have you used intravenous injectable?"), or a combination of both types of queries.

Beyond RTTIs, donors must be assessed for underlying conditions or for medications that could negatively impact the recipient, the collected component, or the donor himself or herself. Donors with medical conditions such as cardiac or pulmonary issues, cancers, and a multitude of other medical conditions are identified to determine if it is safe for them to donate. Unfortunately, there is little consensus regarding which conditions warrant deferral or the length of such deferral. Many deferrals are based on the medical judgement of the day, opinion, or conventional wisdom rather than factual data.<sup>61,62</sup> Furthermore, a wide variety of deferral policies exist globally.<sup>63</sup>

Like deferrals for medical conditions, medication deferrals may be based on several factors: potential teratogenic impact on a developing fetus, an indication of an underlying condition, or potential deleterious effect on a specific component. An example of the latter is the prohibition of collection of platelets from a donor taking medications that inactivate platelets such as aspirin or of plasma from a donor taking an anticoagulant. Medication deferrals also vary widely, as determined by oversight by the local medical director, by national or even international standards.<sup>63</sup>

Acceptable donor age ranges from state to state in the United States. In the United States, most states allow donation by donors aged 17 or older, though some states allow 16 years old to donate with parental consent. In the United States, there is no standard upper age limit. As expected, both lower and upper age limits exist in different countries. The WHO recommends a lower limit of 18 years old with exceptions where permitted by national legislation. An upper limit is recommended at 65 years of age, with the acknowledgment that donors above that age usually donate successfully and have lower adverse reactions.<sup>63</sup>

In the United States, the Donor History Questionnaire (DHQ) developed by the AABB and approved by FDA is used by the large majority of blood collection establishments (BCEs) either in published or modified form. A precursor to the current DHQ, the "donor record card" published by AABB in 1953 consisted of a list of 21 diseases and conditions, and its compulsory use was never intended. Over the years, additional questions were added incrementally and haphazardly. With the recognition of the transmissibility of HIV in the mid-1980s, the number of questions rose dramatically in an effort to identify donors potentially at risk for transmitting HIV; by the 1990s, the questionnaire was both lengthy and complex. By the early 2000s, donors were asked about no fewer than 75 health, travel, medication, and infectious disease risk factors in 29 single-item, 14 compound, and 13 multi-item questions. In recognition that the questionnaire was becoming unwieldy, in 2000 the AABB convened a multiorganization group of experts

from the United States and Canada, including representative from FDA, survey design experts from CDC, and an ethicist, to comprise the AABB Donor History Task Force (DHTF). The assigned task was to revise and validate a new questionnaire, the DHQ.

The resulting DHQ was based on established survey design, including the use of "capture questions," for which a "NO" answer eliminates the need for further inquiry: elimination of "compound and multi-item questions" that require the donor to answer two or more questions, creating confusion; and the use of reverse-chronological formatting that enables the donor to move from recent to remote. The latter approach is based on studies that show people remember recent risks more accurately than remote risks; in addition, going back in time stepwise avoids "mental time-travel" that confounds memory. In addition, medical jargon was removed, and "sexual practices" were clearly defined. Most medications were removed to a separate list, the Medication Deferral List (MDL), so medications and their deferral periods can be added or removed without disturbing the flow of the DHQ.<sup>62</sup> Finally, the document was validated by CDC survey design experts using focus group and cognitive interview techniques. Companion documents developed for use with the DHQ include the MDL, Pre-donation Educational Material (DEM), optional flowcharts, and a User Brochure.

### **The current DHQ**

A comprehensive discussion of donor screening has been published.<sup>61</sup> However, since the DHQ is a dynamic document that is updated as new risks are identified and added, and as sophisticated testing technology shortens the window period, any detailed analysis is by nature short lived. In general, questions address the following factors:

- Current health of the donor
- Pregnancy
- Medication use including aspirin and antibiotics
- History of diseases such as malaria, babesiosis, cancer, and cardiac disease
- Travel history
- Activities that could result in potential exposure to HIV and hepatitis
  - Body piercing and tattoos
  - Receipt of transfused blood components
  - Receipt of allogenic tissues or a transplant
  - Injection drug use
  - Risky sexual practices
  - Accidental needle sticks and other blood exposures
  - History of incarceration
- Activities associated with potential risk of CJD or vCJD
  - Receipt of transfused blood components from the United Kingdom, France, or Northern Ireland
  - Travel or residence in some areas of risk
- History of vaccinations and/or shots
- History of recent donation of blood and components

Subsequent to the approval of the DHQ by FDA, the DHTF developed an abbreviated questionnaire (aDHQ) for frequent donors who donate within a specified period and for whom there have been no changes in their eligibility. The aDHQ resulted from longstanding requests from frequent donors for whom repetitiveness and length of the questionnaire were disincentives to donation, and from studies of behavioral scientists that demonstrated that recall was improved for recent compared with remote events. Companion documents (User Brochure, MDL, Flowcharts, and DEM) similar to those of the DHQ were also developed for the

aDHQ. All AABB-approved donor screening tools for both the full-length and abbreviated versions are available on the AABB website.<sup>64</sup> AABB nonmembers have full access to the documents.

### **Changes to the current DHQ based on history of MSM**

Deferrals for activities considered high risk, specifically men who have sex with other men (MSM), have been in place since the recognition that HIV could be transmitted through blood transfusion. From September 1985 to December 2015, FDA recommended indefinite deferral for MSM behavior. Over time, with the closing of the window period for HIV, the implementation of medications that could mitigate the transmission of HIV through sex, and the recognition that not all MSM activity was equally risky, countries including Australia and Canada have reduced the permanent deferral to shorter periods of time. Following the universal screening of blood with nucleic acid tests (NAT), the risk of HIV transmission was reduced to about 1 in 1.47 million transfusions.<sup>65</sup> Epidemiologic data from countries that changed their deferral policy for MSM indicated no safety concerns,<sup>66</sup> though studies of MSM in the United States indicated that risk would need to be monitored.<sup>67</sup> In December 2015, the US FDA issued Guidance reducing the deferral period for MSM to 12 months.<sup>68</sup> Data from the two years following implementation of the 12-month deferral along with data from countries that further reduced the deferral period to three months (Canada and United Kingdom) showed no safety concerns. Thus, the FDA Guidance was again revised in August 2020 to further reduce the deferral for MSM and other HIV risk behaviors (tattoos, piercing, commercial sex work, and injectable drug use) to three months.<sup>69</sup>

### **Changes to the FDA required deferrals for risk of CJD and vCJD**

Both CJD and vCJD are rare but invariably fatal degenerative diseases of the central nervous system belonging to a group of prion diseases or transmissible spongiform encephalopathies (TSEs). The FDA has required deferral of certain individuals thought to be at increased risk of TSEs including those who had exposures to cadaveric human pituitary growth hormone (hGH) or allogeneic dura mater, a blood relative of a person with genetic CJD, and individuals at risk of exposure to meat or insulin contaminated by vCJD. To date no transfusion-transmitted cases of CJD have been described and the risk remains theoretical. Four potential transfusion-transmitted cases of vCJD have been reported, isolated to the United Kingdom. Based on the theoretical risk of transfusion transmission and the absence of evidence of such transmissions, the FDA issued Guidance in 2020 revising many of the long-standing deferrals including:<sup>70</sup>

- Removal of hGH and bovine insulin manufactured from BSE-affected countries from the MDL
- Discontinuation of questioning about having a blood relative with CJD
- Discontinuation of deferral for residence on a US military base in Europe, with requalification and reentry options
- No changes in deferral policy for exposure to allogenic human dura mater

### **Educational materials and informed consent**

Before each donation, the donor is provided with predonation Donor Educational Materials (DEM) that must be read in entirety. He or she is also asked to sign an acknowledgment that they read

and understood the DEM and consent to donation. Under federal regulation § 21CFR630.10(g)(2), establishments must obtain the donor's acknowledgment that the donor

- has reviewed DEM
- agrees not to donate if a potential risk to recipient exists
- recognizes that a sample of blood will be tested for RTTI
- will be notified of an unsuitable donation
- has been notified of risks and hazards of the donation procedure, as may be provided in the DEM
- has an opportunity to ask questions and withdraw from donation
- must not be required to agree to exculpatory language through which the donor is made to waive legal rights

While both AABB and FDA require donor informed consent (IC), there is no standardized process for obtaining consent. The development and role of the DEM in conjunction with the new DHQ deserves special consideration. Over the years, both AABB and FDA placed requirements on donor screening that inevitably resulted in adding more and more questions. A key goal of the DHTF was to reduce the length and complexity of the questionnaire and to provide standardized donor educational information. In order to remove a number of specific questions about signs and symptoms of HIV from the DHQ, FDA agreed to allow those signs and symptoms to be moved from the questionnaire to the DEM. In addition, since as many as one-third of the questions referenced the terms, "sex, sexual contact, or sexual partner," and since the specific meaning of those terms was often misinterpreted, the DHTF decided to add detailed, graphic definitions of "sex" as it relates to risk factors for HIV, including vaginal, oral, and anal sex. Other information added to the DEM included a brief description of the donation process, lists of HIV risk behaviors, a brief description of testing, and an admonishment to provide accurate and complete answers. Unfortunately, over time as local BCEs added their local information (e.g., risks of donation, information on iron depletion and miscellaneous statements) to that of the AABB in order to house all information on a single document, these documents became anything but "standardized." Of note, many of these elements are considered required elements for donor IC, specifically a description of the process, the risks, benefits, and alternatives, use of the donated product/samples in research, ownership of donated samples/components, notification and possible disclosure of test results, and placement on a deferral registry. Since the essence of IC is the clear and understandable communication of outcomes and risk associated with blood donation, it is imperative that the DEM and IC documents meet this standard. Townsend *et al.* carried out a comprehensive survey of content and reading level of informed consent and related educational materials in US and Canadian centers, and demonstrated wide variation between centers in the length, completeness, and complexity of the documents. Further, documents were written at high reading levels (IC: 7.8–16.0 grade; DEM: 6.7–10.9 grade) using jargon most people would not understand, and were lengthy (DEM 649–2743 words; IC 131–996) resulting in a reading burden that deters donors from reading the critical information contained within, a finding confirmed by Wehrli and others.<sup>35,71</sup>

### **Method of administration**

In response to the HIV epidemic, FDA not only added a large number of screening questions but further required that specific questions had to be asked and answered verbally in a face-to-face setting with the donor. This type of questioning was thought to assure

honesty on the part of the donor, allowing the historian to directly observe the donor to identify any hesitancy or other indications the donor might be confused or less than honest. In contrast, studies by survey experts show that the opposite is true. Donors are more likely to honestly answer questions of a sensitive nature when presented anonymously.<sup>72</sup> It was for this reason that the DHTF designed the new DHQ to be self-administered. This approach was endorsed by a unanimous vote of the FDA Blood Products Advisory Committee. As a next step, the DHTF envisioned the application of computer-assisted self-interview (CASI) to expedite completion of the self-administered questionnaire and provide yet another layer of anonymity. Since the mid-2000s, most BCEs have implemented CASI screening of some sort. In recent years, donor prescreening has further expanded to allow donors to log on to a secure network from their home or work computer where they can self-complete an online questionnaire. The screening must be completed on the day of donation, and typically a bar code or other label may be generated for the donor to bring into the donation site. Using the bar code, the health screener is able to locate and review the completed questionnaire and to verbally follow-up on any questions that may require further clarification. As expected, self-assessment, whether written, by CASI, or online, enhances donor satisfaction with the screening process, reduces errors and omissions, and provides the confidentiality to ensure more accurate information.<sup>73,74</sup>

During the screening interview, if a donor becomes aware of disqualifying information or feels too embarrassed to answer, he may exit the donation site at any time. In addition, donors are provided a secure phone number and encouraged to contact the blood center immediately if they have an adverse reaction, if they become ill, or if they have second thoughts about the safety of their donated blood.

### Vital sign measurement

Although often referred to as a “mini physical exam,” the predonation health check is largely historic, not evidence-based and restricted to measurement of vital signs including temperature (*T*), pulse (*P*) rate and regularity, and blood pressure (BP). Hemoglobin or hematocrit is measured to prevent the collection of blood from an already anemic donor and, in some cases, to set collection parameters in automated collections. There is no uniform assessment of vital signs. In 2009, the 26th edition of AABB Standards for Blood Banks and Transfusion Services (BB/TS) eliminated the requirement for measurement of *P* and BP based on scientific evidence and recommendations from recognized experts in transfusion medicine and cardiology that these vital signs did not correlate with any risk of postdonation adverse events. However, the 2015 Final Rule issued by FDA set strict criteria for vital signs and for hemoglobin/hematocrit (at least 12.5 g/dL or 38% hematocrit for females and at least 13.0 g/dL or 39% hematocrit for males).<sup>75</sup> 21CFR630.10(f)(2) requires that the donor’s systolic blood pressure be between 90 and 180 mm of mercury, inclusive, and diastolic between 50 and 100 mm, inclusive. A donor with measurements outside these limits may be permitted to donate only when the responsible physician examines the donor and determines and documents that the health of the donor would not be adversely affected by donating. The CFR further requires (§630.5(b)(1)(i)(A) and (c)(1)(i)(A)(1)) that this determination may not be delegated and must be performed on site with the donor. Pertaining to pulse, §630.10(f)(4) states that “the donor’s pulse must be regular and between 50 and 100 bpm inclusive. A donor with an irregular pulse or measurement outside these limits may be permitted to donate only when the responsible physician determines and documents

that the health of the donor would not be adversely affected by donating.” A telephonic consultation by a physician is permissible for the evaluation of an unacceptable pulse.

In contrast, BCEs in other countries have widely varying rules ranging from no requirements for pulse and/or blood pressure to measurement only under specific conditions. In 2019, Canadian Blood Services (CBS) discontinued deferring whole blood (WB) donors for high or low BP, but continued measuring BP in first-time donors. In spring 2020, both CBS and Hema-Quebec stopped measuring BP in first-time and apheresis donors. The United Kingdom national health service blood program similarly dropped the requirement for obtaining vital signs other than temperature prior to collection. Other countries either do not require vital signs or make them voluntary, while they require measurement only in select populations.<sup>63</sup>

A final step of the donor assessment is the inspection of both arms for any evidence of previous intravenous drug use; at the same time, the antecubital areas can be assessed for any evidence of a skin condition that could compromise the sterility of the phlebotomy site, such as infections or skin lesions.

## Blood collection

### Whole blood

Whole blood (WB) collection represents the most common type of blood donation. A unit of WB can be easily processed into a number of components including packed red cells (PRBCs), whole blood derived platelets (WBDPs), plasma, and cryoprecipitate. For this reason, it may be the only blood product collected, especially in developing countries where access to automated collection devices is limited. The WB process is fairly quick, taking only around 45–60 minutes from registration, through medical screening, physical assessment, aseptic scrub, venipuncture, and recovery. Following the establishment of donor eligibility by medical and physical screening, the donor is placed in a comfortable position on a donor chair, cot, or bed. The phlebotomist examines both arms to determine which vein(s) appear best suited for a successful needle insertion. The application of slight pressure using a tourniquet or blood pressure cuff inflated to 40–60 mm mercury may assist in enhancing the prominence of the veins to identify the best candidate veins. Once a suitable vein is selected, the antecubital area at and around the needle insertion site is thoroughly scrubbed using an antiseptic solution and allowed to dry. After clamping the collect line, the needle is inserted using a swift, clean motion and secured in place using medical grade tape. The line is unclamped allowing blood to flow by gravity into a diversion pouch. The diversion pouch serves at least two functions: the skin plug, with any resident bacteria, is diverted away from the collection pouch reducing potential bacterial contamination of the product; after being sealed off, the diversion pouch then serves as a reservoir for blood to be collected into tubes for testing. Donor blood is then directed into a closed collection set with very frequent or continuous agitation to mix the blood with anticoagulant. WB collection sets are composed of multiple bags steriley connected. The primary bag contains an anticoagulant; satellite bags contain red cell storage solution and/or are used in the separation of blood into components.

### Postdonation care

Following collection of the designated volume of blood, the tubing is sealed from the donor, and the needle is removed from the arm and discarded without recapping. Gauze is placed over the needle

insertion site and direct pressure is applied. The donor is instructed to keep the pressure bandage on and avoid strenuous activity for a prescribed amount of time and to increase fluid intake. Donors are asked to remain in the refreshment area where they are provided snacks and beverages to begin the rehydration process. Donor adverse events such as vasovagal reactions are most likely to occur in the few minutes immediately postdonation, so donors are typically asked to stay in the area for at least 15 minutes, where they can be observed. Postdonation care instructions are provided describing actions to take if the donor feels unwell after donation, becomes ill in the week or two after donation, or has second thoughts about the safety of his or her donation.

### **Component separation**

WB may generally be stored without separation for up to 21 days. WB may be separated by centrifugation into PRBC, WBDP, and plasma components according to density. This allows longer shelf life, better inventory management, and more choices and resources for patient care. The capacity to provide patients with the different blood components they require is still limited in low-income countries: 37% of the blood collected in low-income countries is separated into components, 69% in lower-middle-income countries, 95% in upper-middle-income countries, and 97% in high-income countries.<sup>63</sup>

Using a “closed” separation system that is never open to outside air is important to prevent contamination with microorganisms during collection and component separation. Centrifuge-separated components are transferred from the original whole blood draw container to satellite bags through integrally connected plastic tubing. Adding nutrients and saline to the RBCs permits storage at 1–6 °C for up to 42 days (see Chapter 12). The preservative solution may be held steriley in one of the integral transfer bags until the plasma is separated off into a satellite bag after centrifugation, after which the solution may be added to the PRBC remaining in the original bag. Beginning in 2008 and moving through 2019, the production and transfusion of RBCs has been on a steady decline, with a 6% decline from 2017 to 2019. On a positive note, the discard of both WB and PRBC declined by almost 22% over that same time period.

Leukocyte reduction (LR) is accomplished by passing whole blood or PRBC through filters that reduce the number of white blood cells (WBCs). Ideally, this should be done as soon after collection as possible, but no longer than 72 hours. Leukoreduction has been shown to be more effective when done before storage because some of the adverse effects of transfusion are caused by factors produced or released by leukocytes during storage. Although LR can reduce untoward events, significant amounts of WBCs remain in LR products (up to  $5 \times 10^6$  WBCs/unit in the United States and  $1 \times 10^6$  in Europe). Automated apheresis collections often have technology that produces LR products during the collection process.

WBDP may be made by several methods. In the United States, an initial “soft” spin (slower speed) separates platelet-rich plasma (PRP) from the RBCs, and then a “hard” (faster speed) spin separates the platelets from the plasma. In Europe and many areas of the world outside the United States, “buffy-coat” platelets are produced by using first a “hard” spin to separate all cellular elements from the plasma. Then, the buffy coat containing the platelets, white blood cells, and a significant contaminant of RBC is further processed to isolate the platelets. In the United States, in 2019 WBDP represented only about 10% of platelets transfused; however, there was an almost 200% increase in the transfusion of WBDP over 2017. Additional information about platelet preparation and storage may

be found in Chapter 17. Traditionally, platelets were stored for 5–7 days at controlled room temperature, with gentle agitation, with the expiration dependent on the bacterial detection and/or removal processes used. In 2019, FDA issued guidance specifying conditions under which processes for mitigation of bacterial contamination of platelets could be considered for extended storage up to seven days.<sup>76</sup> Although currently not generally licensed, other options for platelet storage include the storage of platelets at refrigerator temperature for use in situations such as trauma and active bleeding, and other uses are being actively explored.<sup>77,78</sup>

Plasma separated and frozen within eight hours is called fresh frozen plasma (FFP), whereas plasma frozen up to 24 hours is known as PF24 or FP24. Each contains coagulation factors in quantities adequate for most patient indications. FP24 represented only 15% of the 4.1 million units of plasma transfused in the United States in 2004 but 47% of the 5.9 million units of plasma produced in 2011.<sup>56</sup> Data collected from hospitals no longer distinguish between FFP and FP24 as they are considered equivalent. This increase may be in part due to the concern about transfusion-related acute lung injury (TRALI) and the exclusion of women who have been pregnant from plasma donation. Blood centers are attempting to make plasma from all eligible donors of the needed types, even though the plasma logically cannot always be frozen within eight hours. More volunteer plasma is typically drawn than needed for transfusion. Plasma stored for as long as 72 hours before freezing may be sold for fractionation (discussed further in this chapter).

After separation from the RBCs, plasma may be pooled and treated with solvent and detergent (S/D) to decrease the risk of transfusion-transmitted disease. In addition, the pooling enables quality control of coagulation factors per lot, and the dilution dramatically decreases the risk of TRALI.<sup>79,80</sup> Other pathogen reduction techniques may be applied (see Chapter 42). Only a small amount of S/D-treated plasma is used in the United States due to economic factors and possible safety concerns regarding pooled products.<sup>81</sup> However, some countries in Europe rely on this process for the majority of plasma transfused.<sup>79</sup>

Preparation of cryoprecipitate involves thawing FFP at 4 °C in a circulating water bath, followed by centrifugation so that the supernatant plasma can be drained into an integrally attached storage bag (see Chapter 21). The remaining cold-precipitated material is then refrozen at 18 °C or less and stored for up to 1 year. Approximately 1.7 million units of cryoprecipitate were prepared in the United States in 2011, an increase of 16% since 2008.<sup>56</sup> Cryoprecipitate production in the United States has steadily increased since 2008, increasing to 1.86 million in 2015, 2.16 million in 2017, and 2.18 million in 2019. Cryoprecipitate infusion declined in the recent past, but increased from 2017 to 2019, probably related to use in trauma and massive transfusion situations.<sup>1</sup> In some regions in Europe, cryoprecipitate is not produced, as the use of factor concentrates for inherited bleeding disorders and plasma or fibrinogen concentrate for hypofibrinogenemia has proven satisfactory.

### **Apheresis Collection**

Another common type of blood collection, especially in industrialized countries, is apheresis. In the United States in 2019, 16.5% of red cells and 94% of platelets in the supply were collected using apheresis technology.<sup>1</sup> Apheresis collections provide a number of advantages. Many, though not all components are leukoreduced automatically during collection, alleviating the manpower and cost to filter components after the fact. Apheresis technology provides a

way to collect multiple components in a single setting—either a variety of different components or multiples of the same component. By using selection algorithms, BCEs are able to collect specific components based on inventory needs and donor attributes. Component selection criteria are based on a number of factors including:

- Donor factors—sex, height, weight, hematocrit, and platelet count—used to program the device to determine volume to be collected
- Risk of plasma containing antibodies (anti-HLA and anti- HNA) that can cause TRALI
- Donor blood type—Group O individuals may be selected for red cells and double red cells; Group A and AB individuals are sought after for platelet and plasma donations
- Time—the amount of time a donor has available to donate on any given day

So, for example, a large male with a high platelet count may be able to give three apheresis platelet units or a double RBC depending on blood type, while a female with TRALI risk may be restricted to an RBC or double RBC depending on weight, height, and hematocrit. In 2001, the FDA published a guidance on collecting RBC by automated apheresis methods, providing restrictions on the cumulative loss of red cells and plasma over a rolling 12-month period.<sup>81</sup> In addition, there is a 112-day deferral for donors giving double RBCs, which precludes donation of any component (red cell, platelet, or plasma). For donors giving a combination of a single RBC with any other component type, the usual 56-day deferral applies for another RBC donation, although donations of platelets and plasma may be allowed prior to 56 days (see Chapter 24).

### The collection process for source plasma: screening, phlebotomy, choice of product, collection, and testing of source plasma donors

The handling of source plasma donors differs in several respects from the handling of whole blood and apheresis donors providing blood products for hospital transfusion. Source plasma is collected by apheresis specifically for further manufacture into biological derivatives, in contrast to “recovered plasma,” which is the plasma remaining after RBC production from whole blood donation. About half of the recovered plasma collected for fractionation comes from Europe. Many European countries have maintained national programs that fractionate plasma from recovered plasma from volunteer donors. However, the demand for biological therapies worldwide requires the additional supply from remunerated or compensated (paid) donors. Source plasma is collected (in order of amount per population) in the United States, Austria, the Czech Republic, Germany, and Hungary from compensated donors. Smaller programs are found for collections from noncompensated donors (in order of number per population) in Australia, the Netherlands, Denmark, France, Sweden, and Belgium. In 2010, source plasma accounted for approximately 75% of plasma for further manufacturing worldwide, with recovered plasma accounting for the remainder. In 2012, approximately 26.2 million donations of source plasma were fractionated along with approximately 9 million liters of recovered plasma. Source plasma collections in the United States increased to greater than 53. 5 million donations in 2019; in the midst of the pandemic, it fell to 43.4 million collections in 840 centers in 2020. In 2019, in Europe 88 PPTA-certified centers collected 2.988 million units (website: PPTAglobal.org). The requirement for immune globulins has largely driven this increase

(data from [www.pptaglobal.org](http://www.pptaglobal.org)). The United States provides a large proportion of the world’s source plasma requirements (approximately 67%) for two reasons: first, under FDA regulations, source plasma donors can donate more plasma, more frequently, than is the case in most countries. Second, the United States has a well-developed source plasma industry that has invested in a network of centers that compensate donors for their time and inconvenience.

In both the United States and the European countries, source plasma is collected in fixed sites without mobile collections. Either the Autopheresis-C (Fresenius) or the PCS-2 (Haemonetics) is used for most collections. Recruitment is typically by word of mouth, newspapers, radio, and posters, although more recently social media has been employed. The donor groups are heterogeneous. Sites near campuses that can attract student donors are common, and payment varies. More money is paid for donations from immunized donors for hyperimmune plasma (rabies immune plasma, tetanus immune plasma, hepatitis B immune plasma, anti-D plasma, etc.) and by those from donors with disease-state antibodies needed for diagnostic manufacture.

In addition to specific national regulations and guidance, in the United States and Europe an International Quality Plasma Program (IQPP) of the PPTA is also followed. The program has been judged successful because the major manufacturers in Europe and the United States will only use plasma from these programs. This self-regulation includes the following:

- *Community-based donors:* Donors must have a permanent address in the vicinity of the center.
- *Qualified donor standard:* Each donor’s plasma is used only after two medical screenings, and required viral testings are successful and less than six months has elapsed between the two donations.
- *National Donor Deferral Registry (NDDR):* All donors deferred for viral marker testing are entered into an NDDR that must be checked before each *applicant* (i.e., not qualified) donor is accepted.
- *Viral marker standard:* This requires centers to keep their viral marker rates below established levels.
- *60-day inventory hold:* This is required so that units from a donor with a subsequent positive test or disqualifying information can be removed before being pooled (part of the Q-SEAL Standards for fractionators). FDA had incorporated the hold into the regulations. During the COVID epidemic, it temporarily reduced the hold to 45 days.

The community-based donor standard discourages transient individuals from donating as this population has been associated with an increased incidence of infectious disease markers. In the United States, an electronic current donor registry run by PPTA prevents cross-donation in multiple centers that exceeds the limits. A minimum weight of 50 kg (110 lbs) must be met. At each donation, the hematocrit and total protein are determined from a finger-stick blood sample. The hematocrit must be 38% or greater (recently increased by FDA to 39% for males), and protein must be 6.0 g/dL or greater. The protein is measured using a refractometer (the only device currently available to meet the FDA requirement).

Initially, the donor is subjected to an extensive interview similar to that used for blood donors, followed by a brief physical examination by a physician. In the United States, a physician substitute may be used, such as a nurse, paramedic, or other health professional trained to do the physical exam and operating under the guidance of the center medical director. The physical examination consists of an external eye, ear, and nose exam, with an examination of the throat with a tongue blade and light. Lymph nodes in the neck area

are palpated. Auscultation of the back and front of the chest for lung abnormalities is followed by auscultation of the heart. The abdomen is examined for liver and spleen enlargement. A short neurological and extremity review completes the examination. In some centers, a urine dipstick for protein and glucose is also performed. After passing these initial tests, the donor can donate as an *applicant* donor. Collected units are held until the donor has two successful donations without any positive infectious disease markers. If the donor does not return for the second donation within six months, the plasma is considered an "orphan" unit and cannot be used for injectable product. In Europe, the first donation is for sample only. In the United States, the donor must pass initially, and every four months, tests that include serum protein electrophoresis, total protein, and syphilis. The physical examination with full interview is repeated annually or if the donor has not presented in the past six months. An informed consent must be executed on the first donation and again whenever the consent is changed.

The donor donates according to a nomogram: body weights of 50–67.5, 68–79, and greater than 79 kg can donate 625, 750, and 800 mL plasma, respectively, not counting anticoagulant. This nomogram was promulgated by the FDA in 1992; recently, it has been subject to reconsideration. It has two basic drawbacks: (1) it does not take into consideration the donor's height or hematocrit which influences the total plasma volume; (2) being discontinuous, donors will donate much more plasma at some weights with a very minimal change in weight. A retrospective analysis showed that the ratio of the plasma volume collected compared to the total plasma volume ranged from 15 to 42% in a study of over 100,000 donations. The ratio was inversely proportional to weight which meant that lower weight donors donated a higher percentage of their plasma volume. The authors made two key points: (1) despite the good safety records of the 1992 nomogram, there were significant differences in risk of a hypotensive reaction based on proportion of plasma volume drawn; (2) the efficiency of plasma collection was suboptimal with many large donors donating a small percentage of their plasma volume.<sup>82</sup> Accordingly, the Haemonetics Corporation developed a personalized nomogram that they showed optimized collection yields without increasing adverse events. This nomogram was continuous and used BMI (height and weight) and hematocrit to achieve a collection target of 28.5% of plasma volume with a maximum removal of 1000 mL.<sup>83</sup> This is now becoming available in the United States. Some variation of this might be available in the future from other manufacturers.

Standards from the quality program of the Source Plasma industry group PPTA require infusion of saline at the end of the donation. After a brief rest in the donation chair, the donor is allowed to collect the compensation (usually electronically added to a debit card) and leave. Refreshment and recovery areas are not common in plasma donor centers. In the United States, donors are allowed to donate no more than twice in seven days, with at least two days between donations. A donor could theoretically donate 104 times per year (65–83 L, depending on donor weight).

The Council of Europe recommendations limits the amount of plasma collected per session to 600 mL, not counting anticoagulant.<sup>84,85</sup> There is also a 15 L annual limit. German national guidelines set in 1999 allowed donations of up to 650 mL plasma twice weekly with an annual limit of 25 L. Donors must weigh a minimum of 50 kg and have a hemoglobin level of 12.5 g/dL in females and 13.5 g/dL in males. Immunoglobulin G (IgG) along with total protein is measured at every 5th donation and must be at least 115 g/L and 6 g/dL. CBC is performed initially and at every

15th donation. Donors are also examined by the physician at every 15th donation as opposed to annually by a physician or physician substitute in the United States. Europe also has rules for permanently excluding donors who have a pattern of low protein and/or IgG.

To determine if less restrictive donation standards (closer to those in the United States) were safe, 21 German plasma donor centers participated in a study known as SIPLA. They found that donors weighing greater than 70 kg could donate 850 mL each session up to 60 times per year with appropriate monitoring (up to 51 L total per year). Currently, there is an effort to change European requirements to be consistent with these findings.<sup>86,87</sup>

Each donation by a source plasma donor is tested serologically for HIV antibody, HBSAg, and hepatitis C antibody. Nucleic acid testing is done on each donation for HBV, HCV, and HIV. A positive test results in deferral. This is usually regardless of results of confirmatory tests that are performed for donor counseling. In addition, tests for hepatitis A and parvovirus are also performed. If positive, the units are discarded. Donor and health department notification are required for hepatitis A positivity along with look-back and look forward.

Due to the investment in the applicant's first donation, donor centers cultivate long-term donors who donate often. Some very dedicated donors are given vaccines to provide rabies immune plasma, tetanus immune plasma, hepatitis B, or other immune plasma in order to make specialty immunoglobulins. Rh-negative donors who are not of childbearing potential can become donors of Rh-immune plasma for manufacture into Rh immune globulin. This is done by immunization with carefully "qualified" and characterized D-positive red cells. Long-term consistent donation is particularly important for these specialty donors.

There are additional requirements for donors receiving red cell immunizations, including physician performance of the initial physical examination and a separate informed consent, physician presence when immunizations are given, and a specific physician approval of the red cells to be injected. Meticulous preparation of the red cells for immunization occurs. Red cells from whole blood donors are frozen and collected over a year. Only when a year's viral marker testing remains negative, the cells can be deglycerolized and used to immunize a donor. When the red cells from a specific whole blood donor are first used, they are given to 1–3 recipients for a year. When viral marker testing is negative throughout the year for those recipients as well as the donor, the cells are then "qualified." Plasma from these donors is used to manufacture Rh immune globulin, and the pool must contain a sufficient concentration (titer) of antibody to allow acceptable product to be made.

Rates of viral marker test positivity in donors are monitored, and plasma centers are expected to take steps to reduce levels when they rise above predetermined alert levels. Source plasma donors tend to be more likely male, younger, and larger in size than volunteer donors, probably reflecting the more demanding donation program frequency. In addition, they are more ethnically diverse. Some of these factors act to increase prevalence and incidence of viral diseases. Socioeconomic status and compensation programs might play a role as well. With the push toward voluntary donations worldwide, the payment of plasma donors has been criticized. Nevertheless, monetary compensation of source plasma donors has had an enviable safety record for the last 20 years since many of the additional controls have been put in place. The viral reduction and inactivation treatment of the final product provides additional safety. Thus, the layers of protection operate differently in source plasma programs than in the volunteer programs, but are still

highly effective in preventing infectious units from entering plasma pools and in ensuring safety of the final product.<sup>88–90</sup>

### Blood collection changes with patient needs

The clinical use of blood transfusion has developed tremendously over the last century, and blood collection has changed correspondingly. Transfusion of components, for example, has been both encouraged and facilitated by the development of automated collections that allow precision in the selection of the proper component and the donor to give it. The ongoing improvement of safety has been facilitated by improved donor screening as well as testing. Consideration for the health of the donor as well as the recipient remains critical to the willingness of millions of volunteers to donate blood and plasma freely and repeatedly.

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## CHAPTER 5

# Blood donor testing

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### Background

Each and every donation is tested by an array of assays including ABO and Rh, antibody screen, and infectious disease testing. Other serological testing may include anti-A/B antibody titers of group O whole blood and apheresis platelets to reduce the risk for hemolytic transfusion reactions from passively transfused antibodies when significant amounts of incompatible plasma may be transfused. Routine testing of female platelet donors with history of pregnancy for the detection of human leukocyte antigen (HLA) antibodies helps mitigate transfusion-related acute lung injury (TRALI).

Transfusion-transmitted infection (TTI) has become an extreme rarity due to more effective donor selection, strict US Food and Drug Administration (FDA) regulations, and extensive laboratory testing for transmissible diseases, which has made significant progress over the past four decades. Required testing for transfusion-transmissible diseases includes syphilis, hepatitis B virus (HBV) (in three different assays), hepatitis C virus (HCV) and human immunodeficiency virus (HIV) with two different assays, human T-cell lymphotropic virus (HTLV), Chagas' disease, West Nile virus (WNV) and, until 2021, included Zika virus (ZIKV). The redundancy of assays for HIV, HBV, and HCV includes both serological assays and molecular screening with nucleic acid tests (NATs). As of May 2020, testing of all blood components for babesiosis is required for 14 states or districts considered to be endemic for this transfusion-transmissible infection.

Bacterial testing of all platelet products is routinely performed unless they are pathogen-reduced. Other laboratory testing that may also be performed includes serological testing for cytomegalovirus (CMV), platelet antigen typing, and extended red blood cell (RBC) antigen typing.

### Safety of the blood supply

Although the goal of a "zero risk" blood supply is, by definition, impossible to achieve, risks from transfusion-transmitted infection are quite rare. Each unit of whole blood or apheresis components undergoes an extensive battery of tests (Table 5.1).

### Blood component testing

Testing of voluntarily donated blood is carried out on blood samples obtained for this purpose at the time of donation. Blood is collected for testing in an integrally connected diversion pouch,

and the maximum volume of blood that can be retained for testing is about 50 mL. (Of note, the FDA allows plasma collection centers to perform the infectious disease screening on the collected plasma, thereby minimizing additional red cell loss from these paid donors.) Automated systems with high-throughput testing for RBC typing and antibody detection are typically used in blood centers (Table 5.2).

### ABO typing

The most common cause of a fatal hemolytic transfusion reaction is the administration of ABO-incompatible RBCs.<sup>1</sup> Therefore, the ABO and Rh typing of the donor units are of critical importance. Usually, ABO and Rh typing are carried out together, along with RBC antibody detection testing, because these tests involve RBC antibody–antigen interaction. While ABO and Rh typing maybe done in a variety of modalities, virtually all blood centers use automation (Table 5.2). The choice of the particular method and instrument will depend on throughput requirements at the donor center testing laboratory. Most systems incorporate room temperature testing and centrifuges or other manipulation to foster agglutination. Following a wash or dispersion step, the resulting mixture is observed either visually or by an instrument.

### Rh typing

The Rh type refers to the D antigen (also Rh or RhD). It is the second most immunogenic RBC antigen next to the ABO blood group type. Alloantibodies to the D antigen can cause severe or fatal hemolytic disease of the newborn or transfusion reactions. In particular, the transfusion of Rh-positive RBC-containing products should be avoided whenever possible to Rh-negative women of current or future childbearing age.<sup>2</sup>

Donor centers typically use automated platforms to perform D antigen testing using typing reagents, which must also be sensitive enough to detect a serological "weak D" individual to label their blood products as Rh positive. Additional testing is recommended in the patient setting, particularly for women of childbearing age. In the blood center setting, weak D testing is performed by including the antihuman globulin phase of serological testing, which can be done manually or by certain automated testing platforms. Antisera reagents that are polyclonal high protein, chemically modified low protein, or a combination of polyclonal-monoclonal

**Table 5.1** Donor Testing Performed in the United States

Blood Grouping Tests	Methodology
ABO Rh typing	Agglutination
Red cell antibody detection	Serologic
Transmissible disease screening tests	Microhemagglutination, EIA, or RPR
Syphilis (treponemal or nontreponemal antibody)	EIA or CMIA
Hepatitis B surface antigen (HBsAg)	EIA or CMIA
Hepatitis B core antibody (anti-HBc)	EIA or CMIA
Hepatitis C antibody (anti-HCV)	Pooled PCR or TMA
HBV/HCV/HIV NAT	EIA or CMIA
HIV-1/2 + group O antibody (anti-HIV-1/2 + O)	EIA or CMIA
HIV antigen/antibody combination test	
HTLV-I/II antibody (anti-HTLV-I/II)	EIA or CMIA
WNV NAT	Pooled or IND, PCR or TMA
ZIKV NAT <sup>a</sup>	Pooled or IND, PCR or TMA
<i>Trypanosoma cruzi</i> antibody (anti- <i>T. cruzi</i> ) <sup>b</sup>	EIA or CMIA
<i>Babesia</i> NAT <sup>c</sup>	Pooled or IND PCR or TMA
Bacteria (platelets) <sup>a</sup>	Culture +/- secondary bacterial testing

HCV, hepatitis C virus; HTLV-I, human T-cell lymphotropic virus type I; HTLV-II, human T-cell lymphotropic virus type II; WNV, West Nile virus; ZIKV, Zika virus; EIA, enzyme immunoassay (or similar ELISA, enzyme-linked immunosorbent assay method); CMIA, chemiluminescent microparticle immunoassay; PCR, polymerase chain reaction; NAT, nucleic acid test; TMA, transcription mediated amplification; IND, individual; RPR, rapid plasma reagent by particle agglutination.

<sup>a</sup> U.S. testing requirement for Zika withdrawn by FDA in 2021.

<sup>b</sup> Required on first donation only.

<sup>c</sup> Required in endemic states in accordance with FDA Babesia Guidance to Industry.

**Table 5.2** Automated Systems for Red Cell Typing and Antibody Testing of Donated Blood<sup>d</sup>

Company	Product	Type of Assay
Grifols	Erytra	Gel-phase test
Immucor	NEO Iris	Capture solid-phase test
Immucor	Echo	Capture solid-phase test
Bio-Rad	Tango	Solid-phase test
Ortho	Provue/ID-MTS Gel	Gel-phase test
Ortho	Autovue Innova	Column-agglutination test (glass beads)

<sup>d</sup> All of these systems can also be used for crossmatching.

low-protein reagents are available to detect weak D RBCs, so additional testing of donors who type as Rh negative is no longer necessary. Serological testing, however, can fail to identify rare donors with low antigen expression (weak D and Del) resulting in some weak D+ units being labeled as D-.<sup>3</sup> This may result in inconsistency with historical test results and consideration of *RHD* testing by DNA methods, as well as loss of donor products because of the inability to label donor RBCs that give variable results in serological D testing.

## Red blood cell antibody detection

Serum or plasma from donors is tested for unexpected antibodies to RBC antigens using methods that will detect clinically significant antibodies.<sup>4</sup> Approximately 0.5% of donated units will test positive in the antibody detection test.<sup>5</sup> While most antibodies are due to exposure from previous pregnancy or transfusion, RBC antibodies may rarely be naturally occurring or detected in a donor with autoantibodies. They may also rarely result from residual passive anti-D antibody detectable in a recently pregnant female who received prophylactic Rh immunoglobulin administration.

Transfusion of RBC alloantibodies in components containing donor plasma may result in shortened RBC survival or hemolysis of recipient RBCs, or rarely cause hemolysis of other transfused donor RBCs.<sup>6</sup> Although blood components that contain RBC antibodies can be used for transfusion, they must be labeled with antibody specificity, and the receiving hospital transfusion service would only transfuse to antigen-negative patients or wash the product. Practically, this is usually limited to blood centers offering anti-Rh(D) antibody positive Rh(D) negative units, intended for transfusion to Rh(D) negative recipients, but since the patient may now demonstrate the passive antibody, subsequently requiring full crossmatch, many hospitals will no longer accept such units. Therefore, donors with alloantibodies may be diverted to become reagent blood donors or donate for research.

To demonstrate clinically significant antibodies, the donor plasma is incubated at 37 °C with reagent RBCs in one of the following media: saline, albumin, low-ionic-strength solution, polyethylene glycol, or polybrene. Currently, gel, solid-phase systems, and affinity columns may also be used for RBC antibody detection.<sup>7,8</sup> Use of automated platforms facilitates ABO and D testing and antibody screening on a single instrument. In contrast with the patient setting, the use of either donor plasma or serum may be used to test against either pooled or individual reagent RBCs of known phenotypes.

## ABO antibody titers

Blood products may be tested for the presence of high-titer anti-A (most commonly) and/or anti-B to prevent hemolytic transfusion reactions secondary to ABO incompatibility of isoagglutinins in incompatible plasma. In the United States, this may be performed with a variety of methods and cutoff values as there is no universally agreed upon standard for “low titer.” The two settings where this is routinely applied include (a) the use of group O donors for whole blood and (b) apheresis platelet products.

Low titer group O whole blood is being used increasingly in trauma or acute blood loss.<sup>9</sup> Because group O whole blood has a full unit of plasma, which is incompatible with RBCs of nongroup O recipients, facilities must consider which patients will be eligible for this product, how many units can be received by eligible patients, and how to monitor for adverse events? In addition, transfusion services must have a policy concerning transfusion of significant volumes of plasma containing incompatible ABO antibodies.<sup>10</sup> In practice, however, clinically significant hemolysis following “low titer” WB infusion is rarely observed. Specifically, in a clinical trial using up to four such units in the civilian setting, no hemolysis or complications were observed.<sup>11</sup>

Clinically significant hemolysis is a rare but potentially severe complication of administering ABO-mismatched platelet transfusions. In particular, single-donor platelet products from group O donors (apheresis platelets) are most commonly implicated because of the presence of unusually high titers of anti-A in the plasma.<sup>12</sup>

Blood centers may perform titers on group O platelet components to mitigate risk of hemolysis when non-ABO-identical platelet transfusion is required.<sup>13</sup>

A variety of methods and cutoff values are currently used in the United States to determine which products are high titer and therefore at higher risk for causing a hemolytic reaction. Group O whole blood components may be tested for the presence of high-titer anti-A and/or anti-B using a specified dilution with either group A, group B, or pooled group A and B RBCs. Arbitrary selected titers

range from 1:50 to 1:256 with continued debate on the titer and which antibody class, IgM or IgG, to test to ensure optimal safety. Many centers test only for anti-A titer because there is considerable concordance of anti-A and anti-B titers for most donors. Testing may be performed either manually or on an automated platform. Components that are low titer are labeled appropriately.

Similarly, liquid or thawed group A plasma may be used for emergent transfusions. While some centers offer tiered plasma components, many simply use without tiering; a multicenter survey documented safety of untiered products (from male donors) in this setting.<sup>14</sup>

### **Direct antiglobulin testing**

The direct antiglobulin test (DAT) is not routinely performed on blood donors; however, a positive DAT on a unit of blood is occasionally identified during the course of Rh(D) type confirmation or compatibility testing in the hospital transfusion service. Although this is a rare event in blood donors, it reportedly occurs in about 1 per 7000–14,000 donors.<sup>15</sup> Occasionally the positive DAT result is caused by a viral infection or autoimmune disease, including systemic lupus erythematosus and other disorders associated with antiphospholipid antibodies.<sup>16</sup> Although there is often no explanation for this positive test result in an otherwise healthy donor, there is evidence that this finding is associated with an increased risk for malignancy.<sup>17</sup>

Several methods of testing are available, including tube testing, column agglutination (gel testing), and buffered gel cards.

## **Testing for transmissible diseases**

### **General concepts of infectious disease testing**

The World Health Organization requires screening of all blood donations for HIV-1 and HIV-2 by either a combination of antibody–antigen or HIV antibodies, HBV by screening of hepatitis B surface antigen (HBsAg), HCV by screening of a combination of HCV antigen–antibody or antibodies, and syphilis by screening of *Treponema pallidum* (TP) antibodies.<sup>18</sup> Screening of blood donations for infectious diseases has been in place since the 1950s when syphilis testing became routine. The decision to implement a test and the strategy used in dealing with the test results are complex issues.<sup>19</sup> The presence of an asymptomatic phase of infection, the prevalence of the disease in the donor population, the likelihood of transfusion transmission as well as the viability of the infectious disease agent during component storage are important factors to consider. Over the past several decades, major advances in the quality of laboratory testing and response to emerging infections have significantly improved safety as demonstrated by declining rates of post-transfusion infection and reductions in estimated residual risk for such infections.<sup>20–23</sup>

In the United States, screening tests of infectious disease (pre-release tests) used in blood component manufacturing are classified as “biologics” by the FDA. Test sensitivity and specificity are of great importance with the FDA historically encouraging very sensitive tests. This results in a significant rate of false-positive results because typically there is low prevalence in the blood donor population of the screened analyte, resulting in a low positive predictive value. False-positive screening tests may be addressed by well-defined confirmatory test algorithms and approaches to donor notification, counseling, and follow-up of reinstatement protocols, when allowable per FDA guidelines.

Donated units of blood products must remain in quarantine until testing is completed. If tests are nonreactive, they are considered negative for the infectious disease marker, and the blood products from the associated donation may be released from quarantine and issued.

Serological tests for infectious disease are developed to detect antibody, antigen, or combined antibody–antigen for a particular agent. A commonly used screening test method in this category is the enzyme immunoassay (EIA) or the similar enzyme-linked immunosorbent assay (ELISA). These tests typically use viral lysates to capture the antibody, and adherent donor antibodies are detected with antiglobulin conjugate. For serological testing, the FDA typically allows repeat testing to diminish the rate of false positives. Specifically, serological tests with initially reactive screening results are retested in duplicate. If both repeat test results are nonreactive, the donor sample is classified as negative. However, if one or both repeat tests are reactive (termed repeatedly reactive), the products cannot be released for allogeneic transfusion and must be destroyed, the donor deferred, and confirmatory testing, if available, must be performed.

Direct methods of testing of RNA- or DNA-specific sequences for various infectious agents by nucleic acid testing (NAT) using either a polymerase chain reaction (PCR) or transcription-mediated amplification (TMA) have considerably advanced blood safety.<sup>22</sup> Samples may be tested individually or in pooled aliquots (minipools of six or more samples) or with a multiplex approach (i.e., NAT HIV/HCV/HBV). Because NAT is an expensive, yet sensitive technology, a pooling strategy decreases average cost but requires well-defined algorithms for individual testing, when a positive pool is detected. A combination of NAT and serological testing is required in the United States for HIV, HBV, and HCV as some pathogens may have insufficient concentrations of detectable nucleic acid either due to treatment or in very early or late stages of the infection.<sup>23</sup>

Additional supplemental or confirmatory testing is often required after a positive screening test result to determine the clinical significance of a positive screening result and subsequent donor management. This testing is typically performed using assays with different sensitivity and specificity characteristics compared with the initial screening tests. Not all screening tests have a confirmatory test, such as the test for antibodies to hepatitis B core antigen (anti-HBc), which is problematic for donor counseling and deferral. The results of confirmatory testing improve accuracy and predictive value of the screening test results, whereas supplemental tests may aid with product management and donor notification and eligibility for re-entry or reinstatement, if deferred from donation.

In the United States, most tests are mandated in the FDA Code of Federal Regulations (CFR) or under specific FDA Guidance documents and American Association of Blood Banks (AABB) standards. Blood product testing in general is highly regulated and typically performed by any number of automated testing systems available for infectious disease testing (Table 5.3).

Consideration when implementing new blood donor screening includes sensitivity and specificity of the assay, the disease prevalence in the donor population (which determines positive-predictive value), and the likelihood of transmission of the disease to blood recipients.<sup>19</sup> Tests that perform very well in a patient population have different ramifications in a normal donor population. For instance, a test with a specificity of 99.9% performed on 12 million blood donors will yield 12,000 false-positive test results. However, if the disease preva-

**Table 5.3** Automated Testing Systems for Infectious Disease of Donated Blood

Company	Device	Assay	HBsAg	Anti-HBc	Anti-HCV	Anti-HIV or Ag/Ab combo*	Anti-HTLV-I/II	Anti-T. cruzi	NAT	HBV/HCV/HIV	NAT	NAT	NAT	Syphilis	Anti-CMV
Ortho	VIP	EIA	X	X	X	X	X	X							
Abbott	Alinity	CMIA	X	X	X	X*	X	X							
Abbott	PRISM	ChLIA	X	X	X	X	X	X							
Grifols	Tigris/	NAT							X	X	X	X			
Procleix	Panther														
Roche	6800/8800	NAT							X	X	X	X			
Beckman	PK7300	MHA												X	X
Coulter															
Immucor	NEO	TPHA											X	X	

Ag, antigen; Ab, antibody; HBsAg, hepatitis B surface antigen; Anti-HBc, hepatitis B core antibody; HBV, hepatitis B virus; HCV, hepatitis C virus; HIV, human immunodeficiency virus; HTLV, human T-cell lymphotropic virus; WNV, West Nile virus; ZIKV, zika virus; CMV, cytomegalovirus; EIA, enzyme immunoassay; ChLIA, chemiluminescent immunoassay; CMIA, chemiluminescent microparticle immunoassay; NAT, nucleic acid test; MHA, microhemagglutination assay; TPHA, Treponema pallidum hemagglutination assay.

\*U.S. testing requirement for ZIKV withdrawn by FDA in 2021.

**Table 5.4** Sequence of Testing of Donated Blood

Pathogen	Screening Tests	Confirmatory/Supplemental Tests
HIV	Anti-HIV-1/2 + O; EIA or ChLIA	Positive NAT confirms reactive serology; otherwise, HIV-1 Western blot or immunochromatographic assay. If HIV-1 positivity is not confirmed, further testing may be performed using licensed or approved tests to diagnose HIV-2 infection.
	HIV antibody/antigen combination; CMIA	Positive NAT confirms reactive serology; otherwise, HIV-1 Western blot or immunochromatographic assay. If HIV-1 positivity is not confirmed, further testing may be performed using licensed or approved tests to diagnose HIV-2 infection.
HCV	HIV-1 NAT Anti-HCV; EIA/ChLIA or CMIA HCV NAT	No confirmatory test; correlation with serology indicated. <sup>c</sup> Positive NAT confirms reactive serology; otherwise, alternate EIA/ChLIA testing No confirmatory test; correlation with serology indicated. <sup>c</sup>
HBV	HBsAg; EIA/ChLIA or CMIA Anti-HBc; EIA/ChLIA or CMIA HBV NAT	If NAT negative, ID NAT required if anti-HCV EIA and ChLIA reactive. HBsAg neutralization test Positive NAT confirms reactive serology
HTLV	Anti-HTLV-I/II; EIA/ChLIA or CMIA	No confirmatory test; correlation with serology indicated. <sup>c</sup>
Syphilis	TP test	Western blot (FDA) and live immunoblots (not FDA licensed)
(TP)	Non-TP test	Second FDA-cleared TP screening test
WNV	WNV NAT	TP-specific immunofluorescence or agglutination assays
ZIKV <sup>d</sup>	ZIKV NAT	No confirmatory test <sup>c</sup>
Babesia <sup>a</sup>	Babesia NAT	No confirmatory test <sup>c</sup>
T. cruzi <sup>b</sup>	T. cruzi; EIA/ChLIA or CMIA	ESA (FDA)

Source: Adapted from Fung M.K. *Technical Manual*, 19th edition. Bethesda, MD: American Association of Blood Banks, 2017, pp. 166–167.

HIV, human immunodeficiency virus; HBc, hepatitis B core antibody; HBV, hepatitis B virus; HCV, hepatitis C virus; HTLV, human T-cell lymphotropic virus; TP, *Treponema pallidum*; T. cruzi, *Trypanosoma cruzi*; NAT, nucleic acid amplification test; EIA, enzyme immunoassay; ChLIA, chemiluminescent immunoassay; CMIA, chemiluminescent immunoassay; ID, individual (not pooled) testing; ESA, enzyme strip assay.

<sup>a</sup> Testing required in endemic states in accordance with FDA Babesia Guidance to Industry.

<sup>b</sup> Required on first donation only.

<sup>c</sup> Confirmatory testing not applicable for NAT; supplemental testing may be performed for purposes of donor notification and counseling.

<sup>d</sup> U.S. testing requirement for ZIKV withdrawn by FDA in 2021.

lence is 1 per 500,000 in a blood donor population, for example, only 24 people would truly have the disease in the 12 million tested. This means the positive predictive value (24/12,000) is 0.2% or a 99.8% false-positive rate. When the prevalence of the disease being screened for is low in the donor population, the implementation of tests with less than perfect specificity is fraught with challenges. Confirmatory testing should be available to distinguish those who are truly positive from those who are not, and effective systems must be in place to carry out the supplemental or confirmatory testing necessary to provide this information (Table 5.4). For donors who have a true positive test, complete and accurate information must be available describing the significance of the test result.

### Testing in resource-limited settings

In resource-limited countries or areas of the world highly endemic for an infectious disease agent, it may be more cost-effective to prescreen donors with rapid tests before collecting

their blood.<sup>24</sup> Serial testing strategies such as starting with the most prevalent disease like hepatitis B may then be followed by testing nonreactive donors for HIV, and then further testing for syphilis and HCV.<sup>25</sup> Rapid tests are often used.<sup>26–29</sup> An excellent comparison of rapid tests for hepatitis C has been reported.<sup>29</sup>

### Plasma for further manufacture: source plasma

Source plasma donors are collected by apheresis and must meet the requirements for allogeneic plasmapheresis donors, including infectious disease testing. There are a few notable exceptions from allogeneic blood donors in the United States. Because plasma for manufacture is subjected to pathogen reduction, regulators have not required tests for WNV and ZIKV to be done on source plasma (SP). Hepatitis B core antibody testing has never been required because of the desirability of having that antibody in final

immunoglobulin (IG) product. The current test menu is serology for HIV antibody (now a combination of antigen/antibody on the Alinity), HCV antibody, and hepatitis B antigen. Molecular testing (NAT) includes HIV, HCV, and HBV. The pool sizes may be significantly larger. Source plasma screening also includes in-process testing for hepatitis A virus (HAV) and Parvovirus B19. Although these viruses are usually inactivated by current methods, testing is required for historical reasons because of the nonenveloped nature of the viruses. All units with positive results are removed. While donor notification is not strictly required for in-process testing, the FDA has recently indicated that hepatitis A positive donors should be notified as it is a reportable disease infection to state health departments. There is also lookback of 183 days and look forward or deferral of donors of 120 days for hepatitis A. This is based on European regulations; there are no US regulations related to this. Typically, new infection in repeat donors is detected by NAT. Testing may be performed in a staged series, starting with serology and only advancing to NAT testing when all serological tests are negative. Rules for donor notification and re-entry are the same. All positive tests in the United States must be reported to the National Donor Deferral Registry so that individuals cannot donate in another company's center. Syphilis testing is performed only on initial donation and once every four months thereafter. Historically, it was instituted as a public health measure. SP donors

are typically tested only initially for irregular red cell alloantibody and excluded from further donation if it is positive, unless they have anti-D and are recruited into a program for RH-immune plasma. Blood typing is only occasionally done for products for Von Willebrand disease (VWD).

## Donor management

FDA regulations address donors with reactive infectious disease test results in 21 CFR 610.41. In addition, FDA guidance documents and AABB Association Bulletins provide detailed information regarding requirements for donor testing, notification, and deferral, as well as guidelines for reinstatement following FDA-defined requalification algorithms. Donor management and eligibility for re-entry is dependent on the combined results of the donor screening test and resultant supplemental or confirmatory tests for each specific pathogen targeted for disease marker testing (Table 5.5).

The introduction of HIV testing brought about a major shift in donor notification. The process of notifying donors of abnormal infectious disease testing results is sensitive and may lead to confusion or distress. Care must be taken in any written communication of abnormal results to donors, as well as provision of donor counseling to address questions or concerns<sup>30</sup>

**Table 5.5** Donor Management of Reactive Infectious Disease Results

Reactive Screening Test	Supplemental/Confirmatory Test Results	Deferral Type	Re-entry Eligible
HIV-1/2 Ab RR or HIV Agn/Ab RR HIV NAT positive	Confirmed positive Negative or indeterminate NA	Permanent Indefinite Permanent, if HIV-1,2 Ab RR Indefinite if HIV NAT only None after 1x* Indefinite after 2x	No Yes No Yes NA Yes
HBc Ab RR	NA	Permanent None* Permanent	No NA No
HBsAg RR	Confirmed positive by NAT Not confirmed; HBc Ab– Not confirmed; HBc Ab+	Permanent None* Permanent	No NA No
HBV NAT positive	NA	Permanent, when HBc Ab and HBsAg RR (both positive) Indefinite, IF HBc Ab RR only OR HBsAg RR (not confirmed) Indefinite, if HBc Ab and HBsAg NR (both negative)	No Yes Yes
HCV Ab RR	Confirmed positive (NAT) +/- reactive supplemental	Permanent Indefinite	No Yes
HCV NAT positive	NA	Permanent, if HCV Ab RR Indefinite, if HCV Ab NR None after 1x*	No No Yes
HTLV Ab RR	Negative Negative Confirmed positive	Indefinite after 2x Permanent	Yes No
Syphilis Ab RR	Negative Confirmed positive	Indefinite Indefinite; no previous syphilis Indefinite; h/o syphilis	Yes Yes Yes
WNV NAT positive	NA	Temporary (120 days)	NA
ZIKV NAT positive**	NA	Temporary (120 days)	NA
BABS NAT positive	NA	Temporary (2 years)	NA
<i>T. cruzi</i> Ab RR	Negative Indeterminate or positive	Indefinite Permanent	Yes No

Ab, antibody; Ag, antigen; RR, repeat reactive; NR, nonreactive; NAT, nucleic acid test; BABS, Babesia; HBc, hepatitis B core; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HCV, hepatitis C virus; HIV, human immunodeficiency virus; HTLV, human T-cell lymphotropic virus; *T. cruzi*, *Trypanasoma cruzi*; WNV, West Nile virus; ZIKV, Zika virus.

\* Temporary deferral for 56 days.

\*\*U.S. testing requirement for ZIKV withdrawn by FDA in 2021.

Note: Performing a discriminatory NAT assay on a reactive sample is a required step for those establishments using pooled or certain multiplex tests that do not simultaneously detect and discriminate HIV RNA, HCV RNA, and HBV DNA in the same reaction. Donor re-entry policies must conform with current FDA guidance.

Sources: FDA guidance documents: [www.fda.gov/vaccines-blood-biologics/biologics-guidances/blood-guidances](http://www.fda.gov/vaccines-blood-biologics/biologics-guidances/blood-guidances)  
CFR documents: [www.Ecfr.gov](http://www.Ecfr.gov)

It is imperative to have a robust donor notification system that embraces the following ethical concepts:

- 1 Confidentiality:** Even notification of a false-positive result may be misconstrued by the donor or a close family member. It is vital to have a secure strategy to notify donors in private. This is particularly challenging in the setting of prescreening donors with point of care rapid screening tests in countries with high disease prevalence.
- 2 Informed consent:** Donors should be aware of the potential for and implications of donor testing, including potential for deferral and mandatory reporting of certain positive results to local departments of health.
- 3 Truth telling and deferrals:** Collection and testing centers are obligated to share any information of potential medical significance, including sickle trait testing as well as infectious markers with health implications such as positive tests for HIV, HBV, and HCV.
- 4 Treating donors fairly:** There are ethical obligations to both donor and recipient and there is no specific right to donate. Where directed donation or autologous donation are allowed, they should be used in a medically appropriate and consistent fashion.

## Product management

Blood products are kept in quarantine until all testing is completed. Results of infectious disease testing are necessary prior to labeling and releasing products for transfusion. The screening test results determine product disposition (Table 5.6). In addition, final test results are used to direct retrieval of previously released products and trigger transfusion recipient notification (i.e., lookback).

## Retrieval of in-date products and recipient notification

Results of infectious disease testing not only impact currently collected components, but are also used to retrieve previously released products and trigger notification of prior recipients, when indicated, by a process known as lookback. FDA and AABB recommendations are very specific for each infectious agent depending on the test method to address the concern that a previous donation with a

negative test result could have been in the “window” phase of early infection, even though the screening test results were negative.

Specific requirements and algorithms for managing components from prior blood donations, including recipient notification of prior donations for HIV and HCV test results, are available (21 CFR 610.46 and 610.47). Lookback is required by law for both of these infectious agents. FDA guidance documents and AABB Association Bulletins govern the management of other licensed screening tests. Investigational protocols for unlicensed screening assays outline requirements for product retrieval and recipient notification (Table 5.6).

The lookback process requires notification of consignees who have received components from previous collections from a donor who is now reactive for a particular infectious disease marker. They must be notified in order to begin recipient tracing for the purpose of notification of recipients of previously transfused products for testing and counseling. The transfusion service consignees are responsible for notifying the physician(s) of the recipient(s) to begin notification of the recipient(s). The transfusion service may also notify the recipient(s) directly. The notification process shall include a minimum of three attempts to notify the recipient (or a relative or legal representative of the recipient in the case of HIV) and must be completed within a maximum of 12 weeks for HCV or HIV.

## Donor Re-entry

Blood centers were reluctant in the past to perform donor re-entry after indefinite deferral due to donations associated with positive infectious disease marker test results on a serologic test that is not confirmed by additional testing. In 2010, the FDA eased the pathway for re-entry of previously deferred donors with introduction of specific guidelines.<sup>31</sup> Detailed eligibility is defined in FDA guidance documents.<sup>32</sup> These describe additional testing requirements following a minimum time period of deferral, at which point the donor may be notified to return for follow-up testing. Guidelines for re-entry eligibility are currently available for donors with possible false-positive test results for HIV, HBV, HCV, HTLV, syphilis, and *T. cruzi* (Table 5.5). Prior to allowing the donor to be re-entered into the donor pool, both the original screening test and a licensed

**Table 5.6** In-Date Product Retrieval and Recipient Notification

Reactive Screening Test Result*	In-Date Product Retrieval		Recipient Notification (Lookback)
	Blood Components	Recovered Plasma	
HIV-1/2 Ab or HIV Ab/Ag combo test	All products	Unpooled only	Indefinite or 12 months from most recent licensed negative
HIV NAT	All products	Unpooled only	1 year
HBsAg	All products	Unpooled only	5 years or 12 months from most recent negative
HBc Ab	All products	None	None
HCV Ab	All products	Unpooled only	10 years or 1 year before last known reactive
HCV NAT	All products	Unpooled only	1 year
HTLV Ab	All products (unless frozen)	None	5 years or 12 months from most recent negative (none for frozen components)
<i>T. cruzi</i> Ab	All products	None	All products
WNV NAT	Products in prior 120 days	Products in prior 120 days	120 Days
ZIKV NAT	Products in prior 120 days	Products in prior 120 days	120 Days
BABS NAT	All products	None	12 months from positive draw date

Ab, antibody; Ag, antigen; RR, repeat reactive; NR, nonreactive; NAT, nucleic acid test; BABS, Babesia; HBc, hepatitis B core; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HCV, hepatitis C virus; HIV, human immunodeficiency virus; HTLV, human T-cell lymphotropic virus; *T. cruzi*, *Trypanasoma cruzi*; WNV, West Nile virus; ZIKV, Zika virus.

\* Refers to repeat reactive screening tests by serology or positive tests by NAT based on individual or discriminatory testing, if a pooled or multiplex test was performed.

Sources: Website for FDA guidance documents [www.fda.gov/vaccines-blood-biologics/biologics-guidances/blood-guidances](http://www.fda.gov/vaccines-blood-biologics/biologics-guidances/blood-guidances)  
Website for CFR documents [www.ecfr.gov](http://www.ecfr.gov)

supplemental or confirmatory test must be negative. Specific re-entry algorithms should be developed in conformance with FDA guidance. For example, FDA allows re-entry as early as eight weeks after an initial RR test for HIV if the confirmatory testing was negative on the index donation. In contrast, donors are not eligible for re-entry until six months have elapsed following HCV repeat reactive screening tests.

### HIV testing

HIV is an RNA lentivirus in the subgroup of the retrovirus family. HIV infection may progress to acquired immunodeficiency syndrome (AIDS). With current donor screening and testing, the risk for TTI is less than one in two million blood products in the United States.

### HIV antibody testing

HIV antibody testing generally uses recombinant antigens, but historically viral extracts have also been used. Multiple antigens are typically incorporated to maximize the sensitivity as well as capturing multiple virus strains. The first blood donor screening test for anti-HIV-1 was licensed in 1985. Subsequent HIV assays included antigens from both HIV-1 and HIV-2. Because there is greater than 50% homology between HIV-1 and HIV-2 DNA, the HIV-1 test detects about 90% of HIV-2 strains.<sup>33</sup> However, as cases of transfusion-transmitted HIV infections from HIV-2 began to appear in the United States,<sup>34,35</sup> the FDA requested that HIV test kits include antigens specific for HIV-2. Efforts to improve donor screening also focused on narrowing the preconversion window phase of infection.<sup>36–38</sup> HIV-1 group O infection was later identified in certain African countries, where it was found to be endemic. Currently, licensed HIV-1/HIV-2 plus O EIA antibody tests are routinely used for HIV antibody screening of donors. Traditionally, HIV antibody screening tests have utilized chemiluminescent immunoassays (ChLIA) or enzyme immunoassay (EIAs). More recently, the so-called fourth generation of HIV screening tests for blood donation have introduced a combined antigen/antibody test.<sup>39</sup> Newer chemiluminescent microparticle immunoassays (CMIAs) have been approved for blood donor testing, which use a method for the simultaneous qualitative detection of human immunodeficiency virus (HIV) p24 antigen and antibodies to HIV type 1 (HIV-1 group M and group O) and/or type 2 (HIV-2) in human serum and plasma specimens. It is anticipated that these fourth-generation assays will increase accuracy and reduce the number of donations requiring confirmation, save time and money, and reduce the number of discarded blood donations and allow re-entry processes.<sup>40</sup>

### HIV NAT

NAT for blood donor screening introduced a new era in the field of blood safety.<sup>41–45</sup> HIV NAT testing is performed by either PCR or TMA. Testing may be performed on pools of 6–16 donor samples (minipools), to reduce the expense without significantly decreasing sensitivity, or individually (ID NAT). Currently, donor testing is typically performed in a multiplex assay for HIV/HCV/HBV. If a tested pool is positive, discriminate testing with individual donor (IND) NAT is required. In areas of high endemic disease prevalence, such as South Africa, it is more practical to simply screen all donors individually as the

frequency of required pool resolution would obviate any benefit of starting with pools.<sup>46</sup>

There is recent evidence that the results of diagnostic and screening assays for HIV may be altered in individuals who are taking antiretroviral therapy because of the suppression of circulating HIV RNA below levels of detectability by NAT.<sup>47</sup> Similarly, there is concern that rare individuals may be able to transmit HIV after receipt of prescription preexposure prophylaxis, even though they believe they are protected from infection. In a recent study of HIV antibody-positive, NAT-negative donors in South Africa, approximately 66% had been receiving antiretroviral therapy.<sup>48</sup> Test results in these donors may not reflect likelihood of infection transmission.

### HIV confirmatory testing

For blood samples that test repeat reactive (RR), additional testing must be performed if serology is not confirmed by positive HIV NAT (Table 5.4). Until recent years, confirmatory testing for HIV was routinely performed by HIV-1 Western blot (WB) or immunofluorescence assay (IFA) followed by anti-HIV-2 if HIV-1 is not confirmed. The criteria for HIV-1 positivity on WB are the presence of antibody against any two of the associated proteins. Sera that react with only one protein are considered indeterminate, which can occur for a variety of reasons and typically have no relationship to HIV infection. An immunochromatographic assay (Geenius<sup>TM</sup>) for HIV-1/2 has enabled an improved approach for HIV-1/2 testing.<sup>49</sup> Recently, FDA approved for blood donor testing a supplemental assay, which avoids the significant rate of indeterminates seen on WB and offers a single, simplified approach for HIV-1/HIV-2 antibody confirmation and differentiation, enabling the replacement of previously used FDA-licensed supplemental assays.<sup>50,51</sup> The supplemental results of testing help determine the infectious state of the donor for purposes of counseling and consideration of re-entry, as allowable by the 21 CFR and FDA Guidance for Industry. Components of blood collected from donors with either reactive anti-HIV serology or positive NAT tests must be discarded and the donor indefinitely deferred, except for autologous blood collections.<sup>52</sup>

### Hepatitis B testing

HBV is a DNA virus in the *Hepadnaviridae* family, which is highly prevalent in Asia and Africa. Although most cases (~95%) acquired during adulthood are cleared, perinatally acquired cases may result in chronic infection. The first blood donor screening test for this virus was required in 1972.

### Hepatitis B surface antigen

Testing for HBsAg was first introduced in 1971 shortly after the discovery of the hepatitis-associated antigen. Although test methodology has evolved over the years, currently an EIA system is used. Antibody to HBsAg is coated onto particles, the donor's serum is added, and any HBsAg binds to the particle and is detected by a second anti-HBs (antibody to HBsAg) that is linked to an enzyme. Automated testing methodologies often use chemiluminescent labels, replacing the enzyme conjugates and chromogenic detection methods. Confirmation of a positive test is done by a neutralization step adding anti-HBs antibody and repeating the assay. A substantial reduction in activity signifies a true positive or confirmed positive test.

### **Anti-HBc antibody**

The test for anti-HBc was originally introduced in hopes of identifying cases of hepatitis B with below detectable HBsAg.<sup>53</sup> It was also used as a surrogate assay with ALT testing for non-A, non-B hepatitis in 1987 prior to the identification of HCV.<sup>53-55</sup> The implications of anti-HBc and lack of correlation with HBsAg and HBV DNA have been observed, including rare donors infectious for hepatitis B who have a negative HBsAg test.<sup>56</sup> Hence, donor screening tests are designed to detect both IgM and IgG antibodies. The anti-HBc test is different from the others in that it is an inhibition-type assay. HBc is bound to the solid phase, and the indicator (enzyme-linked) probe is anti-HBc. The anti-HBc in the probe competes with the donor's serum sample, and if anti-HBc is present in the donor's serum there is less binding of the labeled probe and a reduced assay activity, indicating a positive test. A direct antiglobulin assay for anti-HBc is also available. Unfortunately, the inhibition-type assay is subject to greater variability than the direct-type assay used for other tests, and this has resulted in a considerable false-positive rate for the anti-HBc test.<sup>57</sup> Notably, most non-US countries do not use this test.

Unfortunately, no confirmatory test method exists for the anti-HBc test, which has resulted in a unique approach when other tests for HBV are negative. Donors are indefinitely deferred after the second RR anti-HBc donation if the other HBV markers are negative. Due to the concern that such a donor could be at the tail end of an active HBV infection, recent FDA guidance has added the recommendation to perform IND HBV NAT testing to increase the sensitivity for the detection of HBV present at very low levels, which could be below detectable limits in pooled samples.<sup>58,59</sup> Donors who are RR for anti-HBc on more than one occasion, but remain negative for NAT and HBsAg, are eligible for re-entry for blood donation after a minimum of 8 weeks after the last RR anti-HBc donation.

### **HBV NAT**

HBV-NAT is a required screening test for HBV. The slow doubling time of that virus causes low levels of viremia during the window phase.<sup>41</sup> HBV DNA levels in HBsAg-positive and anti-HBc-positive blood donors can be extremely low. HBV may be detected later in infection when HBsAg has already cleared, but those donors would also be detected by the required anti-HBc test. Current minipool HBV-NAT methods would miss about 6% of these donations, and about 3% would not be detected even by single-donor NAT.<sup>58,60</sup> Therefore, the widespread adoption of NAT testing for HBV was very slow.<sup>56</sup> A recent study of the incremental cost utility of NAT after the implementation of serology screening prompts the need for further discussion of these testing strategies in the future.<sup>61</sup>

### **Hepatitis C testing**

HCV is a single-stranded RNA flavivirus, which is transmitted through blood exposure. It is typically asymptomatic during the acute phase, but may result in slowly progressive chronic infection yielding cirrhosis and liver failure.

### **HCV antibody screening**

The primary anti-HCV antibody test used for screening blood donations is a third-generation EIA. Peptides of the HCV are bound to the solid phase, and an enzyme-linked antiglobulin is the detection system. The mix of peptides in the assay system has been adapted to improve test performance and reduce false-positive reactions.<sup>62</sup> The currently available tests have recombinant HCV

antigens representing four viral sequences. A positive screening test is repeated in duplicate, blood from donors with RR results is destroyed, and donor notification and deferral are required. For many years, the confirmatory test performed for anti-HCV antibodies was the FDA-licensed recombinant immunoblot assay against HCV antigens, which became commercially unavailable in the United States in 2012.<sup>63</sup> Since then, supplemental testing has been accomplished using an alternate EIA test method.

### **HCV NAT donor screening**

In the United States, there are two commercially available, licensed NAT assays for volunteer blood donor screening: one is a PCR test and the other is based on TMA technology for the detection of HCV RNA in the donor sample. Both test methods can identify HCV RNA with an average of 11 days after infection and are available in a multiplex format (HIV/HCV/HBV). Multiplex testing occurs in minipools of 6–16. If a pool is positive, discriminate testing of individual samples is required and the donor is indefinitely deferred.

Blood donors with positive HCV NAT results in the absence of anti-HCV are likely to have been recently infected. The most common reason for this is injection drug use.<sup>64</sup> However, immunocompetent HCV-infected individuals may occasionally not have a persistent HCV antibody response.<sup>65</sup> If a donor with a negative HCV NAT has RR anti-HCV test results, they are indefinitely deferred. Results of supplemental testing in this situation assist with donor notification, counseling, and eligibility for donor re-entry following a period of at least 6 months. If all repeat HCV testing is negative, including IND HCV NAT, the donor can be reentered.<sup>66</sup>

### **Human T-cell lymphotropic virus testing**

Testing of US blood donors for antibodies to HTLV-I and HTLV-II using an FDA-approved EIA or ChLIA test began in 1997. Supplemental/confirmatory testing may be performed by repeating the test using an alternate manufacturer's assay or by WB. FDA-approved the MP Diagnostics HTLV Blot 2.4 in 2014, which uses a combination of envelope recombinant proteins and viral lysate (envelope rgp46-I, rgp46-II, and GD21, and gag p24 and p19 antigens) to confirm the presence of antibodies against HTLV-I/II and differentiate between HTLV-I and HTLV-II infection (gag p24 and p19 antigens). A donor who is negative or indeterminate with supplemental testing may continue to donate. However, donors positive with a supplemental test or who are RR on the screening test on two separate donations are permanently deferred.

HTLV-I is a virus that is endemic in certain areas, including Japan and the Caribbean region. It is associated with a slowly progressive myelopathy in a small percentage (<2–4%) of infected individuals. HTLV-II is most likely to occur in donors with either a history of intravenous drug use or sexual contact with an intravenous drug user. Most infections are asymptomatic, but there is a very low lifetime risk (<4%) of development of adult T-cell leukemia/lymphoma. Because these viruses predominantly infect T-lymphocytes, leukoreduction may theoretically prevent infection. The seroprevalence in blood donors in the United States is less than 0.01%.<sup>67</sup> The impact of testing on improved patient safety is questionable and donor counseling is challenging due to the low risk for the development of the disease. Blood collection establishments outside the United States rarely screen for HTLV.

## West Nile virus testing

WNV is a single-stranded RNA flavivirus, which is primarily spread in the United States by the *Culex* mosquito species.<sup>68</sup> After confirmation of transfusion transmission of WNV to 23 patients in 2002, the FDA mandated testing of all components of blood collection in 2003. WNV NAT was rapidly implemented, in part due to existing NAT platforms for HIV and HCV testing. Testing of blood donated from epidemic areas began in early summer 2003.<sup>69–72</sup> NAT testing has been quite effective in reducing the likelihood of transfusion-transmitted WNV infection.<sup>69–72</sup> Routine testing is done in pools similar to HIV and HCV, but due to low-level viremia, WNV is switched to individual testing during the epidemic season based on surveillance for infection in the region.<sup>73</sup> Blood donor WNV testing provides valuable information about community-wide disease prevalence.<sup>74</sup>

## Zika virus testing

ZIKV is a tropical arbovirus of the flavivirus group. Although first discovered in Uganda in 1947, human infections were first recognized in Africa and Asia in the 1960s, with epidemic spread to the Pacific Islands and beyond in the 2000s. An American outbreak was first identified in Brazil in 2015, where it was linked to the alarming observation of babies born with microcephaly presumably due to maternal–fetal transmission. With the recognition of the possibility of transfusion transmission, an FDA Guidance for Industry was issued in February 2016 with recommendations for donor screening, deferral, and product management to reduce the risk for transfusion transmission of ZIKV.<sup>75</sup> As the epidemic evolved and autochthonous (local acquisition) mosquito-borne infection was identified in pockets of southern Florida and Texas, the FDA published a revised ZIKV guidance that included a testing requirement in July 2018.<sup>76</sup>

Based on the FDA guidance recommending either NAT or pathogen reduction of blood products, ZIKV testing of the blood supply is performed by one of the two commercially available platforms, which uses either real-time PCR or TMA to detect ZIKV RNA. Both methods of detection are highly sensitive and specific. Donors with a positive test result are deferred for 120 days, similar to those with a positive WNV result. The first test introduced was ID NAT, with more recent testing allowed in pools with algorithms for conversion back to ID NAT under appropriate circumstances.

A study of ZIKV-infected US blood donors revealed that they were all acquired from travel to affected areas outside the country.<sup>77</sup> A cost-effective analysis estimated that \$137 million per year is spent on donor ZIKV testing at a cost of at least \$5.3 million per RNA-positive test.<sup>78</sup> Given an absence of evidence of autochthonous transmission since 2017<sup>79</sup> and the questionable impact on patient safety,<sup>80,81</sup> the continued need for Zika screening was debated. The FDA has specifically excluded the need for Zika screening in products treated with an approved strategy for pathogen reduction and discontinued the test requirement in 2021 following waning of the outbreak.<sup>82,88</sup>

## Testing for babesiosis

*Babesia*, an intraerythrocytic parasite, can be transmitted by transfusion of red cell containing components. Up to 1% of donors in some endemic areas test positive for prior exposure on a donor screening test.<sup>83</sup> Targeting tested units for only immunocompromised patients at greatest risk would have limited efficacy given

that many of the affected recipients in case series do not belong to obvious at-risk groups.<sup>84</sup> Initially, antibody-based screening assays were trialed but subsequently replaced by more specific NAT-based assays.<sup>85,86</sup>

The impact of *B. microti* blood donation screening has been reported.<sup>87</sup> The FDA Guidance for Industry document recommends the implementation of year-round *Babesia* testing of all donations collected in 14 states (Connecticut, Delaware, Maine, Maryland, Massachusetts, New Hampshire, New Jersey, New York, Pennsylvania, Rhode Island, Vermont, Virginia, Minnesota, and Wisconsin) and Washington, DC, unless the blood products are pathogen reduced using an FDA-approved technology.<sup>88</sup> (Of note, there are no well-documented transfusion-transmitted cases from plasma or platelets collected by apheresis only.)

Currently, two licensed tests are available for donor screening by NAT, the Grifols Procleix *Babesia* Assay and the PCR-based NAT assay by Roche, which are highly sensitive qualitative tests for the detection of *Babesia* ribosomal RNA or DNA. In the United States, testing may be performed individually or in minipools (up to 16 samples), which is allowed because of the high clinical sensitivity of the assay. These test systems detect four *Babesia* species: *B. microti* (most prevalent in the United States), *B. duncani*, *B. divergens* (most prevalent in Europe), and *B. venatorum*. These both require use of a special collection tube containing lysis buffer to disrupt red cells as most organisms are intraerythrocytic.

## Syphilis testing

Syphilis testing has been conducted on donated blood for more than 50 years.<sup>89</sup> However, no well-documented cases of transfusion transmission have occurred in the last three decades. Fortunately, the spirochete is not infectious after approximately 72 hours of refrigeration in stored blood.<sup>90</sup> A study<sup>91</sup> using nucleic acid amplification technology did not find TP DNA or RNA in any of 169 samples from blood donors with positive serological tests for syphilis. Thus, the current testing probably does not identify infectious units.<sup>89,91,92</sup> Consideration of discontinuing syphilis screening has been controversial.<sup>93</sup> However, as the AIDS epidemic began to unfold, the syphilis test was retained as a surrogate marker of risk for sexually transmitted diseases, including HIV. This has not proved to be an effective way to identify HIV-infected donors, and syphilis testing need not be retained for this purpose.<sup>92,93</sup> Room temperature storage of some blood components, particularly platelets, will not inactivate the spirochete. Syphilis is more common in many parts of the world, but many treponemal-specific screening methods are not positive early in infection. While reagin-based methods (RPRs) may have better early sensitivity, they are plagued by a high false-positive rate.<sup>94</sup>

## *Treponema pallidum*: *T. pallidum* and non-*T. pallidum* testing

Antibody screening tests for syphilis may be performed by using either TP or non-TP tests. TP tests identify both current and distant infection, whereas non-TP tests identify active or recent infections and become negative after the treatment of syphilis infection. TP tests target detect antibodies against specific TP antigens, such as the MHA-TP micro-hemagglutination test. In contrast, non-TP tests (such as the rapid plasma reagin test) detect antibodies that react against anticardiolipin phospholipid and may be present in a variety of disorders and inflammatory conditions. Confirmatory or supplemental testing should be performed following a reactive screening test result. Tests in this category

include fluorescent TP antibody absorption (FTA-ABS) or the Syphilis-G EIA.

Donors with a reactive screening test may return for donation if supplemental testing is negative; however, if supplemental testing is positive, the donor is indefinitely deferred but may be eligible for re-entry in accordance with FDA Guidance.<sup>95</sup>

### Chagas' disease testing

*Trypanosoma cruzi* is the causative agent of Chagas' disease, also known as American trypanosomiasis. It is a tropical parasitic disease, which can also be transmitted by transfusion, as well as solid organ transplantation and transplacental transmission. With increased immigration to the United States from Central and South America, seroprevalence of this organism has increased during recent decades. Many patients with chronic *T. cruzi* infection are asymptomatic, and a question only based approach is ineffective.<sup>96</sup> Cases of transfusion-transmitted Chagas' disease are extremely rare in the United States.<sup>97–99</sup> Multiple ELISA-based assays with high sensitivity and high specificity are FDA approved, but blood donors are only required to be screened once. Virtually, all US donors with confirmed positive tests were born in *T. cruzi*-endemic areas, and no seroconversion of test negative donors has been observed.<sup>100</sup>

### CMV testing

CMV testing is an optional test performed on donated blood products. The virus largely remains latent in the donor's leukocytes and hence transfusion transmission may be largely abrogated by effective leukoreduction.<sup>101,102</sup> Several types of CMV antibody detection methods are available, including enzyme-linked assays (EIA), latex agglutination, solid-phase immunofluorescence, complement fixation, and indirect hemagglutination. These methods can be used to detect IgG or IgM antibodies with a specificity of 85–98% and a sensitivity of 95–99%.<sup>102</sup> The two assay methods used most commonly are the EIA and the latex agglutination test.

CMV infection is rather common in the general population, with 30–80% of blood donors testing positive with the EIA or latex tests, but probably only about 1–3% of donors are actually infectious.<sup>102</sup> IgM antibodies may indicate more recent infection. Although PCR is a very sensitive assay to detect viral DNA, this has not been widely applied for CMV in blood donors. Thus, selective screening for susceptible patients has been advocated.<sup>103</sup>

More recently, prestorage leukoreduced (LR) blood components have been widely implemented to prevent transfusion-transmitted CMV infection (TT-CMV) as most clinicians consider them an acceptable and preferable method of providing CMV "safe" blood products.<sup>104,105</sup> A recent study of umbilical cord blood transplant patients receiving prestorage LR, CMV-unselected blood components between 2007 and 2017 in a Japanese pediatric center found no cases of TT-CMV.<sup>106</sup>

### Additional testing for blood derivatives

Plasma not needed for transfusion or directly collected for preparing derivatives is required to have additional screening for Parvovirus B19 and HAV due to the very large pool sizes used in

preparing these products. Plasma from 5 to 20,000 donors may be pooled in making large derivative lots.<sup>107</sup> Most plasma centers use a donor retested policy, i.e., not including a product into a pool until the donor has returned and been retested and found negative for all infectious disease markers a second time. In addition, the process sometimes includes solvent-detergent treatment that effectively inactivates lipid envelope viruses. Since both Parvovirus and HAV are nonlipid envelope viruses, this inactivation step is not effective against these two viruses and breakthrough infusion-related infectious for each have been reported.

### Bacterial testing of platelets

The primary components associated with transfusion-transmitted bacteria are whole blood-derived and apheresis platelets.<sup>108</sup> Platelet concentrates are stored at room temperature; however, *Yersinia enterocolitica* transmission from RBCs stored at refrigerator temperatures is also reported. For both, the source of potential contamination is usually blood or the skin at the venipuncture site, and almost never the blood collection pack. Steps taken to minimize the possibility of transfusion-associated sepsis include effective skin decontamination, diversion of the first 20–40 mL of blood proper storage and handling conditions, and the detection of bacteria before transfusion.<sup>108–110</sup>

The method most widely used currently in the blood center setting for bacterial testing of platelets is an automated culture system frequently used in hospitals for patient blood cultures.<sup>111</sup> Both the BacT/ALERT and BacTec systems can be used for the detection of bacteria in platelet products, with the ability to detect levels of 10 and 100 colony-forming units per milliliter.<sup>112</sup> Another system tests platelets at the time of release for transfusion when the level of organisms is higher and more easily detected.<sup>113</sup> However, current bacterial detection strategies do not detect all contaminated units and have not eliminated all transfusion-related septic fatalities.<sup>114–116</sup> In fact, sepsis attributed to bacterial contamination of platelets despite being treated with pathogen-inactivation technology has also been identified.<sup>117</sup>

Given the remarkable reduction in the viral risk from transfusion in recent decades to improve safety, the low but persistent risk for bacterial contamination has become the greatest residual threat of TTI resulting in fatality.<sup>1</sup> In September 2019, the FDA finalized the bacterial risk-control strategies for blood collection establishments and transfusion services to enhance the safety and availability of platelets for transfusion guidance for industry, which was updated to extend the deadline for implementation in December 2020.<sup>118</sup> The goal is to further mitigate the risk for septic reactions from platelets, which currently has a reported rate of approximately 1 per 100,000 platelet transfusions.<sup>118</sup> In contrast, sepsis from transfusion of bacterially contaminated RBC components is generally regarded as a rare event.<sup>119</sup>

The two main options are pathogen reduction or bacterial testing, which include single-step and two-step strategies. The single-step strategies require large-volume delayed sampling for culture using aerobic and anaerobic cultures of each platelet product, no sooner than 36 or 48 hours, resulting in expiration at 5 or 7 days, respectively. The two-step strategies involve an initial culture no sooner than 24 hours, followed by a secondary method of bacterial testing by either culture or a rapid test method after day 3 of the 5-day platelet shelf life.

## Other laboratory testing

### HLA antibody testing

With antibodies against HLA and human neutrophil antigen antibodies in blood products (usually platelets or plasma) implicated as an etiology of transfusion-transmitted lung injury (TRALI), efforts to mitigate this risk have been implemented.<sup>120</sup> Collection of plasma (largely limited to male donors) and platelets from males, never pregnant females, or HLA antibody-tested females became mandatory in the United States with the goal of further reducing the TRALI incidence.<sup>121</sup>

Two types of assays are commonly used in blood centers for HLA antibody screening of blood donors. Automated methods for testing may be based on either EIA methodology or a multianalyte assay system using an advanced flow cytometer with microbeads coated with purified class I or class II HLA antigens and preoptimized reagents for the detection of class I or class II HLA antibodies in human donor sera.

### Extended blood group antigen typing

Units of RBCs lacking specific antigens may be requested for patients with RBC antibodies or who require prophylactic antigen-matched units. Transfusion recommendations for RBC antigen matching to prevent alloimmunization in patients with sickle cell disease, thalassemia, and other transfusion-dependent anemias have been published.<sup>122-125</sup> Traditionally, antigen-negative units were identified through serological testing. With the advent of RBC genotyping, many blood centers supplement testing by performing extended RBC antigen typing using DNA-based technology, which provides a reliable and cost-effective prediction of the RBC phenotype.<sup>57</sup> The additional benefits of genotyping for further improving transfusion practice have been documented in recent years.<sup>126-129</sup>

Automated high-throughput platforms for mass-scale RBC genotyping will help identify desirable antigen-negative blood donors and improve availability of antigen-negative units.<sup>130</sup>

### Role of platelet antigen typing

Blood and platelet donors are not routinely tested for platelet-specific antibodies; however, some blood centers perform platelet antigen testing to identify those who are negative for the HPA1a (PlA1) or other platelet-specific antigens. HPA-1a is most commonly involved in neonatal alloimmune thrombocytopenia or post-transfusion purpura.

### Role of platelet serology

Blood centers may be requested to perform platelet antibody testing on patients who are refractory to transfusion to rule out an immune-mediated cause. Serological testing for the detection of HLA or non-HLA antibodies can be performed by a variety of serological methods. Solid-phase RBC adherence testing for the detection of platelet antibodies is commercially available. Other methods of platelet antibody detection include flow cytometry and EIA/ELISA techniques. If antibodies are present, platelet compatibility testing can be performed by crossmatching

patient plasma against apheresis platelets available in the inventory for transfusion. Alternatively, HLA-matched platelets can be provided, if the platelet donor has been HLA typed and the platelet refractory patient has known HLA antibody specificity.

### Parvovirus B19 and hepatitis A virus testing

These viruses can be transmitted by transfusion, but the main concern has historically been transfusion transmission via plasma derivatives. NAT for Parvovirus B19 and HAV is now done for SP (intended for the manufacture of derivatives), which has been described earlier in this chapter under plasma for further manufacture.

### Hemoglobin S testing

Many patients with sickle cell disease require blood transfusion therapy. The goal is to decrease the proportion of RBCs containing hemoglobin S to reduce sickling and prevent increased viscosity of their blood. Healthy donors who have the sickle cell trait may be unaware that they have hemoglobin S. In this era of universal leukoreduction, RBCs from donors with hemoglobin S trait often may adhere to the WBC reduction filter resulting in occlusion or failure to completely leukoreduce the unit, resulting in a "filter failure."<sup>131</sup> In addition, blood provided for patients with sickle cell disease requires some degree of prophylactic antigen matching (Rh and Kell) or additional antigen typing if RBC alloantibodies are present. Blood from donors of African ancestry may be preferentially tested for their phenotype characteristics, so testing for hemoglobin S must also be performed. RBC units must be labeled/tagged to indicate that they are hemoglobin S negative. Hemoglobin S-tested blood is also typically requested for intrauterine transfusion and neonatal exchange transfusion.

Most methods of screening for hemoglobin S are performed manually using a phosphate solubility method. RBCs are lysed by saponin, and released hemoglobin is reduced by sodium hydrosulfite in a phosphate buffer. Hemoglobin S is insoluble and will form a cloudy, turbid suspension, whereas other hemoglobins are more soluble and form a clear red solution. It is important to note that increased percentages of hemoglobin F may cause a false-negative test, and patients with abnormal proteins in their blood may have a false-positive test. In addition, Immucor HEA has an automated molecular bead chip test for hemoglobin S, which can be performed with common RBC genotyping.

### Screening donors for IgA deficiency

IgA levels are not routinely determined in donated blood. Some blood centers occasionally screen donors for a limited time to establish a file of rare donors known to be IgA deficient. The donor record can be annotated so that when the individual donates, the plasma from the unit can be frozen and saved for future use for IgA-deficient patients. Also, those donors can be called on to donate platelets if needed for IgA-deficient patients. In the setting of transfusion-related anaphylactic reactions, patients must be evaluated for severe IgA deficiency (<0.05 mg/dL) and anti-IgA antibodies.

## Summary

Laboratory testing of donated blood is composed of a wide variety of tests, focused primarily on blood group typing and tests designed to screen for relevant transmissible infectious diseases. With more widespread implementation of electronic crossmatching, the importance of proper ABO identification and labeling is paramount. Transmissible disease testing has almost eliminated the infectious risks of blood transfusion. The increasing complexity of these tests has led to the consolidation of the testing into larger laboratories.

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## CHAPTER 6

# Acute adverse reactions after blood donation

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A safe and adequate blood supply depends on healthy, altruistic volunteers who are willing to donate blood despite the potential risk of discomfort or an adverse reaction. Blood donation is generally considered safe and most donors have a good experience or have only mild symptoms after donation. About 7.3 million volunteer blood donors gave blood in 2019 in the United States (US), or 4% of the estimated 205 million individuals who are likely eligible to donate blood, to meet the annual demand for blood transfusion.<sup>1,2</sup> Considering the millions of donations each year, even a low risk of adverse reactions will negatively affect the health and well-being of many people and will influence their willingness to donate blood again. Consequently, prevention and mitigation strategies to reduce donor reactions and efforts to improve the donor experience are essential.

Adverse reactions occur immediately or soon after a blood collection procedure or become apparent only over a longer period of time, after several blood donations. Immediate or acute reactions after blood donation are typically minor symptoms such as dizziness and fainting or phlebotomy-related bruises, and less commonly, more serious injuries from resultant syncope-related falls or needle-related nerve injuries.<sup>3</sup> Blood collection agencies must inform prospective donors about the possible risks at each encounter and monitor adverse reactions during and after the collection procedure as part of ongoing continuous improvement efforts to reduce donation-related complications. Although blood centers cannot completely eliminate all risks associated with blood donation, they can systematically analyze their adverse reaction rates, which can lead to changes in collection procedures and policies to improve safety for the most susceptible groups of blood donors.

### Adverse reactions after blood donation

Most acute reactions (>90%) that occur immediately or within a few hours after donating blood are mild or minor symptoms such as dizziness, lightheadedness, or phlebotomy-related bruises and hematomas that resolve promptly but are still unpleasant for the donor. More serious complications are uncommon, but typically result from a loss of consciousness, from injuries after blood donation, or from a needle-related nerve injury.<sup>3</sup> Young (teenage) donors are much more likely to experience immediate adverse reactions after

blood donation than older donors, with loss of consciousness affecting about 4 in 1000 donations and injury resulting in 0.6 in 1000 donations among donors age 16–17 years.<sup>4,5</sup> Not surprisingly, blood donors who have an adverse reaction are less likely to return to donate blood again than those who have an uneventful donation.<sup>6–8</sup> Even minor reactions and transient symptoms discourage return donation by 36%, with more severe reactions further decreasing the likelihood by 66%, compared to the return rates of donors who do not experience reactions after blood donation.<sup>6</sup> The potential loss in annual donations resulting from donor attrition after adverse reactions has been estimated as 1.6% per year.<sup>7</sup> Donor retention has far-reaching implications for the blood supply, not only on availability but also on safety, because repeat donors account for about 75% of the US blood supply<sup>1</sup> and are less likely than first-time donors to have positive infectious disease markers, such as hepatitis and HIV.

Many studies of reactions after allogeneic blood donation published since the 1940s have reported various incidence rates and associated risk factors.<sup>9</sup> Published reaction rates from different blood centers span a wide range (e.g., <1% to >20%). Many factors contribute to this variability, including disparate donor demographics, different reaction definitions, subjective assessments of reaction severity, and various methods of data collection and analysis. Reactions may be captured at the site by collection staff or identified only after the donor calls the center back to report a reaction. Higher reaction rates are generally observed when blood donors complete surveys about mild subjective symptoms or are directly interviewed days to weeks after a donation.<sup>10,11</sup> Many studies of donor reactions are limited by methodological problems, including retrospective design, poorly controlled comparisons, or the use of univariate methods to detect associations of various factors with donor reactions. Consequently, statements about various risk factors should be evaluated in conjunction with an assessment of the study design and analytical methods.

In recent years, blood centers have focused on the practical application of donor hemovigilance, which is an effort to monitor, track, and trend reactions after blood donation, in order to design and implement preventive measures. An example of such a program in the American Red Cross is shown in Table 6.1, which captures immediate reactions managed by collection staff at the blood drives

**Table 6.1** Adverse Reactions after Allogeneic Whole Blood and Apheresis Donations in the American Red Cross, 2006–2007

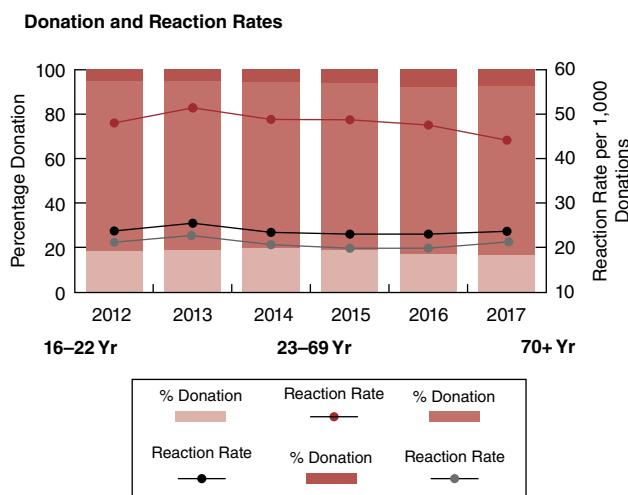
Reaction Category and Description	Whole Blood Donations (12.0 Million)				Automated Procedures (1.42 Million)				
	All Reactions		Outside Medical Care		All Reactions		Outside Medical Care		
	n	Rate	N	Rate	n	Rate	n	Rate	
<b>Systemic (syncope)</b>	Presyncope (prefaint)	324,129	269	69	0.06	14,919	104	4	0.03
	LOC (<1 min)	11,081	9.20	107	0.09	521	3.65	4	0.03
<i>Major (includes callbacks)</i>	LOC ( $\geq 1$ min)	2050	1.70	251	0.21	206	1.44	14	0.10
	Prolonged recovery	4228	3.51	829	0.69	424	2.97	48	0.34
	LOC with Injury	2181	1.81	680	0.57	98	0.69	18	0.13
<b>Phlebotomy</b>	Small hematoma	125,082	104	87	0.07	49,304	345	13	0.09
<i>Major (includes callbacks)</i>	Large Hematoma	4932	4.09	556	0.46	2850	19.9	103	0.72
	Suspected nerve injury	3858	3.20	513	0.43	572	4.00	47	0.33
	Suspected arterial puncture	1644	1.37	112	0.09	82	0.57	1	0.01
<b>Citrate reactions</b>	Citrate (minor symptoms)	—	—	—	—	16,556	116	3	0.02
	Citrate (major, includes callbacks)	—	—	—	—	406	2.84	21	0.15
<b>Allergic reactions</b>	Local (minor) allergic reactions	123	0.10	19	0.02	49	0.34	8	0.06
	Systemic (major) allergic reactions (includes callbacks)	17	0.01	11	0.01	42	0.29	8	0.06
	TOTAL	479,325	398	3234	2.69	86,029	602	292	2.04

Rate per 10,000 donations. LOC, loss of consciousness; small hematoma, 2 × 2 in. or less; large hematoma, more than 2 × 2 in. Prolonged recovery (>30 minutes) after presyncope. Automated collections include plateletpheresis, plateletpheresis with concurrent plasma or other cocomponent, two-unit red cell collections, and plasmapheresis procedures. Minor reactions (e.g., presyncope and small hematoma) are documented at the collection site. Major reactions are documented at the collection site or reported after donation, require follow-up with the donor, and receive a follow-up call, and are reviewed by a blood center physician.

and delayed reactions reported by the donor after whole blood and automated (apheresis) blood donation.<sup>3</sup> AABB also had a voluntary national program for donor hemovigilance in the United States (2012–2017), which encouraged blood centers to use common reaction definitions.<sup>12,13</sup> The general trends in adverse reactions have been consistent among blood centers and over time. The universal observation among all blood centers is that the youngest donors are consistently at the highest risk of reactions (Figure 6.1). According to AABB, young donors (age 16–22 years) accounted for 18% of the total donations, but consistently had the highest reaction rates each year, ranging from 41.41 per 1000 donations in 2017 to 49.09 per 1000 donations in 2013.<sup>13</sup> While the absolute incidence of donor complications varies dramatically among centers for all the reasons mentioned previously, the practical value of hemovigilance activities lies not in the casual comparison or “benchmarking” of reported rates across blood centers, but in the careful analysis of reactions rates within a blood system before and after implementation of measures designed to reduce the risk of reactions. Such efforts are described in greater detail in the following sections.

### Acute reactions after blood donation: immediate symptoms and delayed complications

Most (>85%) systemic reactions to blood donation are acute symptoms such as pallor, lightheadedness, dizziness, diaphoresis, and nausea, occurring in about 2–15% of whole blood donations.<sup>3</sup> The term *vasovagal* is often used to describe these reactions, to refer to physiologic changes that may be associated with syncope (i.e., increased vagal tone and bradycardia). The more general term *presyncope* captures the spectrum of reactions that result from various mechanisms involving not only peripheral baroreceptor activity, but also susceptibility to acute blood loss and orthostatic changes, as well as anxiety and psychological stress. Ultimately, syncope results from an insufficient supply of oxygen to the brain and a transient loss of consciousness. About 1 in every 20 presyncope reactions



**Figure 6.1** Total donations and reaction rates by age, 2012–2017. Reprinted from AABB donor hemovigilance, used with permission from AABB.<sup>13</sup>

(Table 6.1) progresses to loss of consciousness immediately or soon after the blood donation. Seizure-like movements or loss of bladder and/or bowel function might accompany some of these reactions.

Presyncope symptoms typically have rapid onset and short duration. Although most of these reactions are self-limiting and transient, they are still distressing for many donors and some require prolonged recovery periods of more than 30 minutes.<sup>3</sup> Syncope-related falls may be associated with serious injuries. Most (90%) fainting events occur with the phlebotomy or soon afterward at the blood drive, but delayed hypotensive reactions after the donor left the collection site comprised about 10% of the episodes in one center.<sup>13–15</sup> Young donors (16–17 years old) accounted for almost half of all injuries at collection sites among whole blood donors, and some of the injuries (e.g., concussions, lacerations, and dental injuries) required urgent medical care.<sup>4</sup>

## Risk factors associated with reactions after blood donation

Many studies have evaluated donor characteristics and other variables that influence the risk of immediate and delayed reactions after allogeneic blood donation. The strength of the studies' conclusions depends on the trial design and statistical analysis to control for confounding variables. An overview of risk factors and the strength of the available evidence that supports a possible association with syncopal reactions after allogeneic blood donation is summarized in Table 6.2.<sup>9</sup> The strongest, independent risk factors for both immediate and delayed reactions consistently identified in well-controlled studies of whole blood donation are young age (<23 years old), first-time donation status, total blood volume (<3.5 L), and estimated blood loss (>15%), and, in most studies, female sex.<sup>16–18</sup> Young age had the strongest association with complications even after controlling for first-time donation status in one study that showed 16- and 17-year olds were threefold more likely to experience an adverse reaction than older donors.<sup>3</sup> Although regulations in some countries restrict the upper age limit of blood donation, many studies have confirmed the lower observed rates of reactions among elderly donors compared to younger donors.<sup>19,20</sup> A multivariate analysis in 2010 concluded that young donors (<23 years) with low blood volume contributed about 3% of all donations but disproportionately accounted for about 10–15% of presyncope reactions and syncope-related complications after whole blood donation.<sup>18</sup> Many other variables related to the donor, environment, or staff have been reported to have weaker associations with immediate adverse reactions after blood donation, although the supporting data are generally weak and often not consistent among studies.<sup>9</sup> Finally, autologous blood donors might have significant medical conditions that potentially increases their risk for postdonation reactions compared to healthy, allogeneic blood donors.<sup>21</sup>

Most publications report on immediate donation-related reactions or reactions that occur soon after the blood donation; whereas, a few studies are available on delayed or longer-term complications. One such study showed that allogeneic blood donation did not have a long-term effect on the risk of cardiac ischemia.<sup>22</sup> Germain *et al.* compared a group of 50,889 eligible blood donors who made 0.36 donations/year to 12,357 donors disqualified for false-positive infectious disease markers as a control group of healthy donors

during a 17-year study in Canada.<sup>22</sup> There was no statistical difference in the incidence of hospitalizations or deaths attributable to coronary heart disease in the group of donors who remained eligible (3.60/1000 person-years) compared to the control group of disqualified donors (3.52/1000 person-years; rate ratio 1.02; 95% confidence interval, 0.92–1.13).

## Preventing syncopal reactions at blood drives

Focusing on the factors that most strongly predict immediate and delayed reactions, blood centers have reported strategies to improve safety for the youngest and most susceptible group of blood donors. In 2008, an AABB Task Force recommended that blood centers adopt one or more of the measures in Table 6.3 to reduce reactions among young donors and develop monitoring programs to continually assess donation safety.<sup>23</sup> Operational tactics to improve donation safety aim to recruit donors less likely to have reactions, to modify the drive environment, or to use automated (apheresis) procedures instead of whole blood collection. Physiologic strategies may reduce an individual blood donor's risk of a reaction, such as having the donor drink water shortly before the phlebotomy or perform applied muscle tension maneuvers.<sup>24</sup>

Psychological aspects of the donation experience may be addressed by gauging donors' fear and anxiety, providing donors with information about coping strategies before blood donation, or distracting their attention during the phlebotomy. Each measure is supported to varying degrees by controlled trials or by observational data and predictive models.

In 2009, the American Red Cross and Blood Systems Inc. independently made operational changes in their standard practice in an effort to reduce syncopal reactions among young whole blood donors.<sup>25,26</sup> Both centers introduced precautionary measures, which included new donor selection criteria that required individuals to have an estimated blood volume greater than 3.5 L to prevent loss of more than 15% of their total blood volume with a standard whole blood donation. Other preventive measures addressed donor education, drive environment and supervision, predonation water, and muscle tension during collection.

In the American Red Cross, full implementation of the new donor selection criteria and other preventive measures resulted in a 33%, 25%, and 18% reduction in reactions among 16-, 17- and 18-year-old donors, respectively, compared to the baseline rates before the operational changes.<sup>25</sup> The benefit was most pronounced for the youngest, most susceptible donors. Moreover, a significant 14% decrease in syncope was observed among 16-year-old donors with the new selection criteria. The rate of injuries at the collections sites was low in each year, but no consistent changes were observed over time for any age group. Similarly, Tomasulo and colleagues at

**Table 6.2** Variables Associated with Syncopal Reactions after Blood Donation

Independent variables strongly associated with increased risk (strong evidence based on multivariate analysis in multiple studies):
• First-time vs. repeat donation status
• Young (<23 years) vs. older age
• Low body weight/total blood volume (<3.5 L) with standard WB donation (~525 mL)
• Female vs. male sex
• Caucasian vs. African ethnicity
Variables associated with increased risk (weak or no association in some studies, inconsistent or low-quality evidence, and poorly controlled or univariate analysis)
• Admitted anxiety
• Collection volume
• Greater than 4 h since last meal
• Temperature/season
• Wait time
• Duration of phlebotomy
• History of fainting not related to blood donation
• Mobile blood drive
• History of prior reactions after blood donation
Variables not associated with reactions (strong evidence and multivariate analysis)
• Predonation blood pressure

**Table 6.3** Strategies to Reduce Reactions Among Blood Donors

Predonation education
Drive setup and environment
Staff supervision and phlebotomist skills
Selection criteria (e.g., estimated blood volume) for whole blood donors
Automated red cell collection
Interventions
• Water ingestion before donation and within 10–20 min of phlebotomy
• Distraction during phlebotomy
• Muscle tension during phlebotomy
Postreaction instructions to donors and parents

Blood Systems reported that using the new selection criteria to ensure an estimated blood volume of at least 3.5 L for 17–22-year olds and other interventions decreased the aggregate reaction rates in male and female donors by 24%.<sup>26</sup> In addition, the measures were associated with a 25% decrease in delayed reactions occurring more than four minutes after the phlebotomy was completed, and a 38% decrease in off-site reactions among female donors. Falls were infrequent in all donor groups, occurring at a rate of 1.4 per 1000 donations before the interventions.

These data from the American Red Cross and Blood Systems independently support the benefit of the selection criteria for estimated blood volume to mitigate reactions among young donors, largely to the extent predicted by statistical models.<sup>25,26</sup> In both reports, the aggregate effect of the selection criteria for estimated blood volume reduced reactions by at least 20% in susceptible groups. As expected, the measures did not eliminate the risk of reactions after whole blood donation. While the interventions decreased the occurrence of reactions in susceptible groups, the known risk factors (young age, first-time donation status) were still associated with relatively higher reaction rates than the comparison groups.<sup>25–27</sup> Both studies also documented a low rate of falls or injuries at the collection sites in all donor groups, which did not change after introducing the operational changes. These studies were large enough to detect a 20% reduction in these uncommon events but may have missed a smaller difference.

Alternate approaches to predict and prevent presyncopal reactions that show promise in small, uncontrolled trials have evaluated screening measures to identify and educate fearful donors, use of predonation water ingestion and applied muscle tension during collection, predonation measurements of hemodynamic responses to standing before and after water ingestion, collecting smaller volumes (350 mL rather than 450 mL) from young (16–18-year olds) first-time donors, and postdonation salty snacks.<sup>28–31</sup> Unfortunately, none of the preventive measures has been shown to have a consistent effect in practice or a significant effect on the uncommon but potentially serious falls and injuries resulting from syncope after whole blood donation. Future operational efforts should implement evidence-based approaches to reduce the risk of syncope after whole blood donation, especially among the most susceptible, young donors. Notably, AABB Blood Bank/Transfusion Medicine (BB/TS) Standards now require collection facilities to have a process to reduce the risk of adverse reactions in young donors (AABB BB/TS Standard 5.4.3.3, 32nd edition).<sup>32</sup>

### **Phlebotomy-related complications**

Whole blood and apheresis collections utilize large-bore (e.g., 16-gauge needles) for phlebotomy, which achieve rapid blood flow and minimize clotting and hemolysis but also introduce a risk of injury.<sup>33,34</sup> General guidelines for phlebotomy emphasize the importance of staff training, technique, and experience, as well as knowledge of the anatomy of the antecubital area and careful selection of the vein.<sup>34,35</sup> The median cubital vein, in the center of the antecubital space near the elbow crease, is typically prominent and well-anchored between muscles, making it a good first choice for phlebotomy. The basilic vein, located on the medial aspect (i.e., closest to the body) of the forearm, runs close to the brachial artery and nerve, is less likely to be visible or palpable, and more likely associated with a risk of nerve injury or arterial puncture with phlebotomy. Although these generalizations are useful guides, the anatomic relationships are more complex and variable than depicted in

textbooks. Cutaneous nerve branches are closely associated with veins, and anatomic variation is common, making it impossible to avoid them completely during phlebotomy. Needle adjustment after insertion should be limited to no more than one forward-to-backward maneuver, with careful attention to the donor's comfort level. The phlebotomy should be discontinued if the donor cannot tolerate the venipuncture, and the needle should be removed if it is readily apparent that the venipuncture was not successful. Despite adherence to these general guidelines and good technique, phlebotomy as with any invasive procedure carries an inherent risk that cannot be completely eliminated.

Adverse reactions such as sore arms, hematomas, or bruises are relatively common but usually self-limiting; rarely, more severe nerve injuries or chronic complications occur after phlebotomy.<sup>3,36</sup> Hematomas are raised areas of localized swelling, and bruises (contusions) are flat areas of discoloration. Both result from local injury and blood leaking from the punctured blood vessel that accumulates in the subcutaneous tissue and spreads along fascial planes. As the swelling subsides, the extravasated blood is broken down and reabsorbed, and the area can change in shape, size, and discoloration over a spectrum of blue, black, purple, yellow, and green before resolving completely within a few weeks.

Hematomas or bruises may be noted by the staff at the collection site, but more often develop soon after the donor leaves the drive, complicating about 1% of whole blood and 3.5% of apheresis donations (Table 6.1). When donors were questioned about bruises or other symptoms several weeks after a donation, additional information was elicited about minor complications and the rate of bruises reached 8–22%.<sup>10,11</sup> Although this suggests that some minor reactions are not routinely captured when reactions are recorded by staff at the collection site, these data still serve as a relevant gauge of the more severe or concerning reactions, and identify hematomas that cause discomfort, pain, or distress. About 1 in 10,000 whole blood and apheresis blood donors seek outside medical care for phlebotomy-related complications (Table 6.1).

Hematomas may be alarming to donors, especially if they extend along the arm and as they progress through the various stages of discoloration, but typically resolve completely within a few weeks. Treatment of the area with ice immediately and intermittently for 24 hours after the phlebotomy, followed by warm compresses or soaks, may provide symptomatic relief and promote healing. Over-the-counter medications such as acetaminophen may also be taken as directed for pain. In very rare cases, the phlebotomy site may show signs of infection or septic phlebitis (e.g., redness, swelling, or “red streaks” extending up the arm), which might resolve on its own or may be treated with antibiotics. In anecdotal reports, superficial venous thrombosis in the upper extremity has occurred after blood donation, although a causal relationship with the donation may not be clearly established.

Arterial puncture is suspected in about 1.4 in 10,000 whole blood donations (Table 6.1). Signs of an arterial puncture are rapid filling of the collection bag, often within three minutes, bright red blood, and pulsatile flow. All of these signs might not be present, making some arterial sticks difficult to distinguish from venous draws. Possible arterial punctures are more often reported in young, male donors, but can occur in other donors because of anatomical variation in the location of the artery in the forearm. Whenever a possible arterial puncture is suspected, staff should discontinue the collection, remove the needle, and apply direct pressure to the site for 10 minutes or more, until bleeding has stopped. Donors typically recover without rebleeding or further consequences, but about

7% of donors with a suspected arterial puncture sought additional medical care, likely related to the resultant hematoma or bruise (Table 6.1). In extremely rare cases, an arterial puncture has resulted in complications that required surgical intervention, such as pseudoaneurysm (three published reports), arteriovenous fistula (four published reports), or compartment syndrome (two published reports), after whole blood donation.<sup>36</sup>

### **Phlebotomy-related nerve injury**

Phlebotomy-related nerve injuries are uncommon and typically resolve without sequelae within weeks, but in rare cases have long-term debilitating consequences. Most patients experience characteristic symptoms when the needle is advanced in the arm suggestive of direct nerve trauma, often described as sharp, shooting pain or tingling that radiates to the hands or fingers. Donors may also report immediate numbness or tingling that persists after removing the needle or intense pain with burning, lancinating, or electrical sensations. The relationship of hematoma formation to the development of nerve damage and symptoms is unclear, and most hematomas do not cause symptoms suggestive of nerve irritation. However, hematomas may aggravate nerve dysfunction and pain possibly by impinging on damaged nerves or traumatized areas.<sup>35,36</sup>

Based on hemovigilance data from the American Red Cross, suspected nerve injuries occurred in about 3 in 10,000 whole blood donors; of those, 13% sought additional medical care after the event (Table 6.1). Almost all (>90%) donors who report symptoms of nerve injuries will recover completely within three months.<sup>36</sup> Some donors may report mild residual numbness that persists over an extended duration. Permanent nerve injury after phlebotomy is an extremely uncommon but potentially debilitating outcome, and reliable estimates on its incidence are not available because it is so infrequently encountered among blood donors. Given the close association of nerves with veins and the unavoidable possibility of direct injury to nerves with phlebotomy, factors other than direct nerve contact by the needle appear to be necessary for the chronic pain syndrome to occur. Chronic regional pain syndrome type 2 (CRPS II, previously called causalgia or reflex sympathetic dystrophy) describes neuropathic pain after confirmed nerve injuries. CRPS symptoms vary in severity and duration, but most cases are mild and individuals recover gradually with time. In more severe cases, individuals may have long-term disability. Treatment options for CRPS neuropathic pain include rehabilitation and occupational therapy, psychotherapy to treat associated depression and psychological symptoms, medications, sympathetic nerve block, and surgical sympathectomy. No drugs have yet been approved by the US Food and Drug Administration (FDA) to treat CRPS, but several different classes of drugs such as nonsteroidal anti-inflammatory drugs, corticosteroids, opioids, nortriptyline and other drugs for neuropathic pain, and botulinum toxin injections have also been used to effectively treat some patients, especially early in the course of disease.<sup>37</sup>

### **Reactions after automated collection of cellular blood components**

Advances in apheresis (automated) technology allow simultaneous collection of multiple standard blood components during a single procedure, yielding various combinations of plasma and blood components such as two units of red blood cells (double red cell collection [2RBC]) or two or three units of platelets (double or

triple plateletpheresis), often with a concurrent unit of plasma from one donation. Blood centers have increasingly relied on apheresis donors to optimize the collection process and reduce costs. As in prior years, apheresis platelets accounted for over 90% of the platelet units produced in the United States in 2017, with increasing trends in double- and triple-platelet collections.<sup>1</sup> Similarly, 2RBC collections continue to increase each year, now accounting for 15% of the total US supply of red cell units in 2017.<sup>1</sup>

Like whole blood donation, automated (apheresis) procedures are generally well tolerated, but some donors will experience phlebotomy-related or syncopal complications (Table 6.1). In addition, apheresis complications may result specifically from the device or anticoagulant (e.g., citrate reactions), preparatory regimens (e.g., G-CSF and/or corticosteroids for neutrophil collection), or frequency of procedures.<sup>38,39</sup> Hematomas are the most frequent complication and are more commonly reported after apheresis than WB donation because automated collections often use both arms for venous access (Table 6.1). The overall rate of minor complications at the collection site is higher for automated procedures than WB collections, reflecting primarily minor citrate reactions and small hematomas. Automated collection procedures had lower rates of presyncope and syncope than whole blood donation, which likely reflects donor demographics, more stringent selection criteria, and the use of saline replacement with automated procedures.

Medically serious complications are less likely after apheresis than WB collection, with observed rates of major complications at the collection site of 7.4, 5.2, and 3.3 per 10,000 donations for WB, plateletpheresis, and automated red cell procedures, respectively.<sup>3</sup> Similar rates of reactions requiring additional medical care occurred after WB donation (3.2 per 10,000) compared to automated procedures (2.9 per 10,000).<sup>3</sup> Hospitalization after donation was reported for 46 whole blood donors (1 in 130,749 donations) and eight apheresis donors (1 in 84,722 donations); a causal relationship between the donation and the hospitalization was not established in all cases.

As observed for whole blood donation, multivariate analysis reveals that young age, first-time donation status, female gender, and low weight are independently associated with the risk of reactions after automated red cell collection and plateletpheresis. Yuan *et al.* reported moderate to severe adverse events in 47 per 10,000 plateletpheresis or automated red cell collections over a 2-year period in a hospital-based donor center and found that small, female donors with lower predonation hematocrit were at higher risk of moderate to severe vasovagal-type reactions than other donors, especially when RBCs were collected.<sup>40</sup> Reactions had a similar dampening effect on return donation by first-time apheresis donors comparable to that observed for whole blood donors. Among experienced donors, however, reactions had less of an effect on retention and decreased the rate of return by about 28% for whole blood donors but only about 4% for 2RBC donors.<sup>8</sup>

### **Citrate reactions and other immediate complications during apheresis procedures**

Citrate is used as an anticoagulant during apheresis procedures because it effectively chelates divalent cations such as calcium to transiently and immediately inhibit the coagulation cascade. Citrate causes minimal side effects in donor plasmapheresis because the citrate is mostly in the retained plasma. Plateletpheresis, large-volume leukapheresis, and hematopoietic progenitor cell

collection are more likely to expose the donor to the effects of citrate toxicity. Greater exposure to citrate during triple plateletpheresis was associated with an increase in mild citrate reactions compared to double plateletpheresis, but did not substantially affect donor safety or product quality in one study.<sup>41</sup> Symptoms are usually transient and rapidly reversible because citrate metabolism occurs within minutes in the liver, kidneys, and muscles. In addition, the release of parathyroid hormone mobilizes calcium from body stores and increases its absorption from the kidney to restore calcium hemostasis. Despite these compensatory mechanisms, citrate infusion can acutely decrease the concentration of ionized calcium to cause symptoms such as perioral tingling and paresthesias, chills, nausea, twitching, and tremors during the apheresis procedure. If severe, citrate toxicity can progress to carpopedal spasm, seizures, tetany, and cardiac arrhythmia.

Prompt attention to mild symptoms usually requires only pausing the apheresis procedure, slowing the reinfusion rate, or decreasing the amount of citrate infused by increasing the whole blood-to-citrate ratio and allowing for dilution and clearance of citrate. The procedure should be stopped if symptoms persist or worsen. Intravenous calcium is rarely if ever needed to reverse the citrate effect during donor apheresis procedures and should not be used in routine practice. Donors who have had severe or unusual citrate reactions during automated procedures should be evaluated for possible underlying factors or medications such as loop diuretics that could predispose to these adverse events. The propensity for citrate reactions depends not only on donor characteristics but also on device-related factors, such as the citrate infusion rate or extracorporeal volume of the device.

Oral calcium supplementation (e.g., Tums) during automated collection procedures may reduce the severity of paresthesias and improve ionized and total calcium levels.<sup>42</sup> However, multivariate analysis revealed that oral administration of calcium was not associated with a reduction in overall symptom development and did not prevent the occurrence of more severe symptoms during donor apheresis procedures.<sup>42</sup> The possible significance of long-term metabolic effects of repeated citrate exposure on bone mobilization and calcium metabolism is not well characterized and remains an area of study.

Equipment and disposables used in apheresis collections may cause unusual reactions in rare cases, such as allergic reactions among repeat donors linked to plateletpheresis collection sets sterilized with ethylene oxide.<sup>38,39</sup> Preventive measures include avoiding use of certain disposables if sensitization is suspected or minimizing exposure to ethylene oxide by repeatedly priming the disposable collection set or using kits near their expiration dates. Air embolism is a very rare complication of apheresis procedures because the instruments have sensors to detect air within the extravascular circuit that stop the procedure. But symptoms of air embolism are still possible if more than 3–8 mL/kg of air enters the donor's venous system through either a leak in the access, instrument failure, or operator's error. These symptoms of air embolism include dyspnea, tachypnea, cyanosis, tachycardia, and hypotension as air enters the right ventricle and pulmonary artery with obstruction of the right ventricular output and pulmonary artery vasoconstriction. If collection staff expect air embolism, they should stop the procedure and place the donor in the Trendelenberg position (i.e., lay the donor on their back and raise their feet higher than their head) on their left side. If the air does not dissipate or symptoms worsen, surgical

intervention to aspirate the air through a pulmonary artery catheter may be necessary.

### **Procedure-related complications related to donation frequency or multiple component collection**

The high volume and efficiency of apheresis collection procedures, as well as the frequency and allowable interval for repeat donations, pose potential acute and long-term risks to donors, such as cellular depletion, iron depletion, and serum protein loss. The current regulations that govern donor selection define precautions for adequate pre- and postprocedure cell counts and serum protein values before donation, as well as limits on the donation interval and frequency for apheresis procedures. Immediately after plateletpheresis, a donor's platelet count may decrease by 20–30% but quickly returns to baseline within about four days.<sup>31</sup> Current FDA-approved apheresis devices have different methods to ensure that the donor's platelet count remains above a predefined set value at the completion of the procedure, typically 100 platelet/ $\mu$ L, which have been validated in practice by blood centers. The transient platelet count decrease and recovery after serial collection procedures are generally larger and last longer for female donors than male donors, but changes are typically transient and recovery occurs promptly after donation.

Frequent whole blood or double red cell donation is known to cause aggravate iron deficiency (see Chapter 7). Frequent apheresis donation has been associated with longer-term effects in some plasma and platelet donors. Progressive decreases in platelet counts, lymphopenia, and increased risk of infections have been associated with frequent plateletpheresis.<sup>43–46</sup> Serum protein, albumin, and IgG concentration are statistically lower in frequent plasmapheresis donors compared to nondonors; however, their levels may not correlate with the intensity of donation and typically remain stable over time in most donors.<sup>47,48</sup>

### **Special considerations: granulocyte collection**

Granulocyte collection (leukapheresis) poses unique risks to donors.<sup>3</sup> To collect sufficient numbers of granulocytes for an adequate therapeutic dose, healthy donors are given corticosteroids (e.g., dexamethasone) and/or granulocyte colony-stimulating factor (G-CSF) prior to the leukapheresis procedure. G-CSF may cause short-term side effects such as bone pain, myalgia, and headache in granulocyte donors. The rare complication of splenic rupture seen with hematopoietic progenitor cell collection has not been reported in G-CSF-stimulated granulocyte donors, likely because of the lower dose and shorter course of treatment. To date, the available data support the use of G-CSF stimulation in volunteer donors and have not detected long-term cardiac, inflammatory, or malignant consequences among granulocyte donors who received G-CSF on three or more occasions.<sup>49</sup>

The risk of subcapsular cataracts in granulocyte donors who received corticosteroids has been evaluated in two controlled studies. Differences between the treatment and the control (unstimulated) groups were not significant, but the tendency for bilateral occurrence of cataracts was observed exclusively among glucocorticoid-stimulated granulocyte donors. The relatively small size of both studies suggests that ongoing surveillance is

prudent to better define the prevalence of posterior subcapsular cataracts in this donor population.<sup>50,51</sup>

### Conclusion

Whole blood donation and apheresis procedures to collect blood components are generally considered safe, and most volunteer blood donors have uneventful donations and feel good about donating blood to help others. Still, blood donation is an invasive procedure that can cause discomfort and pain, and, in some cases, serious injury. Recent operational trials and research programs identify possible ways to reduce the risk of complications after blood donation, especially among the most susceptible, young donors. Ongoing efforts and continued vigilance are necessary to further improve safety for volunteer blood donors.

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## CHAPTER 7

# Chronic effects of blood and plasma donation

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## Introduction

Stability of the blood supply depends on the ability of blood collectors to recruit new donors and to maintain return visits from established, volunteer donors. While the combination of motivations that inspires a person to donate blood will vary considerably, one consideration is surely an expectation for procedures that safeguard donor health and well-being. Many elements of blood collection, whether encoded in federal or state regulations or required by the blood collector's rules, are specifically designed for the protection of donor health. These include the brief physical exam, parts of the donor health history, minimum hemoglobin concentrations, and a mandated deferral period following a successful donation to allow for physiological recovery. The required minimum length of the interdonation interval is specific to the donation procedure, but for whole blood and apheresis collection of red blood cell components the interval is motivated by the need for hemoglobin recovery.

Donating blood has a remarkable safety record. Adverse reactions are generally uncommon, with low, single-digit rates of vasovagal or other systemic reactions in US donors, albeit higher in selected subpopulations of donors. Donors at higher risk have several identifiable features (younger age, lower body weight, first-time donor status, and female sex), and continuous improvements are made to donor intake and phlebotomy procedures to improve donor safety and comfort.<sup>1,2</sup>

In contrast to acute events, however, long-term outcomes attached to blood donation can be harder to recognize, measure, and ameliorate. The opportunity for blood donation to lead to deficits in hemoglobin and iron has been known for decades, and it informs key eligibility requirements in the United States and elsewhere. This chapter discusses potential chronic effects associated with blood donation, including those for apheresis donation of platelets and plasma.

## Prevalence of and risk factors for iron depletion in blood donors

Nominally a simple question, *How common is iron depletion in blood donors?* is a multivariegated inquiry that requires unpacking. One might reasonably interpret the question to mean any of the following:

- *What is the prevalence of iron depletion in people who present to donate?*
- *What is the prevalence of iron depletion in people who make a donation?*
- *What is the prevalence of iron depletion in blood donors (presenting or successful) that is caused by blood donation?*

From these questions follow several more:

- *How do you define "iron depletion?"*
- *How would you know, i.e., how do you measure it?*
- *What definition or what degree of iron depletion is tolerable, what is actionable, and does this vary for different groups of donors?*

Iron is an essential mineral involved in many important physiologic processes, including oxygen transport, energy production, immune and cognitive function, and support of numerous enzymatic reactions. Iron is found in all cells, and a deficit of iron is the most common nutritional deficiency worldwide. Most iron in the human body, about two-thirds, is found in hemoglobin in red blood cells. There is no active mechanism for iron excretion, so iron homeostasis is maintained by recycling of most of the body's iron and tightly controlled absorption in the gastrointestinal tract to balance what are ordinarily minimal basal losses.<sup>3</sup>

Evidence that hemoglobin recovery following donation varies considerably across individuals and especially by sex was recognized at least by the mid-twentieth century. A publication by Fowler and Barer in 1942 documented the interpersonal variability in regeneration of hemoglobin, the acceleration of recovery with administration of iron, and the average recovery period (in mostly male subjects) of about five weeks.<sup>4</sup> Following development of an assay for measurement of ferritin, the storage form of iron which varies directly with a person's iron stores, additional investigators focused on the relationship between blood donation and iron status. Finch and colleagues demonstrated that men donating three times annually had much higher prevalence of ferritin less than 12 ng/mL than those with one donation per year, 12.7% vs. 0.9%.<sup>5</sup> This threshold, considered the level below which one has exhausted iron stores, is still recognized as clinically relevant though alternate cutoffs of 10 or 15 (such as by the World Health Organization) are also used. Simon *et al.* reported similar findings in 1981, showing that female sex compounded the risk for ferritin less than 12 ng/mL associated with blood donation and that menstruation added

incremental risk.<sup>6</sup> Further, and importantly, they also showed that iron stores correlated strongly with lifetime donations, whereas hemoglobin levels did not; hemoglobin, accordingly, was also shown to be a poor indicator of iron status.

The limited information provided by hemoglobin levels with respect to a donor's iron status has been confirmed repeatedly,<sup>7</sup> and reliance on hemoglobin as an indirect proxy for donor iron status has been a long-term obstacle to developing a comprehensive assessment of the iron status of US donors. Regulatory and industry concern are longstanding, evidenced by multiple workshops and advisory committee meetings. One early meeting was a 2001 workshop focused on the need to maintain the iron status of female donors of childbearing age and how that could be done while maintaining adequacy of the blood supply.<sup>8</sup> Discussion noted the heavy preponderance of females in the 700,000 annual deferrals for low hemoglobin (95%) and the often permanent loss of donors who receive the deferral. Iron supplementation programs were indicated as a promising approach to improve female donor iron status and eligibility for donation, with attendant benefits for blood sufficiency.<sup>8</sup>

Over the subsequent two decades, widespread adoption of iron supplementation programs by blood centers did not materialize. There has been, however, considerable public discussion of blood donor iron depletion in parallel with public funding to elucidate the issue. Specifically, these conversations sought to better understand the prevalence of iron depletion in blood donors, to quantify the role of blood donation as a causal factor, and to identify strategies to mitigate risk for iron depletion while maintaining adequate blood supplies. The REDS-II (*Retrovirus Epidemiology and Donor Study*) Iron Status Evaluation (RISE) Study contributed importantly to these endeavors, demonstrating that the scope of the problem was considerable indeed.<sup>9,10</sup> Enrolling 2425 donors into a prospective cohort study, RISE investigators documented the trajectory of iron status and hemoglobin levels over a 15–24-month follow-up. The study population was balanced by sex and captured donors with distinct profiles, specifically those making their first blood donation ever (or alternately within two years) and those donating at high frequency (defined as two donations in a year for females and three times for males). Assigning ferritin less than 12 ng/mL to “absent iron stores” (AIS) and a ratio of ferritin and soluble transferrin receptor to represent “iron-deficient erythropoiesis” (IDE) or intermediate iron depletion, RISE showed that at enrollment 15% of donors were fully iron-depleted (had AIS) while

41% demonstrated IDE. These aggregate numbers masked notable variability by sex and donation history, ranging from 0% prevalence of AIS in first-time male donors to 67% prevalence of IDE in frequent female donors. Enrollment data also showed that how informative hemoglobin was for iron status varied by donation status. In first-time donors no association was seen, whereas in frequent repeat donors lower hemoglobin was associated with higher prevalence of iron depletion (Table 7.1). Because RISE did not enroll a representative sample of the donor population, it did not yield direct estimates of the overall prevalence of AIS or IDE in the US donor pool. But the data developed from statistical models allowed for a simulation study that projected that approximately 13% of donations in the United States come from a donor with ferritin less than 12 ng/mL, while another 21% are from donors with ferritin less than 26 ng/mL (which correlates strongly with the study's measure of IDE).<sup>11</sup>

The statistical models underpinning this simulation study helped identify and quantify the strongest determinants of blood donor iron status. Donation frequency was confirmed to exert a powerful effect, compounding the risks associated with female sex, while genetic markers for iron homeostasis were significant but of relatively modest predictive value.<sup>12</sup> Time itself was also a critical factor, with adjusted risk for AIS shown to last up to five months following donation, controlling for other risk factors.<sup>10</sup>

The REDS-III Hemoglobin and Iron Recovery Study (HEIRS) further sharpened the understanding of physiological recovery postdonation.<sup>13</sup> HEIRS was a randomized controlled trial of 215 subjects, all of whom had waited at least four months since their last donation, and who were assigned to either take 38 mg of elemental iron daily following a single unit of donated blood or to take no iron pill (unblinded treatment). Donor hemoglobin and ferritin were assayed at seven visits over 24-week follow-up, allowing for documentation of how exogenous iron mediates the effects of time in the regeneration of hemoglobin and the restoration of iron stores. In donors taking iron, the recovery of hemoglobin to preindex donation levels was uniformly quick, taking approximately one month. Ferritin recovery followed the recovery of hemoglobin, but was also considerably accelerated by daily iron. In contrast, in those not taking iron, recovery was highly variable and for two-thirds of participants neither hemoglobin nor ferritin had recovered to baseline levels by the end of 24 weeks. In sum, for donors taking a moderate dose of supplemental iron daily, the mandatory eight-week minimum interval between donations was easily sufficient to restore

**Table 7.1** Hemoglobin and Iron Status at Enrollment in REDS-II RISE Study

Sex	Donor Status	% with Low Iron	12.5–13.4	13.5–14.4	14.5–15.4	15.5–16.4	≥ 16.5 g/dL	All
Female	FT	N (%) Hb	267 (58%)	140 (30%)	46 (10%)	14 (3%)	3 (1%)	470
		AIS (%)	7%	7%	4%	7%	0%	7%
		IDE (%)	26%	26%	13%	43%	335	25%
	RPT	N (%) Hb	436 (59%)	212 (29%)	73 (10%)	18 (2%)	2 (0%)	741
		AIS (%)	32%	25%	12%	22%	0%	28%
		IDE (%)	69%	67%	62%	61%	50%	67%
Male	FT	N (%) Hb	61 (15%)	114 (29%)	105 (27%)	87 (22%)	29 (7%)	396
		AIS (%)	0%	0%	0%	0%	0%	0%
		IDE (%)	2%	3%	3%	4%	0%	2.5%
	RPT	N (%) Hb	172 (23%)	228 (31%)	207 (28%)	95 (13%)	35 (5%)	737
		AIS (%)	33%	15%	13%	3%	6%	16%
		IDE (%)	57%	50%	47%	39%	34%	49%

FT: first-time donor or “reactivated” donor (no donations in two years).

RPT: repeat frequent donor, two RBC units donated in one year (F) and three units (M).

AIS: absent iron stores (ferritin < 12 ng/mL).

IDE: iron-deficient erythropoiesis (log ratio soluble transferrin receptor/ferritin) ≥ 2.07.

physiological levels of iron stores and hemoglobin. For those not taking iron, an interval three times that length was still too short.

Research in other jurisdictions confirms the findings in the United States of a high prevalence of iron depletion in blood donor populations. In Canada, a representative sample of 12,000 blood donors was tested for ferritin, 17% of whom had ferritin less than 12 ng/mL and 42% (an additional 25%) had ferritin less than 25 ng/mL.<sup>14</sup> A smaller national sample of 3000 donors in Australia showed 13.6% had ferritin less than 15 ng/mL,<sup>15</sup> close to the prevalence in the US simulation study of advanced iron depletion.<sup>11</sup> A study in the Netherlands demonstrated another valuable lesson, namely, that the prevalence of iron depletion varies widely by how the investigator chooses to define it, based on the particular biomarker chosen and the relevant cutoff used to denote iron insufficiency. Depending on whether the definition was tied to ferritin, zinc protoporphyrin, hepcidin, or other markers, the estimated prevalence of low iron in both male and female donors varied fivefold, between approximately 5% and 25%.<sup>16</sup> Hence, while serum or plasma ferritin has generally emerged as a consensus biomarker for assessment of blood donor iron status, it is hardly the only one in use and cross-study comparisons require careful attention to tests performed and to terminology used to classify outcomes.

Two more large cohorts, the Danish Blood Donor Study and the UK Interval Study, provide further strong evidence that more frequent donation is associated with higher prevalence of iron depletion in blood donors.<sup>17,18</sup> A large cohort of French donors found subgroups of donors with prevalence of low ferritin (defined as <26 ng/mL) up to 72%.<sup>19</sup>

## **Regulatory considerations: Role of the Food and Drug Administration and AABB**

The US Food and Drug Administration (FDA) has engaged the transfusion medicine community multiple times to characterize the scope of iron depletion in blood donors and to solicit input for ways to best safeguard donor health and well-being. The FDA convened its Blood Products Advisory Committee in 2008, 2010, and 2016, and it sponsored a workshop in 2011.<sup>20-23</sup> These meetings spanned the period between 2007 and 2015 when FDA published proposed and final rules relating to salient donation eligibility requirements.<sup>24,25</sup> The proposed rule sought comment on the possible impact on blood availability and blood donor iron status of the following measures:

- Raising the minimum male donor hemoglobin level from 12.5 to 13.0 or 13.5 g/dL.
- Lowering the minimum female donor hemoglobin level from 12.5 to 12.0 g/dL.
- Extending the minimum interval between whole blood donations from 8 to 12 or 16 weeks.

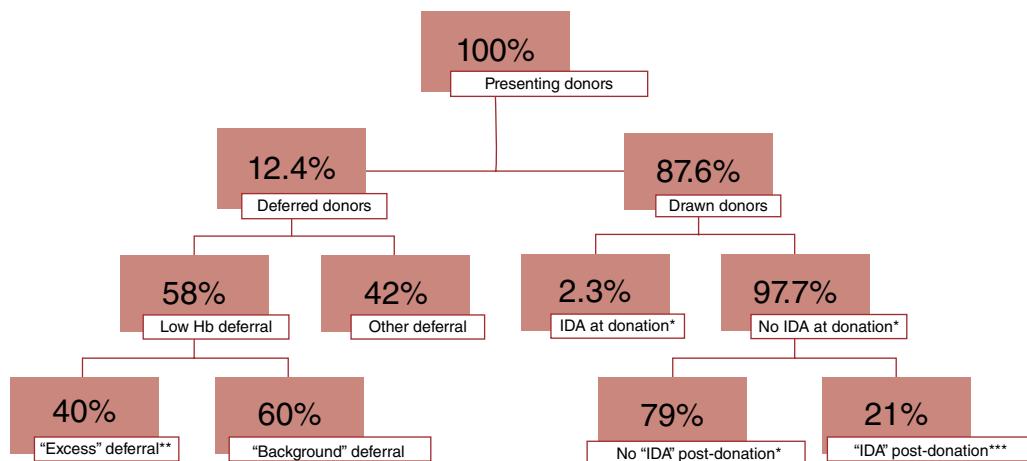
Consideration for increasing male donor hemoglobin acknowledged that the current standard was below the recognized lower threshold for anemia in adult males of 13.5 g/dL,<sup>3</sup> and the possibility of changing only to 13.0 g/dL was a tacit admission of the ever-present tension between eligibility requirements and blood sufficiency. The proposal to lower the female hemoglobin cutoff likewise sought to align donation requirements with the accepted definition of anemia, potentially offsetting diminished collections associated with increasing hemoglobin cutoff for males. The possibly lengthened interval intuitively allowed for additional physiological recovery, as amply documented by the RISE and HEIRS data emerging during this period. The Final Rule published in 2015 left donation intervals unchanged and increased the male donor

hemoglobin minimum by 0.5 g/dL. Female hemoglobin cutoffs were statutorily unchanged, but the text left the door open for blood centers to collect from females with hemoglobin levels of 12.0 g/dL as long as safety measures approved by FDA were implemented.<sup>25</sup> To date, no known approval has been issued for the collection of females with hemoglobin of 12.0 g/dL in the United States.

The AABB (Association for the Advancement of Blood & Biotherapies, formerly the American Association of Blood Banks), which provides accreditation for US blood collectors and publishes industry standards, has also engaged the issue. Communications with membership include bulletins that are often advisory, occasionally mandatory. In 2012, following publication of RISE enrollment data, AABB issued a bulletin to educate blood centers on the prevalence of iron depletion, recognized risk factors, and to suggest remediation measures.<sup>26</sup> Five years later, following presentation of preliminary data from a US study on high-school-aged blood donors at the 2016 BPAC meeting, an updated AABB bulletin was published.<sup>27</sup> Stronger in tone than the 2012 document's educational posture, the new bulletin advised that blood centers "should...take action" with all donors or those donor subpopulations shown to have greater risk for, or from, iron deficiency. Notably, for donors age 16–18 years old, the bulletin recommended limiting collections to a single unit of red cells per 12-month period, unless other mitigation measures were in place. As discussed below, this language helped stimulate a meaningful blood center response.

AABB dedicated further attention and energy to the issue by convening an expert work group that was tasked with evaluating iron depletion in blood donors under a "risk-based decision-making" (RBDM) framework developed by the Alliance of Blood Operators, a network of blood collectors from Europe, North America, and Australia.<sup>28</sup> The paradigm represents a systematic approach for managing complex risks that come up in the context of blood safety. The process seeks to define risks clearly, to assess whether each identified risk is tolerable, and if so under what contingencies. In addition, the process involves delineation of tradeoffs and it expressly seeks to identify and engage all relevant stakeholders. In the case of blood donation, the general public is integral to the process because it represents the source of volunteer blood donors, and public confidence in the institutions providing blood to patients is vital. Though intended as advisory and not an enforceable guideline, the committee's recommended mitigation measures were to encourage iron supplementation in blood donors and to encourage blood centers to conduct ferritin testing for at least higher-risk donor populations (discussed below).<sup>29</sup>

Another notable output from the RBDM workgroup's report was its discussion in the context of defining risk and stating the problem to be addressed. While the relationship between lower levels of iron stores and blood donation has been long evident, a systematic estimate of the occurrence of iron deficiency anemia (IDA) in a representative blood donor population was not available. This mattered because, as discussed more fully below, the adverse consequences associated with iron depletion are more consistently and more reliably observed when low iron is accompanied by anemia. As indicated in the three bulleted questions at the beginning of this chapter, this question could address any or all of the prevalence of IDA in presenting blood donors, the prevalence in donors who successfully donate (conceivably more important), and the prevalence of IDA in presenting blood donors that is caused by blood donation. Sufficient data has been developed in recent years that the RBDM committee was able to begin to characterize this phenomenon, as summarized in Figure 7.1.



**Figure 7.1** Association between iron-deficiency anemia and donation in presenting US donors.

\*Iron deficiency anemia (IDA) at donation is estimable from the proportion of males who donate with hemoglobin <13.5 g/dL and the share who are estimated to have low ferritin, which differs by donation frequency.

\*\*"Excess" hemoglobin deferrals are those attributed to the effects of blood donation and include most hemoglobin deferrals in males and postmenopausal females and a minority share of deferrals in premenopausal females. The "excess" of 40% of hemoglobin deferrals equals about 3% of presenting donors.

\*\*\*"IDA" postdonation represents male and female donors whose laboratory values are consistent with IDA given an assumed loss of 8% of hemoglobin in male donors and 12% in female donors making a whole blood donation and who had low ferritin at the time of donation.

Taking as the unit of analysis presenting donors, the committee assembled data that allowed for identifying a causal association between donation and IDA in a nontrivial share of the donor pool. Specifically, three groups were identified that include:

- 3% of presenting donors who are deferred for low hemoglobin that represents "excess deferral." This figure represents the product of the proportion of presenting donors deferred (12.4%), the proportion of donor deferrals attributed to low hemoglobin (58%), and the proportion of these deferrals attributed to the effects of prior donation (40%). This latter factor is complex and discussed separately below.
- 2% of presenting donors who make a donation despite having IDA. This figure is estimable by taking the proportion of male blood donors whose hemoglobin of record is below the recognized cutoff for anemia, 13.5 g/dL, and adjusting for the proportion of them estimated to have low ferritin, here defined as below 26 ng/mL.
- 18% of presenting donors whose postdonation lab values are consistent with both anemia and iron depletion. They represent the product of drawn donors (87.6%), those without IDA at donation (97.7%), and those whose values are consistent with IDA after donating; they are estimated to occur at a 3:1 ratio of female to male donors.

Identifying the first group, donors deferred for low hemoglobin that can be attributed to donation-associated IDA, leverages data from REDS-II studies including RISE and detailed analyses of donor presentation and deferral outcomes, and other efforts.<sup>9–11,30,31</sup> Data compiled to make these estimates includes the proportion of donations by sex, by menopausal status for females, by first-time or repeat donors, and the proportion of deferrals for males and females that are associated with iron depletion and anemia (below 12.0 g/dL in females and all low hemoglobin deferrals in males). The fact that blood donation contributes to deferral at subsequent visits is an unremarkable observation. However, the virtually identical low hemoglobin deferral rate for first-time and repeat donors (7.7% vs. 7.8%, respectively) reported by Custer and colleagues<sup>30</sup> would

ostensibly support the null hypothesis of no effect. Taking note of the four-time stronger disincentivizing effect of low hemoglobin deferral on first-time compared to repeat donors,<sup>31</sup> one must conclude that self-selection results in a donor pool enriched for donors with relatively lower risk for hemoglobin deferral, by a donor's second visit. With algebra and not terribly speculative assumptions, one derives an estimate that the ≈7.5% low hemoglobin deferral rate represents "background" low hemoglobin for 4% of presenting donors (almost entirely females) and donation-associated deferral for 3% of presenting donors. The clinical relevance of this is discussed below, but in aggregate that an estimated one out of every four or five blood donors presents to donate or leaves the donation site with IDA is indeed notable.

The spotlight on blood donor iron status seen in the United States is mirrored in many countries around the globe. As noted, large prospective studies were funded in the United Kingdom and Denmark, and the Netherlands also has research dedicated to reducing iron depletion.<sup>17,18,32</sup> Australia's study of iron status was cross-sectional rather than prospective, but it was nationally representative and followed by additional research efforts to identify optimal ways of encouraging iron supplementation.<sup>33</sup> More broadly, while virtually no country systematically monitors iron in all donors at each visit, attention and research are expanding in many locations.<sup>34,35</sup>

### Adverse outcomes potentially associated with iron depletion

Though the important causal role of blood donation for creating or sustaining iron depletion in many donors is uncontested, the documentation of serious adverse consequences is generally lacking. The number of clinical outcomes asserted to be due to iron deficiency even without anemia is considerable, including fatigue, lowered exercise tolerance, altered cognitive function, pregnancy-related outcomes, pica, and restless leg syndrome. To date, strong evidence exists only for pica.<sup>36–38</sup>

Expert opinion tends to more consistently find clinical outcomes associated with IDA rather than iron depletion alone, but what one finds in clinical populations might differ from what one documents with systematic examination in healthy blood donors.<sup>39</sup> The literature on the impact of iron deficits early in life is sizable and reflects decades of work, and there is strong documentation that fetal and neonatal deficits create cognitive, behavioral, emotional and metabolic changes that are enduring even if corrected.<sup>39</sup> More recent investigations have extended inquiry into whether iron deficits occurring in adolescence or adulthood have measurable effects, and a few studies have drawn attention with intriguing but not definitive data. In one RCT,<sup>40</sup> Bruner and colleagues studied adolescent females in Baltimore, MD, studying whether double-blinded oral iron treatment of nonanemic, iron-depleted ( $\text{ferritin} < 12 \text{ ng/mL}$ ) school girls impacted standardized test results. At study end, one measure out of three on a test for memory had a  $p$ -value of less than 0.05, and none of the three tests for attention showed a difference. The variability in outcomes attributed to iron was a mere 7%, with 93% ascribed to baseline differences in subjects. Moreover, while the subjects all had hemoglobin within the normal range at study onset, by study end the iron treatment group observed not only an increase in ferritin, but also an increase in hemoglobin of 0.8 g/dL compared to controls. Hence, any difference in memory tests observed, modest as they were, cannot be ascribed to the subjects' changes in iron status given the concurrent improvements in hemoglobin and ferritin levels. Another frequently cited study referenced in support of an association between adult iron status and cognitive function also has an issue with identifiability of the alleged effects.<sup>41</sup> Also an RCT, the study enrolled women into three strata classified as iron sufficient, iron deficient, and iron deficiency anemia, and within each stratum subjects were randomized to 16 weeks' oral iron treatment or placebo. The differences observed at baseline were statistically significant for the IDA group only, with the iron-deficient group being indistinguishable from controls. In longitudinal analysis, one measure (of many tested) was deemed statistically significant for "ferritin responders" while another reported improvement was attributed to "hemoglobin responders," but without disaggregating the IDA group from the iron-deficient-only subjects. This study and others have been discussed in a recent review,<sup>42</sup> and the interested reader can find more detail therein.

In blood donor populations, few studies have addressed whether donation-associated iron depletion affects neurocognitive function. Two large European studies have included the assessment of cognitive function, finding no association with iron status. In the INTERVAL study,<sup>18</sup> a large RCT in England designed to assess the safety implications of relaxing the prevailing minimum donation interval requirements, a battery of standardized tests were conducted at study onset and at the end of two-year follow-up. Expected associations were found between shorter donation intervals and prevalence of anemia and iron depletion, but none of five cognitive function assessments was associated with iron status or treatment group. The Danish Blood Donor Study indirectly addressed this question by evaluating the scholastic achievement of the offspring of female blood donors in comparison to children of nondonors.<sup>43</sup> This study documented the widely acknowledged "healthy donor effect," where donor populations have a favorable health status compared to the general population, but even controlling for that effect the authors found no association between academic outcomes and maternal donation behavior. A recent cross-sectional study in the United States has addressed this issue, also failing to

note any link between cognitive performance and iron status or blood donation in young women.<sup>44</sup>

Aside from concern for cognitive function, industry concern for pregnancy-related outcomes is the other potentially serious adverse outcome that has drawn scientific inquiry. Limited thus far, two large studies, one in Canada and one in Denmark, have not found a negative outcome associated with maternal blood donation. The integrated national health systems in both countries allow for close linkage of female donation behaviors and outcomes from subsequent pregnancies. In Canada, Germain and colleagues reported a lack of association between donation frequency prior to pregnancy and the occurrence of low-birth weight, preterm delivery, or stillbirth.<sup>45</sup> In Scandinavia, Rigas *et al.* did find a modestly lower birth weight from children born to women who were blood donors, but the magnitude was small and there was no difference in the clinically important low-birth weight incidence.<sup>46</sup>

Less serious consequences of donation-associated iron loss include fatigue, restless legs syndrome (RLS), and pica. The literature of fatigue and its relationship with iron and/or hemoglobin levels in blood donors is appreciable. Similar to the studies on cognitive function reported above, teasing out effects related strictly to iron depletion is challenged by the complexity of establishing identifiability of effects. The prevalence of fatigue in the postdonation period is not uncommonly reported,<sup>47,48</sup> but ascertainment would be conducted following loss of both hemoglobin and iron. One recent study evaluated changes in self-reported fatigue and changes in iron status in a prospective cohort of frequent blood donors.<sup>49</sup> Despite sizable changes in both iron and fatigue levels during the study period, fatigue and changes in fatigue were unassociated with iron or changes in iron in multivariable models. Similar findings were reported by Rigas *et al.* who found no association between iron status and self-reported physical or mental quality-of-life measures.<sup>43</sup> Findings from the INTERVAL study indicate a possible signal, with donors assigned to donate more frequently reporting greater levels of tiredness and lower levels of ferritin at study end.<sup>18</sup> Whether the results are derived from changes in red cell mass or from iron is hard to disentangle, however.

Restless legs syndrome (RLS) is a neurological condition with reported prevalence between 5 and 15% in European and North American populations. The most common feature is an irrepressible urge to move one's legs, particularly in the evening, and the condition can significantly compromise sleep and quality of life. The pathophysiology of RLS is complex and multifactorial, with an association with low iron reported 60 years ago and genetic associations being identified over the last 15 years.<sup>50,51,52</sup> Two studies in blood donors in the United States and one in Denmark found expected prevalence of RLS, but in none of the three studies was an association with iron status found.<sup>36,37,53</sup> The success of iron treatment in improving RLS<sup>54</sup> suggests a plausible association with donation that might prove elusive for investigators to confirm. It may be that donors who newly experience RLS as a consequence of blood donation take steps to remedy their condition. If they drop out of the donor pool, they would not be readily available to investigators; if they take iron they might eliminate symptoms and improve iron status, making their affliction short-lived.

Pica, the compulsive ingestion of non-nutritious substances, is one condition that has been clearly associated with iron in nondonor and donor populations. Investigation of pica has its own methodological challenges, in that it is a socially and culturally mediated phenomenon (in terms of what is considered appropriate to consume), it is often conflated with psychiatric conditions, and

eliciting disclosure of socially unusual behaviors is a formidable task. The most frequent manifestation in the United States appears to be consumption of large amounts of ice (pagophagia), but other common substances that are consumed include dirt, raw pasta, starch, or chalk. The association with iron and ice-related pica was identified 50 years ago, including the rapid reversibility of the behavior following treatment with iron.<sup>55</sup> Three studies in US blood donors have shown an association with iron, albeit often without great detail elicited on the specific substances consumed.<sup>36–38</sup>

### Potentially vulnerable populations

As noted above, awareness of the possibility for blood donation to adversely impact pregnancy is long-standing. The 2001 workshop offered solutions including suggestions for expanding iron supplementation,<sup>8</sup> but such programs remain few in number. Research to date does not show empirical support for worse outcomes in babies born to blood donor mothers. Abundant evidence exists that female donors are more prone to hemoglobin deferral than male donors, and that iron depletion is more prevalent in females.<sup>10,11,56</sup> The HEIRS study was not powered to assess differences by sex in the recovery kinetics of hemoglobin or ferritin, so whether sex differences exist in response to blood donation after controlling for menstrual loss remains an open question.<sup>13</sup>

Iron depletion in young blood donors has been a focus of concern for several years. The motivation for investigating whether younger donors might be more vulnerable derives from a couple of factors. First, blood collectors in the United States grew increasingly reliant on high school age donors (16–18 years old), with the share of RBC collections from this age group reaching 12–14% in recent surveys.<sup>57,58</sup> Donors this age show high levels of motivation to donate, and logically high schools represent an opportune locale to efficiently collect blood. State legislatures have accommodated mutual interest by the public and blood collectors to facilitate donation by younger donors, such that 37 states allow for donation by 16-year olds.<sup>57</sup> The second factor motivating the concern in teens is recognition that physical and neurocognitive maturation are ongoing in this population, with both processes requiring iron. The first bulletin issued by AABB in 2012 on iron depletion did not specify young donors as a risk population, but the one in 2017 did list them, given the public availability of a recent study on iron status in young donors.<sup>26,27</sup>

The REDS-III CHILL study (*Comparison of Donation History and Iron Levels in Teenage Blood Donors*) sought to determine the prevalence of iron depletion in young donors and to investigate whether their risk for iron depletion was greater than that for adult blood donors.<sup>59</sup> Two large blood centers systematically measured ferritin levels in 4265 blood donors over a single academic year, mostly subjects from high school blood drives. An adult control population and 1954 follow-up donations allowed for repeated measures, multivariable modeling of risk by age group. Sixteen-to-eighteen-year olds had two to three times the risk for ferritin less than 12 ng/mL compared to adults 19 to 49, after controlling for donation frequency and other factors, and progression to deferral for low hemoglobin was twice as likely in teen versus adult females.

### Potential measures for mitigation of iron depletion

Actions that might reduce the prevalence of iron depletion in blood donors are not hard to conceive. None, however, is cost free in terms of reducing donor availability with the possible exception of

enhanced educational materials. Raising the minimum hemoglobin level to the threshold for anemia would reduce iron depletion in male donors, but as shown previously with probable loss of several percentage points of RBC collections.<sup>11</sup> Extending the deferral period for a low hemoglobin deferral might represent a favorable cost–benefit approach, but despite reported variability in how blood centers manage low hemoglobin deferrals,<sup>60</sup> no systematic evaluation has been performed.

Explicit restrictions of donation frequency, whether by extending the minimum interval between donations, as proposed by FDA in 2007, or by limiting some or all donors to fewer donations on an annual basis, would by definition allow more time for recovery between donations. This was what AABB's 2017 bulletin recommended for teen donors, in fact, capping the donation frequency at a single unit per year unless other mitigation measures were in place. Compared to the current allowable donation frequency of every eight weeks (~6 times per year), this recommendation may seem unduly restrictive. In fact, it is consistent with the findings from HEIRS, which showed that two-thirds of donors not taking iron had not reached predonation levels for hemoglobin or ferritin after 24 weeks. Similarly, the simulation study based on RISE data found that extending the donation interval to 12 or 16 weeks would still leave 80% of prevalent iron depletion uncorrected. Finally, a recent study by Vassallo and colleagues reported that ~20% of male and ~40% of female donors age 16–18 years had insufficient iron stores 12 months after donation, and the share with adequate ferritin after a year was less than half in those whose index donation was also associated with low ferritin.<sup>61</sup> Clearly, longer recovery times in the absence of supplemental iron do not resolve the issue and place blood sufficiency at risk.

As recommended in the 2001 workshop focused on women of childbearing years, and consistent with findings from HEIRS, iron supplementation in donors would accelerate physiological recovery, reducing deferral for low hemoglobin and supporting blood availability. To date, very few blood centers actively distribute iron to blood donors,<sup>62</sup> the reasons for which are discussed at some length in the report from AABB's Risk-Based Decision-Making group on iron deficiency.<sup>29</sup> One consideration is a concern that distributing iron “medicalizes” the relationship between donor and blood center, imposing responsibility for tracking and follow-up that conceivably includes sophisticated hematologic workups. Relevant laws vary by state, and it is possible that in some jurisdictions distributing iron would indeed be considered the practice of medicine, with all the attendant implications for liability insurance and operations that such a transformation implies.

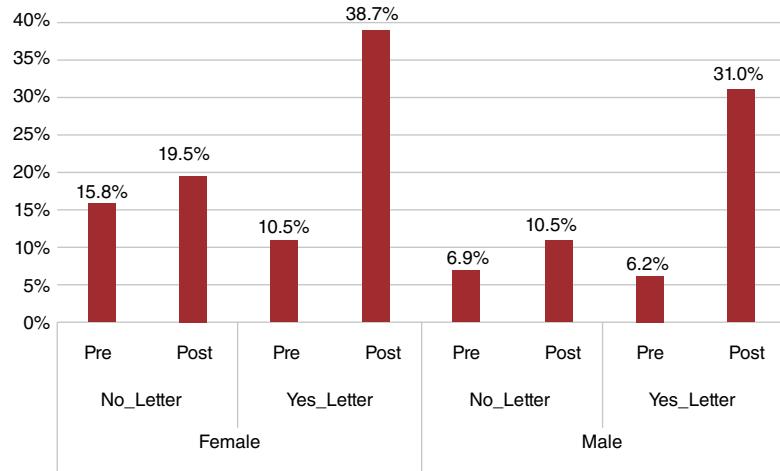
Remarkably, little is known about blood donor habits regarding iron supplementation. Outside of research studies, donors are not asked to report iron-taking behaviors. One study of 53,000 donors in a US blood center found that only 21% of donors reported taking iron in the form of multivitamins or iron supplements, with wide variation by sex, age, and donation history.<sup>63</sup> As little as 3% of first-time, young male donors reported taking iron, while up to 45% of frequent female donors age 50 years or older said they took iron. Motivations were evenly split between those who wanted to support general health and wellness and those who took iron specifically because they were blood donors, with the latter motivation being associated with greater use of separate iron supplements.

Ferritin testing to ascertain donor iron status is one recommendation in the AABB bulletin that is increasingly adopted by US blood centers for their teen donor population. One large center began testing in December 2016, and another large collector began

in January 2018, and unpublished estimates are that at present  $\approx$ 70% of US donors age 16–18 years have ferritin testing performed on their donations. Vitalant (then named Blood Systems, Inc.) was the first large collector to implement ferritin testing, and over the first 18 months they found 27% of tested donors received a low ferritin (LF) deferral, with cutoffs of  $<20$  ng/mL applied to females and  $<30$  ng/mL to males.<sup>64</sup> Their subsequent report showed that 12 months was insufficient for most donors to recover to those thresholds if their ferritin was below that cutoff at the first donation.<sup>61</sup> The experience of the American Red Cross helps explain why that may be the case, assuming comparable iron-taking behaviors across the two systems. In electronic survey of 25,000 teenage donors following donation, Red Cross found that only 11% reported taking iron at the time they donated, half as many as reported by Cable and Spencer.<sup>63,65</sup> Notably, the LF deferral triggered iron-taking, with about one in four deferred male and female donors reporting that they initiated supplemental iron postdonation

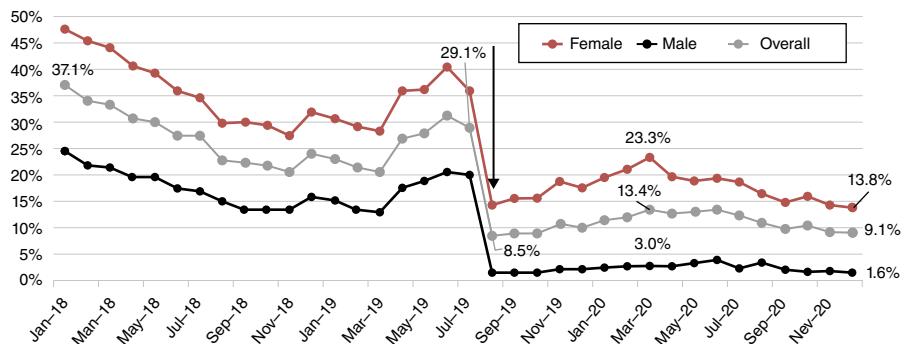
(Figure 7.2). The first 18 months of ferritin testing by the Red Cross was comparable to the experience of Vitalant, with a 6–7-month decline in deferrals followed by a 6–7-month nadir, followed by a climb in deferrals as, presumably, females previously deferred for low ferritin returned to donate after the 12-month deferral (Figure 7.3). Following a change in the deferral trigger to  $<12$  ng/mL in August 2019, deferral rates dropped by 70%.

Compared to restricting teen donation to one unit per year, many US blood centers concluded that ferritin testing provided a stronger balance of protections for donor health and impact on blood availability. Expectations for ferritin testing may, nonetheless, be a net negative contribution to blood collections, reflecting the aggregate impact of temporary removal from the donor pool with deferral, temporary or permanent self-exclusion from donation, and self-initiated iron supplementation. Experience from Canada and the Netherlands supports a decrement to collections as a consequence of ferritin testing.<sup>66–68</sup>



**Figure 7.2** Reported use of supplemental iron in donors 16–18 years old, American Red Cross, by sex and low ferritin deferral.

- “Pre” represents whether donor reported taking supplemental iron prior to most recent donation, as reported by electronic survey 4–8 weeks afterward.
- “Post” represents whether donor reported taking supplemental iron following their most recent donation, reported by the same electronic survey.
- “No\_letter” represents donors whose ferritin values were above the deferral threshold at their most recent donation.
- “Yes\_letter” represents donors whose ferritin values were below the deferral threshold at their most recent donation and who received a letter informing them of their ferritin value and 6-month (male) or 12-month (female) deferral.



**Figure 7.3** Low ferritin deferral rate for 16–18-year-old American Red Cross donors.

- Arrow denotes change in low ferritin deferral trigger in August 2019. Deferrable ferritin values changed from  $<20$  ng/mL (female) and  $<30$  ng/mL (male) to  $<12$  ng/mL for both sexes. Unpublished data.

### Long-term issues for platelet donors

Apheresis platelet donors in the United States can donate up to 24 times a year. While the average platelet donor has many fewer donations annually than the maximum allowable number (as with whole blood donors), a few issues relating to long-term effects of donation have surfaced for this population. Because they usually have a history of whole blood donation, are often recruited to platelet donation on the basis of a high platelet count (a marker for low iron), and can lose  $\approx$ 50 mL whole blood in sample tubes and tubing with each procedure, platelet donors may have unrecognized risk for iron depletion. One recent study in male plateletpheresis donors with borderline hemoglobin found high prevalence of iron depletion and a clear dose-response association with plateletpheresis donation frequency in multivariable models.<sup>69</sup> Another issue that has drawn attention is the possibility of higher risk for fracture in frequent plateletpheresis donors, based on the hypothesis that the citrate anticoagulant might chelate serum calcium and alter bone mineral density. Two studies have examined this question, finding no association between plateletpheresis donation and bone health or fracture.<sup>70,71</sup>

Concern for lymphopenia in frequent platelet donors has drawn renewed attention in recent years, after having been raised as a potential safety concern in the 1980s followed by reassuring findings associated with advancing apheresis technology.<sup>72,73</sup> The leukoreduction that occurs during the procedure has been associated with diminished CD4+ and CD8+ T-cell counts in healthy platelet donors. A recent study of 74,000 donors from Scandat documented potential clinical implications, finding increased risk for bacterial infection in a dose-dependent manner.<sup>74</sup> These results warrant further inquiry and raise questions about additional safety measures prospectively.

### Long-term issues for source plasma donors

Source plasma donors in the United States are permitted to donate twice per week with at least two days between donations with no long-term volume limits. Other countries are more restrictive in frequency and volume donated. Thus, there have been concerns voiced about the safety of source plasma donation in the United States. The long-term record of safety for this industry which has been collecting at this frequency for decades and with the allowed volume allowances since 1992 would seem to allay these concerns.<sup>75</sup> A number of specific issues have been investigated over the years.

When the focus began on iron depletion in blood donors, it was suggested this would be a possible issue for long-term plasma donors due to the accumulated loss of red cells with donation at the maximum frequency. The loss of 10–11 cc of red cells per donation with most of the automated equipment is reduced to about 5 cc when saline reinfusion is employed, as is standard practice. The saline infusion rinses some of the red cells in the tubing back into the donor. Theoretically, a plasma donor could have the equivalent loss of two units of red cells per year. The ferritin levels in plasma donors (FLIPID) study provided data that iron depletion is rare in source plasma donors and is not higher in frequent donors. This study compared male and female donors at different donation frequency to new donors.<sup>76</sup>

Protein depletion is of greater concern since proteins and immunoglobulins are removed with each donation along with other constituents of plasma. Multiple studies of this possibility have been completed since plasmapheresis began. In 1963, studies at NIH showed that chronic plasmapheresis for up to 23 weeks with the

maximum rates not exceeding 1.5 L of plasma withdrawn per week (slightly less than the currently approved maximum) produced no changes in formed elements and only minor changes in serum protein levels. By contrast, five donors subjected to 5 L of plasma removal in five days did have serum protein depletion.<sup>77</sup> A study at the University of Michigan in 1970 showed no major depletion with donations of plasma equivalent to four units of whole blood per week (somewhat less than the maximum currently allowed).<sup>78</sup> Investigators at UCLA and a commercial source of plasma products also showed no adverse effect on plasma protein homeostasis from long-term plasmapheresis at volumes somewhat less than allowed currently.<sup>79</sup> Similar low risk was found by Canadian investigators in 1993, but their protocol was weekly plasmapheresis at volumes about a third of what is currently allowed.<sup>80</sup>

There is clearly protein loss with each donation. In the United States, this is monitored by a protein level before each donation and a protein electrophoresis every four months. In Europe, IgG levels as well as protein levels are monitored. IgG has a delayed recovery after it is removed, which is the basis of this additional test. A comprehensive study of removal and recovery of normal plasma constituents after plasma exchange where much larger volumes are removed showed that immunoglobulin, complement, fibrinogen, and cholesterol have marked reduction in concentrations after high-volume plasma exchange. Interestingly, newly synthesized antibody after the exchange may have increased biologic activity, indicating the immunoglobulin depletion did not necessarily correspond to reduction in antibody activity. Since albumin replacement was used, neither albumin nor total protein declined.<sup>81</sup>

Subsequently, this was investigated in long-term plasma donors. A comprehensive study of proteins and lymphocyte subsets showed low levels of serum protein, globulin, and IgG accompanied by increased percentages of B cells and decreased percentages of suppressor T and natural killer cells.<sup>82</sup>

The evidence of IgG depletion led to concern that immune surveillance might be compromised in long-term plasma donors impacting general health. Industry conducted two studies: one a retrospective review of records of over 500 remunerated donors participating in plasmapheresis programs at various levels of frequency for 10 years or more and a second one determining the reasons for cessation of participation in serial plasmapheresis programs.<sup>83,84</sup> Both studies found no evidence that long-term plasmapheresis impacted the donor's health. The reasons for dropping out of plasmapheresis programs were not medically related.

A study in Austria found bone demineralization presumed to be due to long-term impact of citrate infusion in long-term apheresis donors.<sup>85</sup> Most of the data is related to plateletpheresis donors, but the authors extended their conclusions to plasmapheresis donors. In the plasma collection procedure, most of the citrate goes into the plasma collection bottle or bag and is not returned to the donor. An analysis of this data (unpublished by the Plasma Protein Therapeutics Association) did not support the conclusion, and no other data has indicated bone mineral density losses in plasmapheresis donors.

The concern about the health of source plasma donors, particularly in the United States, relates both to the frequency and amount collected at each donation and the fact that the donors are compensated in cash. The data available does not indicate that there are health-related issues with this donor population. However, the Plasma Protein Therapeutics Association is addressing this concern in the United States with short- and long-term studies of the donor

population's health similar to their FLIPID study mentioned above. These studies are ongoing, and no data have yet been presented or published.

## Summary

As demonstrated repeatedly in research studies, blood donors tend to enjoy stronger health by many measures than the general population. Blood centers and regulators collect data and deploy many measures to protect the health and safety of persons giving the "gift of life." The industry has a strong safety record but continues to evaluate the clear risk for iron depletion in many donors and whether that risk extends to actual health manifestations. Platelet and plasma donors enjoy robust safeguards for frequent donation, but risk for lymphopenia is a recent issue warranting further scrutiny for plateletpheresis donors.

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## CHAPTER 8

# Global perspective: ensuring blood and blood product safety and availability through regulation and certification

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Blood and blood products are considered an essential part of medical therapies worldwide. National systems are seen in much of the developed world (e.g., Europe, Canada, Australia, and New Zealand), with the United States as an exception. In Canada, there is one national system and a second one for the province of Quebec. Less developed countries often have hospital-based systems (in some cases with a small national system, as in Mexico). Many are run by the International Red Cross and are government subsidized.

In the United States, a majority of the blood is collected and distributed by community blood centers that generally are part of a trade organization called America's Blood Centers (ABC). The American Red Cross, which is separate from ABC, collects and distributes about 40% of the blood, and a small percentage of blood is collected and processed by hospital-based blood banks.

A number of organizations impact blood product safety and availability. The AABB (formerly the American Association of Blood Banks) is a professional society of individuals in the field, community blood centers, and hospital transfusion services from around the world. It publishes the *AABB Technical Manual* and *Standards for Blood Bank and Transfusion Services* (as well as several publications containing standards for various specialized services) that both serve as voluntary guides to procedures and quality requirements and tend to establish a standard of care. The AABB through its accreditation program inspects blood banks and transfusion services to ensure that they provide quality care in line with that standard of care.

Plasma collection and manufacturing organizations are certified by the PPTA (Plasma Protein Therapeutics Association or PPTA Source for collections), which is the global and standard setting organization for plasma collection and fractionation. Their International Quality Plasma Program (IQPP) provides independent, third-party evaluation and recognition of a plasma center's adherence to global industry standards for source plasma.

There is also an international blood transfusion society, the International Society of Blood Transfusion (ISBT). Founded in 1935, the ISBT has grown into an international society where transfusion medicine professionals from across the globe come together and share knowledge to improve the safety of blood transfusion worldwide. In addition, the World Health Organization (WHO) has an

ongoing interest in blood availability and safety worldwide as part of its efforts to ensure quality health care is available worldwide.

Because of their importance as medical therapies, blood and blood products are heavily regulated throughout the world. The primary regulator for the United States is the Food and Drug Administration, although in addition there are applicable state and/or local regulations. Blood establishments are also regulated by other federal and state organizations such as the Department of Transportation (DOT), the Occupational Safety and Health Administration (OSHA), and the Nuclear Regulatory Commission (NRC). Europe has multiple regulatory groups within member states and outside Europe, and regulations are often country specific. Regulatory bodies outside the United States are discussed later in this chapter.

It is important to differentiate between regulation and accreditation/certification. Regulations are laws and must be followed whereas accreditation/certification is a choice and is achieved through adoption of voluntary standards. Regulations differ from country to country and even within a given country (e.g., state-specific regulations in the United States). When there are differences between regulations, the more stringent regulation usually applies so that there is full compliance. Many of the standards applicable to blood establishments are applicable worldwide.

It is important to note that blood centers, transfusion services, plasma centers, and laboratories that perform testing on donor blood are known collectively as blood establishments. Depending upon the activities that a blood establishment performs, differing regulations and standards apply.

## US perspective on ensuring blood and blood product safety and availability

### History of safety and efficacy requirements for drugs and biologics in the United States

Regulatory approval of drugs and biologics in the United States is subject to federal law. Safety and efficacy requirements related to drug and biologics approval have evolved through the twentieth and twenty-first centuries. In 1906, President Theodore Roosevelt, appalled by what he read in an Upton Sinclair novel, *The Jungle*, included a

recommendation for food and drug legislation in his annual message to Congress, which resulted in passage of the Pure Food and Drug Act (Public Law 59-384; 34 Stat. 768). The intent of the law was to prevent the manufacture, sale, and transportation of misbranded and adulterated foods and drugs. The passage of subsequent public health laws followed several therapeutic tragedies. In 1937, the S.E. Massengill Co. introduced sulfanilamide dissolved in diethylene glycol. Diethyl glycol, a chemical normally used as antifreeze, is a deadly poison. No drug safety testing was required by law at the time. The drug was promoted to treat streptococcal infections, and 633 shipments were distributed throughout the United States. Before its use could be stopped, more than 100 people in 15 states died.<sup>1,2</sup> This and similar incidents led to the enactment of the 1938 Food, Drug, and Cosmetic Act (FDCA) (Public Law 75-717; 52 Stat. 1040).<sup>3</sup>

The law, which became effective in June 1939 differed substantially from the 1906 Act that it replaced. It extended coverage to include cosmetics and therapeutic devices. It required clearance of new drugs for safety prior to distribution and provided authority to establish tolerances for potentially poisonous substances in foods and drugs. It added the sanctions of injunction and emergency permit control to the seizure and prosecution authority in the previous act. As the main purpose of the law was to prohibit the movement of mislabeled and adulterated food, drugs, devices, and cosmetics in interstate commerce, it authorized inspections of factories, warehouses, establishments, and vehicles used in manufacturing, processing, packing, storing, and transporting these products. The act also established a requirement that a new drug could not be introduced into interstate commerce unless an application was made. The application needed to include a full report of investigations to show whether or not the drug was safe for use; an explanation of the drug's composition; a description of the methods, facilities, and controls used in manufacturing, processing, and packing; as well as samples of the drug and label. Grounds for application rejection included inadequacy of tests, failure of test results, and other information to help determine the product safety, as well as inadequacy of facilities, methods, and controls used in manufacturing, processing, and packing for preserving the product's identity, strength, purity, and quality.

In the 1940s, the FDCA was amended with requirements of batch certification for certain drugs (e.g., penicillin; Public Law 79-139; 59 Stat. 463) to verify product identity, quality, purity, and strength, and thus try to ensure their safety and presumed effectiveness.<sup>4</sup> Thalidomide caused another public health disaster in the late 1950s and early 1960s.<sup>1,2</sup> Introduced in late 1957 to treat insomnia, colds, coughs, and headaches, the drug was found to relieve symptoms of so-called morning sickness, and was prescribed for this indication as well. At the time, drugs were not tested for their effects on the fetus. As a result, thousands of children, born in 46 countries, suffered severe congenital deformities. Although some pregnant women in the United States used this medication as it was distributed to physicians during the clinical testing program, the United States experienced fewer cases of birth defects, as the FDA refused to approve thalidomide. The enactment of the 1962 Amendments to the 1938 FDCA (Public Law 87-781, 76 Stat. 780; commonly known as the Kefauver-Harris Amendments) came as a response to the thalidomide disaster.<sup>1,2</sup> This law made regulatory approval a mandatory prerequisite for product marketing, strengthened safety regulations, and added the requirement for applicants to provide evidence of product effectiveness through adequate and well-controlled studies, including clinical investigations. Additionally, the Drug Amendments of 1962 stated that drug advertising should contain a label with the prominently printed established product

name, formula with ingredient quantification, as well as information related to side effects, contraindications, and effectiveness.

Biologics-related requirements also developed gradually and roughly in parallel with drug legislation. As defined in the PHSA Section 351(i), a biologic product means a virus, therapeutic serum, toxin, antitoxin, vaccine, *blood, blood component or derivative*, allergenic product, protein (except chemically synthesized polypeptide), or analogous product, or . . . applicable to the prevention, treatment, or cure of a disease or condition of human beings.<sup>5</sup> In 1901, 13 children in St. Louis, MO, and nine in Camden, NJ, died of tetanus after receiving contaminated diphtheria antitoxin and smallpox vaccine, respectively. In 1902, Congress passed the Biologics Control Act (Public Law 57-244; 32 Stat. 728), also known as the Virus-Toxin Law.<sup>1,2</sup> The law authorized the promulgation of licensing regulations for establishments involved in the production of toxins, antitoxins, viruses, sera, and similar products. The act also authorized inspections at manufacturing facilities. Companies needed to be licensed for the manufacture and sale of the products. Inspectors were required to study product purity and potency. In 1934, new regulations, published under the title "Regulations for the Sale of Viruses, Serums, Toxins and Analogous Products in the District of Columbia and in Interstate Traffic," explicitly indicated that licenses for new products would not be granted without acceptable evidence of therapeutic or prophylactic efficiency. In 1944, the Public Health Service Act (PHSA) was enacted (Public Law 78-410; 58 Stat. 682). Section 351 of this law incorporated, with few changes, the Biologics Control Act. The document stated that licenses would be issued only upon a showing that the establishment and the products for which a license was being sought met the standards intended to ensure the continued safety, purity, and potency of the products.<sup>5</sup>

As noted, regulatory approval of drugs and biologics in the United States is subject to federal law. Drugs are approved under Section 505 of the Food, Drug, and Cosmetic Act (21 U.S.C. 355), as amended. An order to approve an application is issued if none of the following applies: the investigations are found to be inadequate; the results either do not prove product safety or prove its unsafety; the data submitted as part of the application and other available information do not provide sufficient evidence of product safety and effectiveness; the methods, facilities, and controls used in manufacturing, processing, and packing are found to be inadequate to preserve the product's identity, strength, purity, and quality; and the application lacks the required patent information.<sup>5</sup> Although biologics are also considered to be drugs, because most of them meet the definition of the term drug in FDCA Section 201(g)(1) (21 U.S. C. 321), they are usually licensed under authority of Section 351 of the PHSA (42 U.S.C. 262), as amended. Under Section 351, which has been in effect since 1944, biologics license applications are approved following the applicants' consent to the inspection of the facilities and on the basis of a demonstration that the products are safe, pure, and potent, and the facilities in which the biologics are manufactured pass an on-site inspection.<sup>5</sup>

The FDCA of 1938 has been amended dozens of times. Major amendments include the FDA Modernization Act (FDAMA) of 1997, the Food and Drug Administration Amendments Act (FDAAA) of 2007, and the Food and Drug Administration Safety and Innovation Act (FDASIA) of 2012.

## FDA regulation of blood collection establishments

### Overview

Blood and blood components are used in the treatment of disease. Therefore, blood and blood components meet the definition of a drug in Section 201(g) of the FDCA; and blood products, including

source plasma, must meet all statutory requirements of the FDCA. FDCA sections applicable to blood include Sections 201, Definitions; 301, Prohibited Acts; 501, Adulteration; 502, Misbranding; 510, Registration; and 704, Factory Inspection.

The fundamental principles of the regulation of blood establishments are to ensure the safety, effectiveness, and availability of blood and blood products; protect the health of the donors and recipients; and ensure that the blood collected is safe for transfusion or further manufacture. The Food and Drug Administration is the primary regulator of blood establishments in the United States. The FDA has three major functions:

- 1 Development and promulgation of regulations.
- 2 Registration and licensure of blood establishments.
- 3 Inspection and compliance actions.

The FDA's Center for Biologics Evaluation and Research (CBER) works closely with other parts of the Public Health Service (PHS) to establish blood regulations and to identify and respond to potential threats to blood safety or supply. Regulations promulgated by the FDA are binding both on the agency and the blood establishment industry. The FDA also issues guidance documents that represent the FDA's current thinking on the handling of a particular subject. The FDA licenses and/or registers blood establishments, inspects them routinely, and monitors reports of errors, accidents, and adverse clinical events. If noncompliance is found during inspections, the FDA can take actions (sanctions) against blood establishments.

In addition to its major functions, the FDA conducts a wide range of compliance and surveillance activities during the "life cycle" of biological products. These include:

- Conducting prelicense and preapproval inspections as well as postlicensure and postapproval inspections of manufacturing facilities and products under clinical study.
- Monitoring the safety, purity, and potency of biological products through review of:
  - Biological product deviation reports,
  - Investigations into transfusion- and donation-related fatalities and other adverse events, and
  - Product recalls.
- Monitoring reports of biological product shortages.
- Initiating regulatory action to address noncompliance with FDA laws and regulations.
- Monitoring of research conducted on biological products and assessing the protection of the rights, safety, and welfare of human research subjects and the quality and integrity of research data.
- Monitoring import and export activities.
- Reviewing product advertising and promotional labeling.

### Good manufacturing practices

The requirements found in the current good manufacturing practices (cGMPs) are the major regulations that the FDA enforces on blood establishments. These represent the minimum requirements an establishment should follow. Applicable cGMP regulations are found in the US Code of Federal Regulations (CFR) within Title 21 Part 210, cGMP for Manufacturing, Processing, Packing, or Holding of Drugs; Title 21 Part 211, cGMP for Finished Pharmaceuticals; and 21 CFR 606, cGMP for Blood and Blood Components. Table 8.1 lists the subparts of 21 CFR Part 211, cGMP, and Table 8.2 lists the applicable sections for blood and blood products in 21 CFR Parts 600–640.

The PHSA requires licensure for biological products in interstate commerce. In this regard, each package of a biological product is clearly labeled; the biological product is required to be safe, pure, potent, and effective; the facility where the product is manufactured

**Table 8.1** 21 CFR Part 211 cGMP for Finished Pharmaceuticals

- 
- Subpart A: General provisions
  - Subpart B: Organization and personnel
  - Subpart C: Buildings and facilities
  - Subpart D: Equipment
  - Subpart E: Control of components and drug product containers and closures
  - Subpart F: Production and process controls
  - Subpart G: Packaging and labeling control
  - Subpart H: Holding and distribution
  - Subpart I: Laboratory controls
  - Subpart J: Records and reports
  - Subpart K: Returned and salvaged drug products
- 

Source: Code of Federal Regulations Title 21. Public Domain.

**Table 8.2** 21 CFR Parts 600–640: Applicable Sections for Blood and Blood Products

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- 600: Biological products: general
  - 601: Licensing
  - 606: Current good manufacturing practice for blood and blood components
  - 607: Establishment registration and product listing for manufacturers of human blood and blood products
  - 610: General biological product standards
  - 630: General requirements for blood, blood components, and blood derivatives
  - 640: Additional standards for human blood and blood products
- 

Source: Code of Federal Regulations Title 21. Public Domain.

meets or exceeds standards set in the regulations; and the applicant consents to inspection. Licensure is intended to ensure the safety, effectiveness, and availability of blood and blood products, thereby allowing shipment of product(s) in interstate commerce. It also protects the health of the donor and recipients.

FDA licensure is required to ship products in interstate commerce or internationally. Registration is required for all blood establishments, including those located in hospitals, that manufacture (collect, store, process, or distribute) blood components for transfusion or further manufacture. Registration requirements for manufacturers of human blood and blood products are outlined in 21 CFR Part 607; license requirements are outlined in 21 CFR Part 601. Elements of cGMP are listed in Table 8.3. The division into elements is arbitrary but is based on an analysis of commonality of the requirements of Title 21 as they apply to all aspects of blood establishment operations. Thus, it is irrelevant whether they are applied to blood collection or to infectious disease testing; each element is applicable for each discrete blood establishment operation. Because blood and plasma centers are required to follow their own standard operating procedures (SOPs), each SOP creates unique regulatory requirements for that operation. From a regulatory standpoint, this concept is significant in that compliance standards, and regulatory requirements are created by individual establishment SOPs. cGMP applies to both the production methods used by blood establishments to manufacture components and the manufacturing process controls in place. Each provision of an establishment's SOPs must be in line with the manufacturers' instructions for licensed or approved systems used in that organization.

Meticulous records of compliance with cGMP elements are required to ensure both executive management and the FDA that a

**Table 8.3** Elements of Current Good Manufacturing Practice

- Standard operating procedures
- Recordkeeping
- Personnel management
- Calibration
- Validation
- Labeling
- Error management
- Quality control and auditing
- Facilities and equipment
- Process and production change control

blood establishment's manufacturing processes are under control. Control, in this context, can be defined as compliance in every respect with the establishment's manufacturing SOPs and FDA requirements. It is worth emphasizing that if a firm's SOPs are more stringent than FDA requirements, it is not acceptable to deviate from the SOP, even if the FDA requirement is met, unless appropriate change control is applied, including appropriate approvals, communication of the change, and reporting to FDA of the change, if required. During inspections of blood establishments, whether by the private sector or regulatory bodies such as the FDA, the overriding investigational concern is whether an establishment has this control. Records should be designed to document and provide evidence of control of manufacturing systems and allow for complete trackability (follow logical steps of the process) and traceability of a process (details such as who, when, how, and where).

Although not required by the CFR, it is generally held that the quality function should organizationally be distinct from manufacturing to ensure its independence, and this de facto requirement appears in FDA guidance documents.<sup>6,7</sup> In many blood establishments, the director of the quality reports directly to the chief executive officer. The role of the quality function should be defined in writing as part of the Quality Management System. All matters that relate to quality should funnel to a single person or group with broad knowledge of the blood establishment's operations and access to the information necessary to assess the impact of proposed changes. These principles emphasize the importance with which the FDA views both quality and SOPs. However, case law holds that upper management cannot avoid adverse consequences of noncompliance with regulatory requirements because a lower ranking employee is designated the authorized official (the individual(s) designated to speak for the organization in regulatory matters and to work with the FDA).<sup>8</sup> Quality staff can be deployed to enhance the quality of blood establishment operations in a variety of ways. They can assume responsibility for personnel training, especially in cGMP, maintain calibration and validation records, design validation protocols, and identify trends through statistical analysis. A well-directed quality function can be of enormous value to both the authorized official(s) and the staff of the facilities in which it is established. However, quality staff should not review their own work as their objectivity cannot be ensured. Table 8.4 lists selected responsibilities of the quality function. Although internal audits are also not specifically required by regulations, procedures to detect problems are required and internal audits are an important means of ensuring that processes remain in control. Internal audits are considered confidential by the FDA and the results of such audits are generally not available to FDA investigators unless fraud is suspected or there appears to be an imminent threat to public health. The reason for this policy is to encourage audits that are complete and detailed with no information withheld.

**Table 8.4** Quality Function Responsibilities

**Quality Function Responsibilities Should Include, but Not Be Limited to, the Following:**

- Approve specifications
- Approve test procedures, including process controls
- Approve validation plans, protocols, or equipment
- Review changes in product, process, or equipment, and determine if revalidation is required
- Approve specification changes, sampling plans, and test procedures
- Approve sampling procedures
- Approve reference standards
- Conduct analytic investigations and evaluate results
- Approve testing materials
- Provide analytical reports
- Approve or reject intermediates and active pharmaceutical ingredients manufactured, processed, packed, or held under contract by another establishment
- Gather data to support retest dates (stability testing)
- Evaluate and approve contractors
- Review batch records
- Review complaints
- Perform internal and external audits
- Perform periodic assessments of procedures, policies, and responsibilities within the establishment's manufacturing and control operations

The FDA also respects supplier audits as confidential internal audit information. However, it is critical that the blood establishments have readily available SOPs for audits, schedules demonstrating that all elements of operations are reviewed regularly (at least annually), processes for ensuring and documenting corrective and preventive action and follow-up, and formal closure notices to complete the records. It is also essential that any deviations discovered in the course of audits be properly documented in records that are available to FDA investigators. The Quality Management System, including a review of internal audits and their outcomes, must be assessed for effectiveness by management at regular intervals.

**FDA structure: Office of Blood Research and Review (OBRR)**

FDA regulatory review of devices, drugs, and biologics is conducted by the Center for Drug Evaluation and Research (CDER), the Center for Devices and Radiologic Health (CDRH), and the Center for Biologics Evaluation and Research (CBER). All submissions and applications (including combination products) are assigned to one of these centers for review. The Office of Blood Research and Review (OBRR) within CBER regulates blood and blood products while the Office of Tissues and Advanced Therapies (OTAT) regulates plasma derivatives (except for albumin) and their recombinant analogues. OBRR is also responsible for the regulation of blood donor screening assays and retroviral diagnostic tests. There are two divisions within OBRR, the Division of Emerging and Transfusion Transmitted Diseases (DETTD) and the Division of Blood Components and Devices (DBCD). DETTD has oversight of blood donor screening and supplemental tests, source plasma donor screening tests, and retroviral diagnostic tests. The DBCD has oversight of blood grouping reagents; reagent red cells; anti-human globulin; molecular erythrocyte typing tests; HLA, HNA, and HPA antigen and antibody test kits; and bacterial detection tests.

**FDA guidance documents**

Guidance documents represent FDA's current thinking on a topic. They do not create or confer any rights for or on any person and do not operate to bind FDA or the public. A firm can use an alternative approach if the approach satisfies the requirements of the applicable statutes and regulations. These documents usually discuss specific

products or issues that relate to the design, production, labeling, promotion, manufacturing, use, and testing of regulated products. Guidance documents may also relate to the processing, content, and evaluation or approval of submissions as well as to inspection and enforcement policies.

### FDA inspections

Section 704 of the FD&C Act authorizes FDA to conduct inspections at reasonable times, within reasonable limits, and in a reasonable manner. Facility inspectors are instructed that the facility must have knowledgeable and trained staff, appropriate equipment, and adequate procedures to ensure that, as applicable, donors are screened appropriately and units are collected, stored, and shipped in accordance with FDA regulations. The establishment's procedures must address all manufacturing steps. For example, in a blood collection facility, SOPs should cover donor screening; blood collection; unit identification; operation of all screening and collection equipment; handling of donor reactions; management of post donation information reports; storage of product and supplies; quality control of reagents, supplies, and equipment; transport of collected units; and documentation and follow-up of any unexpected incidents. Inspections should include observations of the manufacturing process and an examination of the physical layout of the facility to ensure that there is limited public access to biohazardous areas, proper disposal of biohazardous materials, accessible restrooms and handwashing equipment, and clean and organized storage areas. Supplies should be examined to make certain they are appropriately licensed and used within their expiration date.

Inspections may be conducted for several reasons: routine, pre-license, for cause, and follow up. Upon arrival at a blood establishment, the inspector presents credentials and an FDA Form 482 that explains the reason for the inspection.

Observations during an inspection are recorded on an FDA Form 483. These are presented to the blood establishment being inspected, and normally the blood establishment writes a written response to each observation and may write responses to any discussion points. The observations represent an inspector's observation of noncompliance. Following the inspection, an Establishment Inspection Report that provides a full narrative of the inspection results is prepared by the inspector(s) and forwarded to the establishment for review.

### FDA reportable deviations

Apart from the requirement to report and investigate adverse reactions, as stipulated in 21 CFR 606.170, an organized error management process is not specifically required by the provisions of Title 21, although 21 CFR 606.171(c) mandates the reporting within 45 days of all biologic product deviations on distributed products. It is important to note that for something to be reportable it must meet the criteria in Table 8.5.

The investigation and follow-up of deviations is critical to the successful and continuing improvement of blood establishment quality. In fact, appropriate management of deviations forms the centerpiece of continuous improvement. Each blood establishment deviation should be treated as an opportunity to be used to improve processes. All facilities must develop SOPs for detecting, reporting, managing, and correcting deviations, including errors and accidents. Error management efforts and results should be documented as quality records. Monitoring corrective action outcomes to ensure effectiveness is an essential part of the system for addressing corrections.

Although error management systems are not defined in Title 21, some attributes of good systems include:

**Table 8.5** Reportable Event Criteria

**For a Deviation Event to Be Reportable, It Must:**

1. Either
  - (i) represent a deviation from current good manufacturing practice, applicable regulations, applicable standards, or established specifications that may affect the safety, purity, or potency of that product; or
  - (ii) represent an unexpected or unforeseeable event that may affect the safety, purity, or potency of that product.
2. Occur in your facility or another facility under contract with you.
3. Involve *distributed* blood or blood components.

- 1 Employees at all levels in the organization are encouraged to report errors. Punitive policies discourage reporting, resulting in lost opportunities for systems improvements.
- 2 Employees involved in the process in which an error was made should be involved with the investigation and resolution of the error and corrective actions taken. It is essential that the investigation be sufficient to determine the underlying root cause for the error rather than identifying mere "symptoms" of the root cause. The FDA also requires that staff are educated concerning the effect their errors have or potentially have on product quality, that is, it is required that they understand how their responsibilities have an effect on ensuring that only safe products are released.
- 3 Confirmation of the effectiveness of the corrective action is vital to ensure that the improved process continues to yield the expected results. Post change monitoring is essential, and long-term evaluation should be performed at appropriate intervals (e.g., 3–6 months after a process improvement is completed).

Deviations must be reported only if the product was distributed. This is defined as having left the control of the establishment. If the product was not distributed, the incident still must be recorded in internal records [21 CFR 606.160(b)(7)(iii)]. If the product was distributed, a report must be submitted to CBER within 45 calendar days from the date information is acquired that reasonably suggests a reportable event occurred. The incident must also be recorded [21 CFR 606.160(b)(7)(iii) and 21 CFR 211.198] and investigated [21 CFR 606.100(c) and 21 CFR 211.192]. The FDA has stated that the purpose of this reporting system is to provide early warning of faulty processes as an indicator for potentially immediate problems that may be related to recalls and as surveillance for improving training and establishing guidance.<sup>9,10</sup> Table 8.6 contains a synopsis of the final rule for reporting.

**Table 8.6** Synopsis of Final Rule on Reporting Biological Product Deviations

- The rule applies to all establishments: donor centers, blood banks, transfusion services.
- Reporting time is not to exceed 45 days.
- Report by mail: Director, Office of Compliance and Biologics Quality, Food and Drug Administration, 10993 New Hampshire Avenue, Silver Spring, MD 20993, USA; or electronically via CBER's website: [www.fda.gov/cber/biodev/biodev.htm](http://www.fda.gov/cber/biodev/biodev.htm).
- If the answers to the questions below are affirmative, the event is reportable:
  - Was the event associated with manufacturing?
  - Did the deviation affect safety, purity, or potency?
  - Did it occur in a licensee's or a contract facility?
  - Did the facility have control over the product when the deviation occurred?
  - Was the product distributed?

## Device regulatory controls

All classes of medical devices are subject to what are known as general controls. General controls are the basic provisions of the May 28, 1976, Medical Device Amendments to the FDCA. They provide the FDA with the means of regulating devices to ensure their safety and effectiveness. General controls in the FDCA apply to all medical devices. They include provisions that relate to adulteration; misbranding; device registration and listing; premarket notification; banned devices, including repair, replacement, or refund; records and reports; restricted devices; and good manufacturing practices. The FDA considers three classes of devices.

- Class I devices are subject to general controls and are typically exempted from submission of a premarket 510(k) notification. They are viewed as posing the lowest risk to the patient and/or user.
- Class II devices are subject to what are known as special controls in addition to the general controls provision of the FDCA. Special controls may include compliance with a recognized standard, warning statements in the instructions for use, specific performance requirements, and/or other controls necessary to ensure a reasonable assurance of safety and effectiveness. Class II devices typically require FDA clearance of a premarket 510(k) notification to permit the device to be marketed and sold in the United States.
- Class III devices are viewed as presenting a higher level of risk. They may be first-of-a-kind devices for which general and special controls are not adequate to ensure safety and effectiveness. Class III devices require FDA approval in the form of a premarket approval application prior to marketing, as well as compliance with device general controls.

The 510(k) premarket notification is a submission made to the FDA to demonstrate that the device to be marketed is at least as safe and effective as (i.e., is substantially equivalent to) a legally marketed Class I or II device of that same generic type. When determined to be substantially equivalent, the subject device may be legally marketed and sold in the United States.

The legally marketed device to which substantial equivalence is determined is known as the predicate device. A predicate device can be a preamendments device (legally marketed prior to the May 28, 1976, Medical Device Amendments to the FDCA) or a postamendments device that is, or was, legally marketed in the United States following the device amendments. A claim of substantial equivalence does not mean the new device must be identical to the predicate device. Substantial equivalence is based on a comparative assessment with respect to intended use, design, energy used or delivered, materials, performance, safety, effectiveness, labeling, biocompatibility, standards, and other applicable characteristics that would demonstrate the device is as safe and effective as the predicate device.

## Blood establishment computer software

Blood establishment computer systems (BECS) are currently subject to the 510(k) premarket notification provisions of the FDCA. Currently, these devices are regulated as Class II devices, subject to 510(k) premarket notification requirements.

Medical device data systems (MDDS), regulated under Section 880.6310, are Class I medical devices exempt from 510(k) premarket notification and were not included for consideration under the BECS and BECS accessories classification. By definition, Medical Device Data Systems (MDDS) are hardware or software products intended to transfer, store, convert formats, and display medical device data. The MDDS does not, in any way, manipulate or alter the data.

## Regulation and accreditation of hospital transfusion services

### Overview

The Clinical Laboratory Improvement Act and Amendments (CLIA) stipulate requirements for the qualifications of staff who perform or supervise the testing conducted within a transfusion service. AABB standards and FDA cGMP regulations also require that the transfusion services have a process for personnel training and competency evaluation.<sup>11</sup>

Written SOPs are required by the FDA in 21 CFR 606.100. A system must be in place to ensure process control for the validation of processes and procedures, introduction and change of processes and procedures, proficiency testing, quality control, and the use of materials and other aspects of performance of procedures. A defined system of documentation and record retention is required by 21 CFR 606.140 and 21 CFR 606.160 and also may be found within the AABB standards.<sup>12</sup>

AABB standards state that the blood bank or transfusion service shall have a medical director who is a licensed physician and qualified by education, training, and/or experience. The medical director shall have responsibility and authority for all medical and technical policies, processes, and procedures—including those that pertain to laboratory personnel and test performance—and for the consultative and support services that relate to the care and safety of donors and/or transfusion recipients. The medical director may delegate these responsibilities to another qualified physician; however, the medical director shall retain ultimate responsibility for the medical director duties. However, the standards do not require that overall executive management to be under the control of the medical director. The Joint Commission (discussed later in this chapter) also does not require that the overall direction of the laboratory be performed by a physician but does state that “a pathologist or physician qualified in immunohematology, hemotherapy, and blood banking directs blood transfusion services” (HR 1.15).<sup>13</sup> Thus, the medical director may or may not have authority in personnel (other than policies and procedures), purchasing, budgeting, and other administrative matters. The regulation states that the laboratory director must be a doctor of medicine or doctor of osteopathy licensed in the state where the laboratory is located (42 CFR 493.1443).

The FDA, AABB, and the College of American Pathologists (CAP) all have requirements regarding the evaluation and reporting of adverse effects of blood transfusion. CAP is an organization of board-certified pathologists that serves patients, pathologists, and the public by fostering and advocating excellence in the practice of pathology and laboratory medicine worldwide. The FDA requires that records be maintained of any reports of adverse reactions to blood transfusion and that a thorough investigation of each reported reaction be conducted. All transfusion services must report deaths confirmed as being caused by a transfusion. The applicable regulation [21 CFR 606.170(b)] reads

When a complication of blood collection or transfusion is confirmed to be fatal, the Director, Office of Compliance and Biologics Quality, CBER, must be notified by telephone, facsimile, express mail, or electronically transmitted mail as soon as possible. A written report of the investigation must be submitted to the Director, Office of Compliance and Biologics Quality, CBER, by mail, facsimile, or electronically transmitted mail (for mailing addresses, see 600.2 of this chapter), within seven days after the fatality by the collecting facility in the event of a donor reaction, or by the facility that performed the compatibility tests in the event of a transfusion reaction. (<http://www.fda.gov/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/Blood/ucm074947.htm>)

The AABB requires that a transfusion service has a process for the detection, reporting, and evaluation of suspected complications of transfusion and that all suspected transfusion complications are evaluated and reviewed by the medical director. CAP requires that all transfusion reactions or incidents be reported immediately to the laboratory, that documented procedures exist for actions to be taken in the event of a transfusion reaction, and that the results of the investigation be recorded in the patient's chart.

### FDA regulation of hospital transfusion services

Total quality systems have been required for blood establishments since 1975 when the FDA incorporated cGMP into the Code of Federal Regulations. Although the regulations were aimed primarily at blood and plasma centers, many of the provisions apply to transfusion services as well. In 1995, the Center for Biologics Evaluation and Research produced a Guidance to assist establishments in developing quality programs in accord with applicable regulations.<sup>6</sup>

In laboratories where clinical samples are tested, the Clinical Laboratory Improvement Amendments (CLIA) requirements for quality control (QC), listed in 42 CFR Part 493, must also be followed. All personnel shall be trained in its application. The quality system shall be under the supervision of a designated person who reports to executive management. For reagents for which there are no QC requirements in Title 21, the QC testing described in the manufacturer's package insert must be followed. It is mandated that "each laboratory establish and follow written policies and procedures for a comprehensive quality assurance program. . . . The laboratory's quality assurance program must evaluate the effectiveness of its policies and procedures; identify and correct problems. . . . All quality assurance activities must be documented."<sup>11</sup>

For any hospital quality program to work successfully, physicians must give priority to the process, and hospital administration must make the necessary resources available to ensure its development, implementation, refinement, and continuation.

The FDA requirement that errors or accidents affecting the safety, quality, integrity, purity, or potency of a blood product be reported extends to all blood establishments, including the transfusion services (21 CFR 606.171). However, this statutory authority extends only to the blood product itself and does not permit FDA oversight of the actual transfusion episode. Although FDA MedWatch provides a voluntary venue for reporting adverse events, most hospitals do not voluntarily report errors to MedWatch (<http://www.fda.gov/Safety/MedWatch/default.htm>). Therefore, the FDA's purview extends only as far as the laboratory door, and there is no requirement to report a patient misidentification error unless it results in a death. (A transfusion-related death, however, must be reported to CBER within 24 hours.)

The FDA considers establishments that perform certain activities that it defines as manufacturing steps to be hospital blood banks, which are required to register annually using Form FDA 2830. A hospital blood bank is an entity that routinely collects or processes whole blood or blood components. These components may be collected by means of apheresis or prepared from whole blood. Processing includes freezing, deglycerolizing, washing, irradiating, rejuvenating, or removing leukocytes from components. However, the collection and processing of blood and blood components in an emergency situation, therapeutic collection of blood or plasma, preparation of recovered plasma for further manufacture, or preparation of red blood cells for transfusion do not require registration (21 CFR 607.65(f)).

Because blood and blood components are drugs under the Federal FDCA, the FDA cGMP regulations (21 CFR Parts 210 and 211) apply to the manufacture of these products. All of these regulations apply to FDA-defined hospital blood banks. FDA registration allows the agency to plan and perform routine cGMP inspections.

Although the FDA does not routinely inspect hospital transfusion services, these services also engage in manufacturing in the view of the FDA because compatibility testing, blood storage, labeling, and recordkeeping are considered steps in the manufacturing process. Thus, transfusion services are also subject to cGMP regulations. Inspection of hospital transfusion services is overseen by the Centers for Medicare and Medicaid Services (CMS) through a 1980 memorandum of understanding with the FDA that addresses inspection of these establishments. In an effort to reduce duplication of inspections, it was agreed that inspection of hospital transfusion services that are approved for Medicare reimbursement and that engage in compatibility testing but that neither routinely collect nor process blood components would be subject to inspection by the CMS. This agreement pertains to responsibility for inspection only. No statutory authority transferred between the agencies. As part of the agreement, the CMS adopted FDA regulations in 21 CFR Part 606 titled "Current Good Manufacturing Practice for Blood and Blood Components" and 21 CFR Part 640 titled "Additional Standards for Human Blood and Blood Products." These are the FDA requirements that have been incorporated into the CLIA regulations. Observations made by the CMS may be communicated to the FDA, which has the authority to directly inspect a hospital transfusion service if deemed necessary.

All transfusion services, registered or unregistered and regardless of FDA nomenclature, must also comply with the regulations in 42 CFR Part 493 in accord with CLIA 1988. For the purposes of CLIA certification, CMS retains responsibility for inspection of all transfusion services.

Many transfusion services may not be surveyed directly by the CMS. Some are in an exempt state or have been accredited by an organization that has been granted deemed status (discussed in the subsections below on AABB, the Joint Commission, and CAP).

### FDA and transfusion service error reporting

Both registered and unregistered blood establishments, including transfusion services, must report errors and accidents in manufacturing to the FDA. As defined in 21 CFR 600.3(u), manufacture means "all steps in propagation or manufacture and preparation of products and includes but is not limited to filling, testing, labeling, packaging, and storage by the manufacturer." The term includes packaging, labeling, repackaging, or otherwise changing the container, wrapper, or labeling of any blood product package in furtherance of the distribution of the blood product from the original place of manufacture to the person who makes final delivery or sale to the ultimate consumer (21 CFR 607.3(d)). Errors and accidents (termed biologic product deviations) and unexpected events in manufacturing that can affect the safety, purity, and potency of a product are deemed reportable (21 CFR 606.171 and 21 CFR 600.14). Form FDA 3486 is used for reporting these deviations. The requirement to report applies only to manufacturing errors and not to transfusion errors occurring in clinical areas outside of the transfusion service.

The deviation or unexpected event must occur in the facility or another facility under contract with the controlling facility. If a facility under contract to the hospital blood bank or transfusion service is responsible for a deviation, the hospital blood bank or

transfusion service is responsible for reporting the problem if the product is distributed. The contract facility must perform an investigation but is not required to report. For example, if a test laboratory under contract to a hospital blood bank fails to provide viral marker testing and the unit is subsequently distributed, the blood bank must report this. If a transfusion service discovers that a unit is mislabeled with an extended outdate, the transfusion service must notify the blood center responsible for reporting to the FDA. The transfusion service would report this incident only if it further distributed the unit without correcting the label.

Deviations and unexpected events occurring within the facility or a facility under contract must be reported if they may affect the safety, purity, or potency of either licensed or unlicensed products that have been distributed. However, as noted, an error occurring after a product has left the facility need not be reported. Examples of events that would not require a report include a unit not being held at the appropriate temperature before transfusion after release from the blood bank, transfusion of a unit to the wrong patient, or failure by hospital staff to use a filter issued by the transfusion service. Reportable, unexpected events may occur even if all established procedures are followed within the transfusion service itself. An example of this would be a patient sample used for compatibility testing that was collected from the wrong patient.

### FDA and defective product reporting

If the transfusion service determines that the transfused blood or blood component was at fault in causing the adverse event, a summary of the transfusion services' investigation and conclusions must be sent to the manufacturer or blood collection establishment, who must then maintain such copies (21 CFR 606.170(a)).

### AABB

The AABB assessment incorporates evaluation of the quality system at an institution and of each operational system. The quality system assessment is based on the same criteria for every facility. The operational systems, however, are identified by the activities performed within an individual facility. This voluntary assessment is conducted every two years. AABB Standards for Blood Banks and Transfusion Services apply equally to member blood establishment. AABB policy includes the provision that although some requirements are based on the FDA's regulations, a committee with international expertise can review requests for variance from facilities both in and outside the United States that involve a departure from US regulations.

In May 2014, AABB was granted deemed status for CLIA to meet CMS requirements. This status means that the CMS has determined that the AABB accreditation process provides assurance that facilities meet or exceed conditions required by federal law and regulations. A laboratory accredited by the AABB that designated AABB as its CLIA provider does not need to be inspected routinely by the CMS. However, these facilities are subject to validation surveys and surveys performed in response to complaints to the CMS or state agencies on behalf of the CMS. This deemed status applies to the following AABB Standards: Blood Banks and Transfusion Services; Cellular Therapy Services; Immunohematology Reference Laboratories; and Molecular Testing for Red Cell, Platelet, and Neutrophil Antigens.

The AABB works with CAP to coordinate the AABB assessment and CAP inspection at the same time if the institution falls under both AABB and CAP, but the activities are still separate and each organization makes their own determination of accreditation.

AABB does not work with the Joint Commission in the area of accreditation. Many hospital transfusion services, especially if they are in smaller hospitals, are not members of AABB, but nearly all fall under the Joint Commission.<sup>13</sup>

### The Joint Commission

Founded in 1951, the Joint Commission (TJC) is a global driver of quality improvement and patient safety in health care. It is the oldest and largest standards setting and accrediting body in health care in the United States. Since 1961, review of blood use has been an element of the accreditation process of the Joint Commission which is voluntary but widely used. By 1970, the Joint Commission required review not only of blood utilization but also of transfusion reactions. The 1986 Standards were more comprehensive, mandating review of transfusion policies and procedures, ordering practices, and adequacy of the transfusion service generally. The standards also required evaluation of all transfusions. After repeated blood utilization reviews consistently documented appropriate blood use, sampling became acceptable. Although only a minority of hospitals complied with this requirement for 100% evaluation, this standard was nevertheless the driving force behind blood utilization review in the United States.

Since 1991, the Joint Commission has made a series of modifications to this standard, and 100% review is no longer required. The actual number of transfusions to be reviewed is not mandated, although recommendations are provided. Additionally, as part of an effort to reduce medical errors, the Joint Commission has developed National Patient Safety Goals; the number one goal is to "improve the accuracy of patient identification." Although this safety goal was implemented because of the recognition that patient misidentification affects all aspects of medical care, its relevance to transfusion therapy was clearly recognized by the Joint Commission. One of the implementation expectations for the goal clearly states that "Two patient identifiers are used when administering medications or blood products" and "when collecting blood samples . . . for clinical testing."<sup>13</sup> There are also explicit statements that specimens collected from a patient must be labeled at the bedside and that the patient's location may not be used as an identifier. The patient safety goal is reiterated in the body of the standards themselves.

The Joint Commission views transfusion services from a dual perspective. Standards are devoted both to the entire process of blood transfusion from blood ordering through infusion and to the laboratory procedures and practices. Two accreditation manuals have standards regarding blood transfusion. The *Comprehensive Accreditation Manual for Hospitals* provides specific standards for hospitals that transfuse and monitor blood components. The *Comprehensive Accreditation Manual for Pathology and Clinical Laboratory Services* contains technical standards that are patterned after the CLIA 1988 requirements and AABB standards. This dual approach provides the Joint Commission with the opportunity to assess the entire spectrum of clinical and laboratory blood transfusion practices. The Joint Commission Laboratory Accreditation Program has deemed status for CLIA to meet CMS requirements.

Standards related to blood transfusion are included in the *Comprehensive Accreditation Manual for Hospitals* in those sections addressing the medical staff, provision of care, treatment and services, management of information, improvement of organization performance, environment of care, and sentinel event review. Among the salient standards, MS 3.10 states that the medical staff must have "a leadership role in hospital performance improvement activities to improve quality of care."<sup>13</sup> One of the specific elements

of performance for MS 3.10 requires that the medical staff be “actively involved in measurement, assessment and improvement in the use of blood and blood components.”<sup>13</sup> A similar provision, PI 1.10 in the section on performance improvement, states that the hospital must “collect data to monitor its performance,” and this requirement includes collecting data on blood and blood product use. PI 2.20 requires that “undesirable patterns and trends in performance are analyzed,” including all confirmed transfusion reactions.<sup>13</sup> In addition, hemolytic transfusion reactions involving the administration of blood having major blood group incompatibilities are identified as reviewable sentinel events subject to specific review by the Joint Commission.

The *Comprehensive Manual for Laboratory and Point of Care Testing* contains more specific provisions governing transfusion services. HR 1.15 requires that the director of the blood bank must be a physician, either a pathologist or other physician qualified in immunohematology and hemotherapy. Sections QC 5.10 through QC 5.260 provide guidance on what the Joint Commission regards as critical elements comprising an acceptable transfusion service. Section QC 5.10 requires that the transfusion services have written policies and procedures that “are acceptable in format, content, review process and availability.” These policies and procedures must be consistent with AABB standards and must be reviewed annually. There must also be policies and procedures governing transfusion reactions and adverse events. Every adverse event must be evaluated by the medical director and documented in the patient’s medical record.

### College of American Pathologists

CAP has an established accreditation program for transfusion services. This program examines preanalytical, analytical, and postanalytical aspects of quality management in the laboratory. These include the performance and monitoring of general quality control, test methodologies and specifications, reagents, controls and media, equipment, specimen handling, test reporting and internal performance assessment, and external proficiency testing. In addition, personnel requirements, safety, document management, and other administrative practices are included in the inspection process. The CAP laboratory accreditation program expects a participant laboratory to demonstrate that it is in compliance with the CAP standards for laboratory accreditation. These standards relate to requirements for laboratory direction, physical facilities and safety, quality control and performance improvement, and inspection. Assessment of whether a laboratory meets the standards is accomplished through a series of checklists. Any applicable question that cannot be answered “yes” is considered a deficiency and must be corrected within 30 days with the submission of supporting documentation for accreditation to be achieved. The inspector does not grant or deny accreditation but makes a recommendation for the outcome. The accreditation decision is made by the CAP Accreditation Committee. In addition to the on-site inspection program, the CAP laboratory accreditation program monitors the proficiency testing performance of its participant laboratories. The CAP has deemed status with the Joint Commission and for CLIA to meet CMS requirements. As requested, CAP assesses hospital blood banks and transfusion services as well as blood collection establishments every two years.

Inspection of transfusion services is not limited to the contents of the Transfusion Medicine Checklist but includes the All Common Checklist (COM) and all applicable portions of the Laboratory General Checklist (GEN). All sections of the laboratory must be familiar with and in compliance with the requirements of the COM

and GEN Checklists. The Transfusion Medicine checklist contains the following: “Note: Many of the requirements in this Checklist reflect United States regulatory requirements, particularly those of the US Food and Drug Administration (FDA). These requirements may not be applicable in other countries for purposes of CAP accreditation.”<sup>14</sup>

### Transfusion Medicine Committee

A clinical staff committee concerned exclusively with practices and policies related to transfusion and the transfusion service is not mandated by federal regulation, the Joint Commission, or other accreditation entities. However, it has become routine to have such a committee in most hospitals because a standing transfusion committee is an efficient way of meeting QA and peer review requirements. In fact, one of this committee’s principal activities in many institutions has been the utilization review of transfusion: both over transfusion and under transfusion. Assessment of transfusion practices has the potential to enhance the knowledge and judgment of health-care professionals; provide significant information about patient care; reduce the risk of litigation; decrease costs; ensure compliance with regulatory and accreditation requirements; conserve the blood supply; provide an opportunity to demonstrate quality and value to the public; and help create, sustain, and document excellence in patient care. Many committees have also been engaged in developing informed consent practices for transfusion as well as policies related to “lookback” to find patients who may have previously been infected by a blood transfusion at the committee’s institution.

This committee typically reports to the health-care evaluation office or committee, or directly to the medical policy committee of the institution.

### Conclusion: US structure

FDA and the voluntary accrediting agencies seek to advance and protect public health. Federal regulations that carry the force of law, federal guidance documents that contain nonbinding recommendations, and voluntary accreditation standards issued by the organizations addressed in this chapter are the instruments used to achieve these goals. Blood donor qualification and the collection of blood and blood components as well as their processing, storage, transport, and subsequent manufacturing at hospital transfusion services and plasma manufacturing facilities are FDA-regulated activities to which certain biologic and drug laws apply.

The regulations, guidance documents, and standards are crafted so that compliance by the affected institutions is feasible while providing the greatest protection possible to blood donors and patients.

### International perspective on ensuring blood and blood product safety and availability

From a global perspective, WHO, the European Union (EU), the Council of Europe (CoE), and the FDA are the most prominent organizations that develop guidance documents, regulations, directives, and standards, used nationally or internationally to ensure the quality, safety, efficacy, and availability of blood and blood products. Although these organizations do not codevelop their regulations, they do collaborate on common initiatives, comment on each other’s public documents, and exchange ideas, all of which promote a general convergence of their regulatory and advisory activities. The information and standards that these organizations provide are employed by many developed and developing

countries to establish their own national blood programs. International trade associations, patient and professional organizations, and professional societies also contribute to global blood and blood product safety. This section describes the international programs and the organizational structures related to the regulation and/or accreditation of blood establishments and cite other major international organizations that advance global blood and blood product safety and availability.

### **WHO programs for blood and blood component transfusion safety**

WHO is the authority within the UN system that is responsible for the coordination of health policy. It has a mandate for “providing leadership on global health matters, shaping the health research agenda, setting norms and standards, articulating evidence-based policy options, providing technical support to countries and monitoring and assessing health trends.”<sup>15</sup> WHO is in the forefront of providing guidance for safe blood collection and transfusion. “The objective of the WHO program on Blood Transfusion Safety is to ensure provision of universal access to safe, quality and efficacious blood and blood products for transfusion, their safe and appropriate use, and also ensuring blood donor and patient safety.”<sup>16</sup>

WHO has a 50-year history of involvement in improving blood safety and availability. In 1975, a World Health Assembly resolution urged member states to promote the development of national blood transfusion services based on voluntary nonremunerated blood donations and to take other actions to promote and protect the health of blood donors and recipients of blood and blood products. These objectives are further elaborated in WHO’s strategic directions for 2008–2015 to build a conducive political, social, and economic environment for the effective integration of sustainable national blood programs in health systems; respond to country needs to enhance national blood programs and improve clinical transfusion practice; build effective collaboration and partnerships for coordinated action; and strengthen systems for assessing, surveillance, alerting, monitoring, and evaluating.<sup>17</sup>

WHO has developed an extensive program to promote access to safe blood transfusion products, particularly to address the needs of less developed and transitional countries. This includes giving advice on improving blood systems by establishing a national blood system recognized through a national blood policy. Functions of the national blood system should include “policy formulation and standard setting, strategic and operational planning, provision of sufficient resources and national coordination and management to ensure an adequate supply of blood and blood products and safe clinical transfusion.”<sup>17</sup>

WHO recommends the implementation of a quality system that provides a framework within which activities are established, performed in a quality-focused way, and continuously monitored to improve outcomes. The risk associated with blood transfusion can be significantly reduced through the introduction of quality systems, external quality assessment, and education and training for staff.

Other programs supported by WHO include voluntary blood donation, donation testing, blood processing, proper clinical use of blood transfusion products, and hemovigilance.

### **Implementation of WHO programs for access to safe blood transfusion**

WHO has established a number of collaborations and partnerships to share knowledge, coordinate technical support to increase blood donations, support blood system strengthening, and promote universal

access to blood transfusion. One means that WHO uses to coordinate activities related to blood is through the work of WHO Collaborating Centers (WCCs) on Blood Transfusion Safety and Blood Products, whose members currently (2014) include Iran, Thailand, the United Kingdom, China, Slovenia, Brazil, Tunisia, and Germany.

Another WHO partnership is through the WHO Global Safety Network comprised of members of the WHO Expert Advisory Panel on Blood Transfusion Medicine, the WCCs on Blood Transfusion, nongovernmental officials in official relations, key developmental and implementing partners for blood safety, WHO regional focal points for blood safety, and WHO Blood Transfusion Safety staff. This group shares information from the WCCs and develops mechanisms of working together to enhance WHO strategies and objectives in the area of blood transfusion safety.

In 1998, WHO established a Global Data Base on Blood Safety to address global concerns about the availability, safety, and accessibility of blood for transfusion. The database reports the number of blood transfusions and donations in countries throughout the world and provides information that can be used to assess where deficiencies lay and identify where progress is being made. A fact sheet from June 2020 provides examples of the information contained in this database.<sup>18</sup>

Of the 118.5 million blood donations collected globally, 40% of these are collected in high-income countries, home to 16% of the world’s population.

In low-income countries, up to 54 % of blood transfusions are given to children under five years of age; whereas in high-income countries, the most frequently transfused patient group is over 60 years of age, accounting for up to 75% of all transfusions.

Based on samples of 1000 people, the blood donation rate is 31.5 donations in high-income countries, 15.9 donations in upper-middle-income countries, 6.8 donations in lower-middle-income countries, and 5.0 donations in low-income countries.

An increase of 7.8 million blood donations from voluntary unpaid donors has been reported from 2013 to 2018. In total, 79 countries collect over 90% of their blood supply from voluntary unpaid blood donors; however, 56 countries collect more than 50% of their blood supply from family/replacement or paid donors.

Only 55 of 171 reporting countries produce plasma-derived medicinal products (PDMP) through the fractionation of plasma collected in the reporting country. A total of 90 countries reported that all PDMP are imported, 16 countries reported that no PDMP were used during the reporting period, and 10 countries did not respond to the question.

### **WHO QA and safety programs for blood products and related biologicals**

In addition to programs focused on ensuring safe blood components for transfusion, WHO has an interest in providing technical guidance and QA tools to regulatory authorities, national control laboratories, and manufacturers to support implementation of quality and safety systems for the production and control of blood products and related in vitro diagnostic devices worldwide. Input for the development of these tools is provided by technical experts from academia, industry, national regulatory authorities, professional societies, and WHO’s Collaborating Centers for Biological Standards and Standardization (i.e., the National Institute of Biological Standards and Control [NIBSC], United Kingdom; the Paul Ehrlich Institute [PEI], Germany; and the Center for Biologics Evaluation and Research [CBER], Food and Drug Administration [FDA], United States).

As an example of these resources, WHO has issued a document entitled *Assessment Criteria for National Blood Regulatory Systems* to assist “capacity building of national regulatory authorities for the regulation of blood and blood products. The document is intended to help Member States identify gaps and priorities when developing capacity building programs, and to support the introduction of regulation of blood products.” This document outlines “elements and functions which may support the creation of an appropriate blood regulatory system where none exists so far, and which may also be used as a tool to assess strengths and gaps of established systems.” It “identifies the essential elements and core regulatory functions that should be present in an effective national regulatory authority to assure the quality, safety and efficacy of blood and blood products, as well as associated substances and medical devices including in vitro diagnostics.”<sup>19</sup>

In addition to this document, WHO has produced a series of technical reports that give guidance on topics such as GMPs for blood establishments; recommendations for the production, control, and regulation of human plasma for fractionation; and guidelines on viral inactivation and removal procedures intended to ensure the viral safety of human blood plasma products.

#### **Major WHO advisory groups relevant to blood product safety, quality, and standardization**

The Expert Committee on Biological Standardization (ECBS) and the WHO Blood Regulators Network (BRN) are two advisory groups associated with WHO whose work directly promotes blood product safety and quality.<sup>19,20</sup>

ECBS was established in 1947 to provide detailed recommendations and guidelines for the manufacturing, licensing, and control of blood products and related in vitro diagnostic tests, biotechnology products, and vaccines along with the establishment of WHO Biological Reference Materials. The ECBS meets annually and reports directly to the Executive Board, the executive arm of the World Health Assembly.

Members of the ECBS are scientists from National Regulatory Agencies, academia, research institutes, and public health bodies. The decisions and recommendation of the committee are based entirely on scientific principles and considerations of public health.

Written guidelines and recommendations submitted to the ECBS are drafted through a consultative process during which WHO brings together experts from around the world on a given topic. Written guidelines and recommendations describe procedures for the manufacture and quality control testing of biological medicinal products to ensure safe and effective products. Guidelines provide more general information on a range of topics of interest to National Regulatory Authorities (NRAs) and manufacturers, whereas recommendations establish the technical specifications for manufacturing and quality control of specific products. By adopting these guidance documents in their pharmacopoeias or equivalent legislation, national governments ensure that the products produced and used in their country conform to current international standards. Regulatory guidance documents also advise NRAs and manufacturers on the control of biological products, with the aim of establishing a harmonized regulatory framework for products moving in international markets.

In addition to guidelines and recommendations, WHO has played a key role for over 50 years in establishing the WHO

Biological Reference Materials necessary to standardize biological materials. Reference materials are required to standardize potency, purity, and identity measurements for complex biological materials. “The WHO Biological Reference Materials provide a global standard against which experimental values can be compared and expressed, thereby allowing direct comparisons between products and measurements across different methodologies and assays in use around the world.”<sup>20</sup> Reference materials are established through scientific studies involving participation of a large number of laboratories worldwide.

The proceedings of the meetings of the ECBS are published in the WHO Technical Report Series (TRS). They provide information on the establishment, discontinuation, and replacement of the WHO Biological Reference Materials as well as on the adoption of Guidelines and Recommendations. The TRS are available electronically as well as publications, and relevant topics can be searched either by the TRS number or by topic.

The WHO Blood Regulators Network (BRN) is a group whose work helps to ensure blood product safety in a timely manner. BRN was established in 2006 and is composed of leading international regulatory authorities that have responsibility for the regulation of blood, blood products, and related in vitro diagnostic (IVD) devices.

Members of the BRN exchange information and opinion on blood-related issues. The BRN focuses on scientific assessment of current and emerging threats to the safety and availability of blood and blood products, assesses the impact of new blood-related technologies, and also explores opportunities for regulatory cooperation and collaboration, where possible.

Member organizations have legal standing and well-established, demonstrated institutional capacity to regulate blood and blood products, and the necessary expertise to address emerging global public health challenges.

BRN recommendations and considerations are communicated to the ECBS through WHO. Documents published by the BRN contain the collective views of members and do not necessarily represent the decisions or the stated policy of WHO or of the participating regulatory authorities.

Examples of the work of BRN are the publications *Position Paper on Collection and Use of Convalescent Plasma or Serum as an Element in Filovirus Outbreak Response* and *Potential for Use of Convalescent Plasma in Management of Ebola*. These documents were published in August and September 2014, respectively, and demonstrate BRN’s ability to respond quickly to emerging threats to the blood supply.

#### **Regulation of and guidance on blood products in Europe**

The regulation of blood components for transfusion, and blood plasma derivatives and analogous recombinant analogs, in Europe is complex because of historical considerations and the balance that is needed between the role of a centralized authority and the involvement of the many independent member states that have their own medicines regulatory authorities. The functionality of the system depends on the exchange of information among the member states and their acceptance of common standards and practices. The standards, guidance documents, and authorized medicines produced within the European framework stretch beyond its borders and have been adopted in many other parts of the world.

Organizations involved in European regulatory processes include the following (see Figure 8.1).

### The European Union and associated organizations

**European Union:** The European Union was founded in 1948 to promote stability and economic cooperation among member states. It consists of 27 member states and operates through a system of supranational independent institutions and intergovernmental negotiated decisions by EU Member States. The European Union has developed a single market through a standardized system of laws, and the same rules and harmonized procedures apply to all the 27 Member States regarding the authorization of medicines and the supervision of the safety of medicines.

**European Commission:** This executive arm of the European Union proposes legislation, sets objectives and priorities, and grants centralized marketing authorization.

**European Economic Area (EEA):** This includes the European Union plus Norway, Iceland, and Liechtenstein.

**European Medicines Agency (EMA):** The agency within the European Union is responsible for the scientific evaluation of applications for marketing authorizations for human and veterinary medicines in the centralized procedure. Its main responsibility is the protection and promotion of public and animal health through the evaluation and supervision of medicines for human and veterinary use.

**Committee for Medicinal Products for Human Use (CHMP):** The committee at the EMA that is responsible for preparing opinions on questions concerning medicines for human use. The members and alternates of the CHMP are nominated by EU member states in consultation with the Agency's Management Board. They are cho-

sen on the strength of their qualifications and expertise with regard to the evaluation of medicines.

### Council of Europe and associated organizations

The Council of Europe (CoE) is an international organization founded in the wake of World War II to uphold human rights, democracy, and the rule of law in Europe.<sup>21</sup> It has 47 member states.<sup>22</sup> The organization is distinct from the 27-nation European Union (EU), although it is sometimes confused with it, partly because the EU has adopted the original European Flag that was created by the Council of Europe in 1955, as well as the European Anthem. No country has ever joined the EU without first belonging to the Council of Europe. The Council of Europe is an official United Nations Observer. The CoE regularly reviews and updates technical requirements in its Guide to the Preparation, Use and Quality Assurance of Blood Components and Good Practice Guidelines for blood establishments. In addition, it prepares ad hoc guidelines on different topics, in regard to the safety and quality, including a Directive last amended in 2004 that sets requirements for blood collection and processing. The Council of Europe cannot make binding laws, but it does have the power to enforce select international agreements reached by European states on various topics. Organizations associated with the COE are briefly described below.

**European Directorate for the Quality of Medicines (EDQM):** The EDQM is a Directorate of the CoE. Its mission is to establish and provide official standards that apply to the manufacture and quality control of medicines to member states of the CoE. As it specifically relates to blood, it serves to propose ethical, safety, and quality standards for blood transfusions, including the collection, preparation, storage, distributions, and appropriate use of blood components.

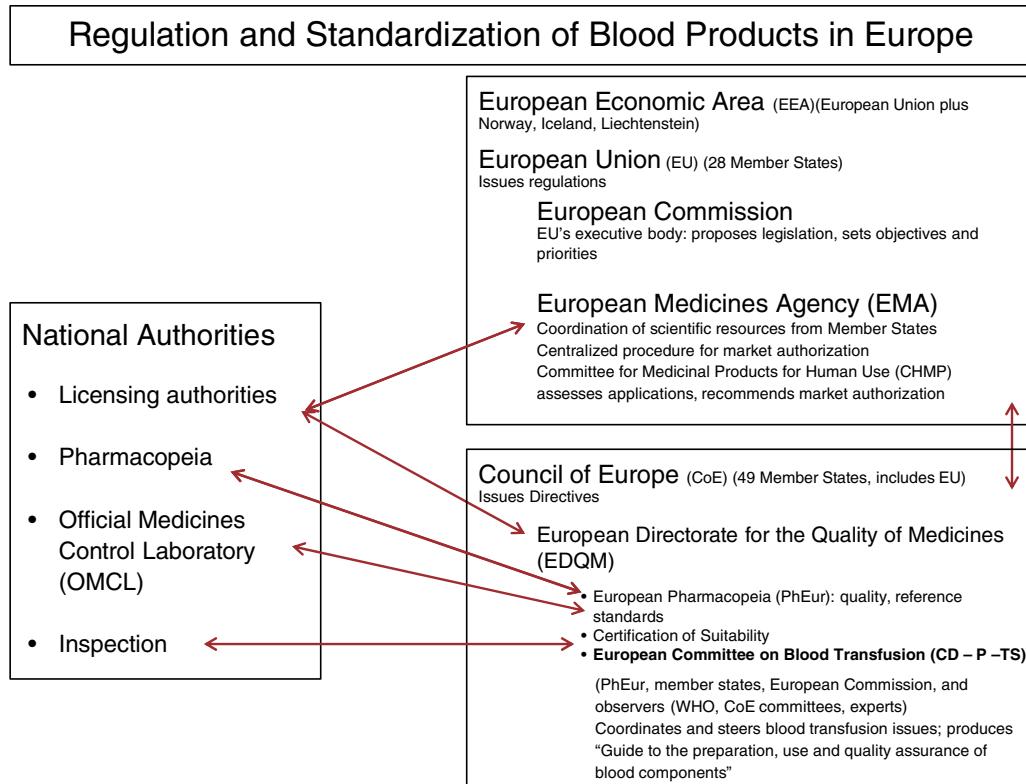


Figure 8.1 Regulation and standardization of blood product in Europe.

**European Pharmacopeia (PhEur):** Within the EDQM, the PhEur sets common standards for all the national pharmacopoeias of the member states. It provides standardization of analytical methods used for the control of substances used in medicines for human and/or veterinary use. The Expert Group 6B is the entity within the PhEur that is involved with standards for blood products. Importantly, the European Pharmacopeia standards are legally binding for member states.

**Official Medicines Control Laboratory (OMCL):** OMCLs support regulatory authorities in controlling the quality of medicinal products for human and veterinary use available on the market. Within Europe, OMCLs are nominated by the national authority responsible for the quality control of medicines in their country. OMCLs test products in the EEA, where appropriate, and are independent of manufacturers.

**European Committee on Blood Transfusion (CD-P-TS):** This coordinates and steers blood transfusion issues, and produces a publication entitled *Guide to the Preparation, Use and Quality Assurance of Blood Components*. Its members include PhEur, member states, the European Commission, and observers (WHO and CoE committees).

**European Medicines Agency (EMA):** The European Medicines Agency is an agency of the European Union in charge of the evaluation and supervision of medicinal products.

**Committee for Medicinal Products for Human Use (CHMP):** The CHMP plays a vital role in the authorization of medicines in the European Union (EU). The CHMP is responsible for:

- conducting the initial assessment of EU-wide marketing authorization applications;
- assessing modifications or extensions (“variations”) to an existing marketing authorization;
- considering the recommendations of the Agency’s Pharmacovigilance Risk Assessment Committee on the safety of medicines on the market and when necessary recommending to the European Commission changes to a medicine’s marketing authorization, or its suspension or withdrawal from the market.<sup>23</sup>

**Paul Ehrlich Institute (PEI):** German regulatory authority that has a major influence on the development of blood products in Europe and is a WCC.

## Authorization in Europe of plasma derivative and analogous recombinant products

The regulation and authorization of manufactured blood products in Europe involve an interplay among the EMA, national competent authorities (NCAs), CHMP working parties, and the EDQM that includes the PhEur and OMCLs. There are three pathways for authorizing medicines in Europe. In the “centralized” procedure, pharmaceutical companies submit a single marketing-authorization application to the EMA. For blood products, the EMA’s CHMP carries out a scientific assessment of the application and gives a recommendation on whether or not to grant a marketing authorization. Once granted by the European Commission, the centralized marketing authorization is valid in all EU member states. Most innovative medicines go through this procedure.

Most medicines are not authorized through the centralized procedure. Instead, they are authorized by NCAs in member states. When a company wants to authorize a medicine in several member states, it can use one of the following procedures:

- The decentralized procedure, through which companies can apply for the simultaneous authorization of a medicine in more than one EU member state if it has not yet been authorized in any EU country and it does not fall within the mandatory scope of the centralized procedure; or

- The mutual-recognition procedure, through which companies that have a medicine authorized in one EU member state can apply for this authorization to be recognized in other EU countries.

This process allows member states to rely on each other’s scientific assessments. Rules and requirements applicable to pharmaceuticals in the European Union are the same, irrespective of the authorization route for a medicine.

## Safety monitoring of medicines in Europe

EudraVigilance is an EU web-based information system within the EMA that collects, manages, and analyzes reports of suspected side effects of medicines, including blood. Information is obtained from EEA members, and these data are continuously monitored in order to identify any new safety information.

The EMA has a committee dedicated to the safety of medicines for human use—the Pharmacovigilance Risk Assessment Committee (PRAC). If there is a safety issue with a medicine that is authorized in more than one member state, patients and health-care professionals in all member states are given the same guidance by the committee, and the same regulatory action is taken across the European Union.

The PRAC has a broad remit covering all aspects of pharmacovigilance. In addition to its role in risk assessment, the committee provides advice and recommendations to the European medicines regulatory network on risk management planning and postmarketing benefit–risk assessment for medicines.

## Activities to promote blood component safety in Europe

The CoE has been actively contributing since the 1950s to the implementation of standards for blood transfusion. In 2007, the secretariat responsible for blood transfusion activities was transferred to the EDQM. Within the EDQM, the European Committee on Blood Transfusion (CD-P-TS) is in charge of steering and coordinating the actions of the CoE in this area. The membership of CD-P-TS includes the CoE member states and parties to the Convention on the Elaboration of a European Pharmacopoeia. The European Commission, WHO, and other CoE Committees (European Public Health and Bioethics Committees) are special observers to the CD-P-TS.

Activities of the CD-P-TS include, among others, addressing issues about quality and safety standards for blood transfusion, including collection, storage, distribution, and use of blood components; improving blood transfusion services; promoting the principle of voluntary nonremunerated donations; and establishing good practices in transfusion medicine and monitoring their use in Europe. These objectives are attained by setting standards and preparing guidance on professional practices (e.g., *Guide to the Preparation, Use and Quality Assurance of Blood Components*); organizing and evaluating surveys on blood components; and using resolutions to promote continuous improvement of an ethical, organizational, and regulatory approach to blood transfusion.

## Other major international organizations involved in the regulation or standardization of blood, blood products, and their biotechnology analogs

**America’s Blood Centers/European Blood Alliance (ABC/EBA):** This works to harmonize requirements and decision-making processes to promote safe and high-quality blood products in North America and Europe.

*World Federation of Hemophilia (WFH)*: This works on issues involving hemophilia and other bleeding disorders, including supply, affordability, safety, and regulatory harmonization.

*European Hemophilia Safety Surveillance (EUVASS)*: This is a pharmacovigilance program to monitor the safety of treatments for people with inherited bleeding disorders in Europe.

*Plasma Protein Therapeutics Association (PPTA)*: This is the international trade association and standards-setting organization for the world's major producers of plasma-derived and recombinant analog therapies, collectively referred to as *plasma protein therapies*.

*International Plasma Fractionation Association (IPFA)*: This is an international association for nonprofit organizations involved in the manufacture of blood products made from blood collected from nonremunerated donors.

*International Society of Blood Transfusion (ISBT)*: The ISBT promotes research, new developments, and changing concepts in blood transfusion medicine.

### FDA role in the global regulation of blood and blood products

The activities of the FDA have a large impact on the global regulation of blood and blood products. FDA's international standing is bolstered by the substantial medical and scientific resources that it devotes to ensuring the safety, efficacy, and availability of blood and blood products.

FDA is strongly supportive of harmonization efforts that will maximize national and global health. A convergence of thinking on regulatory and guidance issues is important for trade as well because many of the companies involved in blood product manufacture are international in scope; the US market for these products is extensive; and a large amount of plasma, intermediates, and manufactured products are distributed abroad.

FDA influences global regulatory and guidance norms for blood and blood product safety, through its interactions with all of the organizations cited in this chapter. For example, FDA representatives

are members of the WHO's ECBS and the BRN, have Observer status with EDQM's Expert Group 6B, and routinely communicate with the EMA and Health Canada through Blood Cluster meetings. In addition to interacting with organizations that are composed of multiple international partners, FDA has bilateral memoranda of understanding and confidentiality arrangements with a number of individual countries. All of these relationships facilitate cooperative activities and the sharing of information, which support the common goal of enhancing the global safety and availability of blood and blood products.

### Acknowledgement

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## **SECTION III**

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# **Blood groups and pretransfusion testing**

## CHAPTER 9

# Carbohydrate blood groups

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Blood group antigens of the H, ABO, Lewis, and historical “P” blood groups are defined by small carbohydrate epitopes expressed as posttranslational modifications on glycoproteins, mucins, and glycolipids. Their synthesis is complex and dependent on several distinct glycosyltransferases and interrelated biosynthetic pathways. In many cases, carbohydrate antigens share features of more than one blood group (e.g., ABO and Lewis).

### ABO system

Discovered in 1900 by Karl Landsteiner, the ABO blood group system remains the most important blood group system for blood transfusion and transplantation. ABO antigens are widely expressed on human tissues and fluids, leading to their designation as *histoblood group* antigens.<sup>1</sup> ABO antigens are differentially expressed during cellular development and can be detected as early as human embryonic stem cells.<sup>2</sup> This can be observed in bone marrow, where hematopoietic progenitor cells strongly express H-antigens (H, Le<sup>y</sup>), but not A/B antigens, which are restricted to developing erythroblasts and megakaryocytes.<sup>3,4</sup> ABO can also be detected on vascular endothelium, skin, and tissues arising from embryonic endoderm and mesoderm, including epithelial cells of the respiratory, gastrointestinal, and genitourinary tracts.<sup>1</sup>

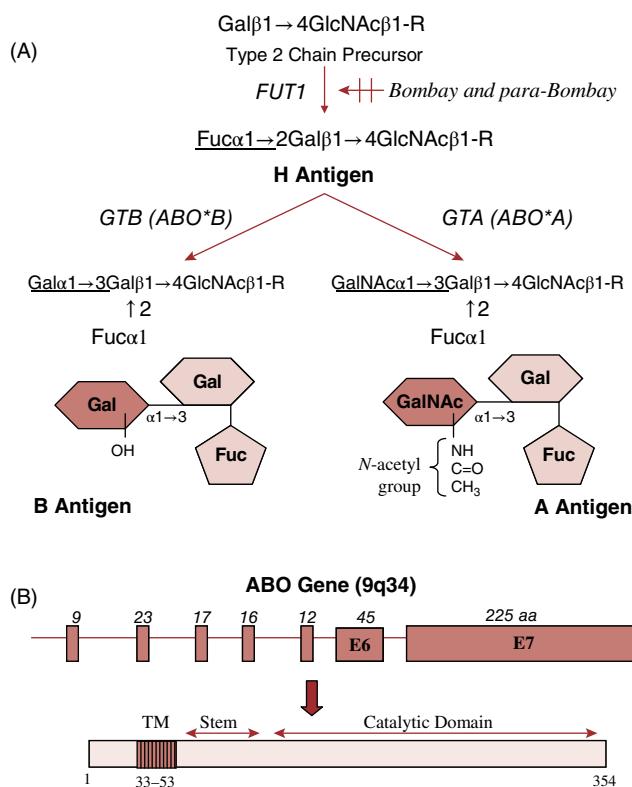
### Chemistry and biosynthesis

The ABO system (ISBT 001) contains two structurally related carbohydrate antigens, A and B. The O or H antigen is the biosynthetic precursor of A and B antigens and is listed under a separate blood group system (ISBT 018). All three antigens consist of 2–3 terminal oligosaccharides on glycoproteins and glycolipids. As shown in Figure 9.1, the minimum A-antigen epitope is a trisaccharide composed of a subterminal  $\beta$ -galactose (Gal) bearing both an  $\alpha 1 \rightarrow 2$ fucose and  $\alpha 1 \rightarrow 3$  *n*-acetylgalactosamine (GalNAc). The B antigen resembles the A antigen except for the presence of an  $\alpha 1 \rightarrow 3$ Gal. Not surprisingly, group A red cells can be converted to group B-active cells through loss of the acetyl group. Clinically, this is observed in the acquired B phenotype, in which group A patients transiently type as group B due to infection by deacetylase-producing bacteria.<sup>5</sup>

In red cells, the synthesis of H and A/B antigens proceeds in a stepwise fashion from a type 2 chain or lactosamine (Gal $\beta 1 \rightarrow 4$ GlcNAc-R) precursor (Figure 9.1). Initially, the H-enzyme, FUT1, adds an  $\alpha 1 \rightarrow 2$  fucose to the terminal galactose to make type 2 H-antigen. H-antigen can then serve as a substrate for A and B synthesis. In group A individuals, A-transferase (GTA) recognizes UDP-GalNAc to transfer an  $\alpha 1 \rightarrow 3$ GalNAc to H-antigen. Likewise, B-transferase (GTB) recognizes UDP-Gal to transfer an  $\alpha 1 \rightarrow 3$ Gal to synthesize group B antigen. Group AB, in which both A and B antigens are expressed, reflects coinheritance of GTA and GTB. The group O phenotype, on the other hand, reflects the complete absence of ABO enzyme activity due to ABO-null alleles. As a result, these individuals only express the H-antigen precursor. Because A/B expression is dependent on H-antigen synthesis, the absence of FUT1 activity due to mutations (e.g., Bombay phenotype) or transcriptional repression is also associated with absent A/B expression.

On red cells, it is estimated that 65–75% of ABH antigens are expressed as N-linked glycans on glycoproteins, especially Band 3 (Diego blood group, AE1).<sup>6</sup> At one million copies, Band 3 is the major glycoprotein on red cells and accounts for 50% of all ABH antigens.<sup>7</sup> The remaining ABH antigens are present on O-linked glycans (10%), glycosphingolipids (GSL, 5%), and polyglycosylceramides (10–15%), also known as *erythroglycans*.<sup>6</sup> The latter are massive, branched, multivalent type 2 chain glycolipids composed of 40 or more oligosaccharides and potentially 4–6 ABH epitopes.<sup>8</sup>

ABH antigens can be expressed on different oligosaccharide scaffolds, which often demonstrate developmental and tissue specificity (Table 9.1). Furthermore, the oligosaccharide backbone can influence ABH valency and spatial presentation, as well as contribute to the immune epitope. On red cells, platelets, and endothelium, ABH is primarily expressed on type 2 chain or lactosamine-based structures. Genitourinary and gastrointestinal tissues, on the other hand, are rich in type 1 chain ABH antigens.<sup>1</sup> Type 1 chains are structurally similar to type 2 chains except for linkage of the galactose residue (Gal $\beta 1 \rightarrow 3$ GlcNAc-R). In addition, the synthesis of type 1 chain H-antigen is dependent on *Secretor* or *FUT2*, a related  $\alpha 1,2$ -fucosyltransferase. Secretor-positive (Se) individuals also secrete type 1 ABH antigens in body fluids, including saliva and plasma.



**Figure 9.1** (A) Synthesis of group A and group B antigen. (B) Structure of the ABO gene and ABO glycosyltransferase.

**Table 9.1** Group A Structures in Humans

Name	Structure
Type 1 A (A-1)	$\text{GalNAc}\alpha 1 \rightarrow 3\text{Gal}\beta 1 \rightarrow 3\text{GlcNAc} \rightarrow \text{R}^b$ ↑ 2 $\text{Fuc}\alpha 1$
Type 1, ALe <sup>b</sup>	$\text{GalNAc}\alpha 1 \rightarrow 3\text{Gal}\beta 1 \rightarrow 3\text{GlcNAc} \rightarrow \text{R}$ ↑ 2      ↑ 4 $\text{Fuc}\alpha 1$ $\text{Fuc}\alpha 1$
Type 2 A (A-2)	$\text{GalNAc}\alpha 1 \rightarrow 3\text{Gal}\beta 1 \rightarrow 4\text{GlcNAc} \rightarrow \text{R}$ ↑ 2 $\text{Fuc}\alpha 1$
Type 3 A (mucinous A)	$\text{GalNAc}\alpha 1 \rightarrow 3\text{Gal}\beta 1 \rightarrow 3\text{GalNAc}\beta 1 \rightarrow 3\text{Gal}\beta 1 \rightarrow \text{R}$ ↑ 2      ↑ 2 $\text{Fuc}\alpha 1$ $\text{Fuc}\alpha 1$
Type 4 A (globo-A, A <sub>1</sub> )	$\text{GalNAc}\alpha 1 \rightarrow 3\text{Gal}\beta 1 \rightarrow 3\text{GalNAc}\beta 1 \rightarrow 3\text{Gal}\alpha 1 \rightarrow 4\text{Gal}\beta 1 \rightarrow 4\text{GlcCer}$ ↑ 2 $\text{Fuc}\alpha 1$

Fuc, fucose; Gal, galactose; GalNAc, *n*-acetylgalactosamine; Glc, glucose; GlcNAc, *n*-acetylglucosamine; GlcCer, glucosylceramide or Glcβ1-1Ceramide.

Type 3 and type 4 chain structures share a terminal  $\text{Gal}\beta 1 \rightarrow 3\text{GalNAc-R}$  (Table 9.1). Type 3 chain A, also known as *mucinous A*, is a polymer of repeating A motifs. Type 4 chain ABH, or *globo-ABH*, is a GSL-specific antigen related to the GLOB blood group system (see the “P Blood Group System” section). Although both type 3 and 4 ABH antigens are found in trace amounts on red cells, their synthesis is relatively tissue-restricted to gastrointestinal and/or genitourinary epithelium.<sup>1</sup> There is evidence that both FUT1 and FUT2 are able to recognize type 3 and type 4 chain substrates;<sup>9</sup> however, it is believed that FUT2 is primarily responsible for type 3 and type 4 ABH synthesis in epithelial tissues.<sup>10</sup>

### ABH antibodies

The ABH system is unique for the presence of naturally occurring antibodies against missing ABH antigens (Table 9.2). Specifically, a group A individual will possess anti-B, whereas anti-A will be produced by group B individuals. These antibodies are not present at birth but gradually develop after 4–6 months of age, with adult titers reached by 5–10 years of age.<sup>11</sup> Bacteria in the diet and the gastrointestinal microbiome appear to be the primary immune stimulus for the development of anti-A and anti-B: many bacterial species express ABH-like structures on lipopolysaccharide that can crossreact with ABH antibodies and lectins.<sup>12</sup>

Antibodies against ABH and other carbohydrate antigens are considered to arise via a thymic-independent mechanism.<sup>13</sup> Specifically, carbohydrates presented as multivalent, repetitive epitopes can directly stimulate B-cells, independent of accessory T-cells, with the production of IgM antibodies. More recent studies in mice also implicate invariant natural killer cells (iNKTs) in ABH antibody formation.<sup>14</sup> Finally, the absence of ABH antibodies against self-antigens may occur through antigen–antibody complexes with specific deletion of antibody-producing B splenocytes.<sup>15</sup>

### Serology of the ABO system

The ABO system contains four major phenotypes, based on the inheritance of A and B antigens: A, B, AB, and O (or none).<sup>11</sup> ABO is unique among all other blood groups in that both red cells and plasma/serum testing are required (Table 9.2). The forward-type or red cell grouping is performed by directly testing red cells for the expression of A and B antigens with commercial antisera. The back-type or serum grouping involves testing plasma or serum against commercial A<sub>1</sub> and B red cells for the presence of anti-A and anti-B. Both the forward and back type must agree for a valid ABO phenotype.<sup>11</sup>

### Weak ABO subtypes

The ABO system contains several weak subtypes. All are characterized by decreased A/B and a parallel increase in H-antigen expression. Weak subtypes can be associated with ABO typing

**Table 9.2** ABO Serology

ABO Type	GT Genes*		RBC Grouping (Forward/Antigen Type)			Serum Grouping (Reverse/Back Type)		
	FUT1	ABO	Anti-A	Anti-B	UEA-1†	A <sub>1</sub> RBC	B RBC	O RBC
A <sub>1</sub>	+	+	++	0	0	0	+	0
A <sub>2</sub>	+	+	+	0	+	+/0	+	0
B	+	+	0	++	0	+	0	0
O	+	0	0	0	++	+	+	0
O <sub>1</sub> (Bombay) (hh)	0	+	0	0	0	+	+	+

\* Inheritance of at least one functional FUT1 or ABO gene.

† Testing for H-antigen with lectin *Ulex europeus* (UEA-1). Not routinely performed except to resolve ABO typing discrepancies.

discrepancies during serum grouping due to the presence of unexpected anti-A or anti-B activity. In some instances, A/B expression is so weak that red cells can forward type as group O.

The most common ABO subtype encountered is A<sub>2</sub>, which occurs in 20% of group A donors in the United States and Europe. A<sub>2</sub> red cells have 75% less A antigen than A<sub>1</sub> cells and can possess an anti-A.<sub>1</sub> Because of the inefficiency of the A<sub>2</sub>-glycosyltransferase (GTA), A<sub>2</sub> red cells also differ in the type of A antigen synthesized. Specifically, A<sub>2</sub> red cells lack globo-A.<sup>16</sup> These differences are more pronounced in other tissues such as platelets and endothelium, where there is little or no A-antigen.<sup>17,18</sup> Studies in A<sub>weak</sub> subtypes confirm a profound decrease in the array and complexity of A-antigens synthesized with weak GTA activity.<sup>19</sup> A<sub>2</sub> and A<sub>weak</sub> red cell subtypes are serologically distinguished from A<sub>1</sub> red cells by their lack of reactivity with the lectin *Dolichos bifloris* (DBA).<sup>11</sup> Weak A/B subtypes have increased reactivity with *Ulex europeaus* (UEA), an anti-H lectin.

### Bombay and para-Bombay

Bombay and *para*-Bombay are autosomal-recessive, H-deficient phenotypes due to homozygous inheritance of amorph *FUT1* alleles (*hh*). As a result, these individuals are unable to synthesize type 2H antigen, the precursor of A and B antigens. Serologically, red cells are negative for H, A, and B antigens, accompanied by the presence of naturally occurring anti-A, anti-B, and anti-H in plasma. In routine testing, these individuals may initially type as an apparent group O but will have a strong positive antibody screen due to allo-anti-H, which recognizes group O screening cells.

The Bombay and *para*-Bombay phenotypes are distinguished by the presence or absence of soluble type 1 ABH substance, which is under the control of *FUT2/Secretor*. The classic Bombay phenotype is an H-deficient nonsecretor (*hh, se/se*), with an absence of both type 1 and type 2 chain ABH antigens. As nonsecretors, these individuals will also type as Le(b-) (see the “Lewis Blood Group” section). In contrast, *para*-Bombay individuals are typically H-deficient secretors (*hh, Se/Se*, or *Se/se*) and retain synthesis of type 1 H antigen on mucosa and in secretions. Unlike Bombay cells, *para*-Bombay red cells may have trace amounts of ABH antigen on red cells due to adsorption of soluble type 1 ABH from plasma. There are also examples of *para*-Bombay arising from an H-deficient, weak Secretor (*hh* and *Se<sup>w</sup>/se*) as well as an H-weak nonsecretor (*H<sup>w</sup>/h* and *se/se*).<sup>20</sup>

## Molecular biology

### ABO gene

The ABO system contains two autosomal, codominant consensus alleles, A<sup>1</sup> (ABO'A1) and B (ABO'B), responsible for GTA and GTB, respectively. The active enzyme is encoded over seven exons, spread over a 19 kb region on chromosome 9q34.1.<sup>20,21</sup> This is in stark contrast to most blood-group-related glycosyltransferases (H, Secretor, Lewis, P1PK, and GLOB), in which the active enzyme is located within a single exon. Most mutations effecting ABO activity arise within exons 6 and 7, which account for 75% of the active enzyme. Exon 6, which encodes the stem region, is a hot spot for recombination. ABO'A1 is generally considered the ancestral gene, with ABO'B and ABO'O alleles arising through convergent evolution.<sup>22</sup> A more recent study, however, suggests that ABO is an ancient balanced polymorphism originating early in hominid history and maintained in multiple primate lineages.<sup>23</sup>

ABO belongs to the GT6 family of glycosyltransferases, a large family (184) of animal and bacterial α-galactose/α-galactosaminyltransferases that includes the enzymes for Forssman antigen and linear B—two histo-blood group antigens found in animals. The enzyme is a 354-amino-acid, type II transmembrane glycoprotein with a short amino-terminal cytosolic (amino acids 1–32) and transmembrane (33–53) domains, followed by a “stem region” and a large, globular catalytic domain (64–354).<sup>21</sup> The enzyme has a single N-glycan site (Asn<sup>113</sup>), five cysteines, and a DxD motif (Asp<sup>211</sup>–Val<sup>212</sup>–Asp<sup>213</sup>). DxD motifs are found in many glycosyltransferases that require Mn<sup>2+</sup> or other cations for catalysis.<sup>24</sup> Crystallography of the ABO protein shows it has a complex secondary and tertiary structure, composed of two functional domains separated by a catalytic cleft that contains the DxD motif and a highly conserved Glu<sup>303</sup>.<sup>25</sup> The H-antigen acceptor binds to the carboxyterminal half, whereas the UDP-nucleotide sugar donor binds to the more amino-terminal half of the enzyme. Initial binding of the UDP-nucleotide sugar leads to conformational changes in the enzyme necessary for H-antigen acceptor binding and catalysis.<sup>25,26</sup>

A comparison of GTA and GTB enzymes shows they are virtually identical, differing by only four amino acids at 176, 235, 266, and 268 (Table 9.3).<sup>21</sup> Using recombinant enzymes, Yamamoto *et al.* demonstrated that only three polymorphisms define whether the enzyme has GTA or GTB activity: Gly235Ser, Leu266Met, and Gly268Ala.<sup>21</sup> Because both GTA and GTB recognize and transfer a

**Table 9.3** Examples of ABO Alleles

Allele Name	RBC Type	Amino Acid Position						Gene Type
		88	176	234	235	266	268	
ABO'A.01	A1	Thr	Arg	Pro	Gly	Leu	Gly	AAA
ABO'AW.08	A <sub>weak</sub>	Thr	Arg	Pro	Gly	Leu	<b>Arg</b>	AAX
ABO'B.01	B	Thr	<b>Gly</b>	Pro	<b>Ser</b>	<b>Met</b>	<b>Ala</b>	BBB
ABO'BW.18	B <sub>weak</sub>	Thr	<b>Gly</b>	Pro	<b>Ser</b>	<b>Met</b>	<b>Thr</b>	BBX
ABO'cisAB.01	cisAB	Thr	Arg	Pro	Gly	Leu	<b>Ala</b>	AAB
ABO'cisAB.02	cisAB	Thr	<b>Gly</b>	Pro	<b>Ser</b>	Leu	<b>Ala</b>	BAB
ABO'cisAB.03	cisAB	Thr	<b>Gly</b>	<b>Ser</b>	<b>Ser</b>	<b>Met</b>	<b>Ala</b>	BBB
ABO'cisAB.04	cisAB	Thr	<b>Gly</b>	Pro	Gly	<b>Met</b>	Gly	ABA
ABO'cisAB.05	cisAB	Thr	<b>Gly</b>	Pro	<b>Ser</b>	<b>Met</b>	Gly	BBA
ABO'BA.02	B(A)	Thr	<b>Gly</b>	<b>Ala</b>	<b>Ser</b>	<b>Met</b>	<b>Ala</b>	BBB
ABO'BA.03	B(A)	Thr	<b>Gly</b>	Pro	Gly	<b>Met</b>	<b>Ala</b>	ABB
ABO'O.01	O	fs118stop	—	—	—	—	—	
ABO'O.02	O	Thr	<b>Gly</b>	Pro	Gly	Leu	<b>Arg</b>	AAX
<b>GWA Studies</b>								
Ref SNP ID (rs)		8176719			8176743	1876746	8176747	

\* Gene type refers to amino acid positions 235, 266, and 268. These three positions strongly influence substrate specificity and differ between A (AAA) and B (BBB) alleles. Amino acids that differ from the ABO'A.01 consensus allele are highlighted in bold.

<sup>†</sup> Deletion mutant (261ΔG) leading to a frameshift and premature stop codon.

sugar to H-antigen, any differences in the two proteins must reflect binding of the nucleotide sugar donor. The mechanism underlying how these three amino acids differences determine whether the enzyme will utilize UDP-Gal or UDP-GalNAc appears to be the size and geometry of the UDP-binding pocket. In general, the amino acids associated with GTA are biochemically “smaller” than those in GTB and are thus able to accommodate the larger UDP-GalNAc donor.<sup>25</sup> Evidence to support the latter includes crystallography studies, where Leu<sup>266</sup> in GTA directly interacts with the GalNAc acetoamido group, whereas no binding or interaction is observed with Met<sup>266</sup>.<sup>25</sup> The adjacent Gly268Ala polymorphism is also believed to be critical for UDP-donor preference,<sup>21</sup> however, this has been challenged. Crystallographic data show both Ala<sup>268</sup> and Gly<sup>268</sup> interacting with galactose hydroxyl groups of the H-antigen acceptor regardless of UDP-donor specificity.<sup>25</sup> Other amino acids interacting with the H-antigen acceptor include Tyr<sup>126</sup>, Glu<sup>303</sup>, and amino acids 233–245, which includes the Gly235Ser polymorphism. The DxD motif near the nucleotide binding pocket binds Mn<sup>2+</sup>, which helps coordinate the UDP-phosphate group.<sup>25</sup>

Mutations in *ABO* are responsible for weak ABO phenotypes. To date, over 100 *ABO* weak alleles have been identified.<sup>20</sup> Most weak alleles possess missense mutations affecting enzyme activity; however, mutations affecting transcription, mRNA processing, translation, and Golgi trafficking are also known.<sup>20,27–30</sup> The most common mutant allele is *ABO'A2.01* associated with the A<sub>2</sub> phenotype.<sup>20</sup> *ABO'A2.01* has a single nucleotide deletion (1061ΔC) near the carboxy terminus that leads to a frameshift and translation of an additional 20 amino acids. Some alleles contain mutations within or proximal to the DxD motif (Val212Met, Met214Arg, and Phe216Ile) and highly conserved Glu<sup>303</sup> (Asp302Gly). A few alleles contain mutations affecting amino acids directly interacting with the H-antigen acceptor (amino acids 233–245: Arg241Trp), including mutations at amino acid 268 (*ABO'AW.08*; Table 9.3). *ABO'AW.08* is a common cause of ABO discrepancies due to extremely weak GTA activity.<sup>31</sup>

The ABO system also contains hybrid *ABO* alleles able to synthesize both A and B antigens. The *cisAB* phenotype was initially identified following apparent anomalous inheritance of both A and B antigens. These individuals typically type as A<sub>2</sub>B with unusually weak B expression, often accompanied by an allo-anti-B.<sup>32</sup> For many years, it was assumed that *cisAB* was the result of unequal crossover events, leading to a hybrid A–B gene. It is now clear that *cisAB* is a consequence of polymorphisms at or near key amino acids (234, 235, 266, and 268) necessary for UDP-donor and H-antigen acceptor binding.<sup>20,21</sup> As shown in Table 9.3, *cisAB* alleles share features of both GTA and GTB enzymes. Similar features are observed in *ABO'BA* alleles responsible for the B(A) phenotype. The B(A) phenotype is observed in rare group B individuals who have small amounts of A antigen when tested with certain anti-A monoclonal antibodies.

Group O, on the other hand, is an autosomal-recessive phenotype due to inheritance of amorph *ABO* alleles. There are several group *ABO'O* alleles documented, but most can be classified as belonging to either the O<sup>1</sup> (*ABO'O.01*) or O<sup>2</sup> (*ABO'O.02, O03*) families (Table 9.3).<sup>20</sup> O<sup>1</sup> accounts for 95% of group O alleles and is characterized by a nucleotide deletion (261ΔG, fs88stop118), leading to a nonfunctional truncated protein. O<sup>2</sup> alleles share a missense mutation at amino acid 268 (Arg<sup>268</sup>), which profoundly impacts enzyme activity. As noted, this same mutation is present in two weak A and B alleles. In general, heterozygous inheritance of an *ABO'O* allele (ex. A<sup>1</sup>/O) does not impact expression on red cells but may be associated with weaker ABO expression on other tissues.<sup>33</sup>

Given the complexity and number of allelic variants, molecular typing is not used for routine donor or recipient ABO typing. Molecular testing is used, however, in genome-wide association (GWA) testing. For these studies, 3–4 single nucleotide polymorphisms (SNPs) are used as a surrogate for ABO typing (Table 9.3). The most common SNPs cover the three amino acid polymorphisms that distinguish GTA from GTB (235, 266 and 268) plus the O<sup>1</sup> allele (261ΔG). Any O<sup>2</sup> alleles should also be identified using this scheme. SNP rs8176704 is sometimes added to screen for the common A<sub>2</sub> allele.

Finally, *ABO* mRNA expression shows evidence of tissue-specific regulation.<sup>34</sup> Like many genes, the *ABO* 5' UTR is CpG rich (82%) and is highly susceptible to transcriptional regulation by methylation.<sup>35</sup> In fact, the loss of A/B expression in leukemia patients is frequently attributed to hypermethylation of the *ABO* promoter region.<sup>36</sup> Methylation patterns are heterogeneous between different tissues; however, the methylation of the core proximal promoter region (-117 to +31) strongly suppresses *ABO* transcription in both epithelial and erythroid cell lines.<sup>34,35</sup> A second CpG island is located over intron 6 and exon 7.<sup>34</sup>

The CpG-rich, proximal promoter contains sites for SP1, which is required for transcription, as well as other transcription factors.<sup>34,37</sup> Mutations affecting the Sp1 site lead to decreased transcription in all cell lines.<sup>34</sup> In erythroid cells, transcription is significantly enhanced by GATA and RUNX1 sites located within intron 1 (+5.8 kb site).<sup>30,34</sup> The weak B<sup>m</sup> phenotype has been associated with mutations within this 5.8 kb site. Likewise, RUNX1 mutations may lead to weakened ABO expression in myeloid leukemia patients.<sup>34</sup> An N-box containing a Hes-1 site is adjacent to the proximal promoter region and is reported to repress ABO expression.<sup>38</sup>

Binding by Sp1 and RUNX1 appears necessary for transcription in erythroid cells but not in other tissues. In epithelial cells, ABO transcription is dependent on Sp1 and ELF5, which is located downstream of exon 7 (+22.6 kb site).<sup>34</sup> This can lead to discrepant expression of ABO on red cells and secreted molecules. For example, the B<sup>m</sup> phenotype has extremely weak B expression on red cells but normal levels of B antigen in secretions. Finally, a CBF/NF-Y minisatellite enhancer region composed of 1–4 copies of a 43-bp sequence is located nearly 4 kb upstream of the translation start site (-3650 to -3931).<sup>39</sup> In some tissues, there is a correlation between the number of tandem repeats, ABO type, and transcriptional activity.<sup>29,39</sup> There is conflicting evidence as to whether this enhancer region plays a role in red cell ABO expression.<sup>34</sup>

## H gene

The *H* gene or *FUT1* resides on chromosome 19q13 in conjunction with the *Secretor* gene and *Sec1*, a pseudogene.<sup>20</sup> *FUT1* contains four exons with the active enzyme located within exon 4. *FUT1* is highly specific for type 2 chain polylactosamine substrates and is solely responsible for H antigen synthesis on red cells.<sup>40</sup> In hematopoietic lines, *FUT1* is expressed by stem cells and erythroid-megakaryocytic progenitors but is classically absent from lymphoid and myeloid cells.<sup>3,41</sup> *FUT1* mRNA is commonly expressed in epithelial tissues and other tissues.<sup>42</sup> *FUT1* is not expressed by salivary and parotid glands, which exclusively utilize *FUT2/Secretor*.<sup>42</sup> This is the basis for testing saliva for *secretor status*.

The H enzyme is a 365-amino-acid glycoprotein composed primarily of a large 240-amino-acid catalytic domain containing two conserved N-glycans, two cysteines, and three α-fucosyltransferase motifs.<sup>40,43</sup> Motif I resides at amino acids 214–224 and is likely involved in recognition of the GDP-fucose donor. Motifs II and III

may be involved in the recognition of the galactose acceptor and are located at 256–269 and 309–318, respectively. The enzyme shares 80% homology with FUT2/Secretor, especially along the catalytic domain.<sup>44</sup>

Loss of FUT1 activity and ABH expression is associated with the Bombay and *para*-Bombay phenotypes.<sup>20</sup> The International Society for Blood Transfusion (ISBT) currently lists 25 null alleles (*h*) and 34 weak-H alleles (*H<sup>w</sup>*) due to missense, frameshift, and nonsense mutations. Several mutations occur at conserved fucosyltransferase and N-glycan motifs or introduce new cysteine residues with the potential for aberrant folding. Nearly all missense mutations involve amino acids conserved between *FUT1* and *FUT2*.<sup>44</sup> In several instances, mutations in the same homologous amino acids in *FUT2* are associated with weak and null *FUT2* alleles.

Although widely expressed, *FUT1* is transcriptionally regulated with evidence of tissue-specific promoters and alternate splicing. In erythroid cell lines, up to four different mRNA transcripts can be identified from two different, developmentally regulated transcription initiation start sites.<sup>3,41,45</sup> In undifferentiated K562 cells, an early pluripotent hematopoietic cell line, *FUT1*, is transcribed primarily from exon 1A. In bone marrow and more differentiated erythробlastic cell lines (HEL, TF1), however, transcription proceeds from exon 2. Several transcription factor binding sites are located immediately upstream of exon 2 (AP-2, Sp1, Ets-1, c-Rel, and Elk), including a retroviral long-terminal repeat sequence that lies within the basal promoter region.<sup>45</sup> Retroviral-type sequences have been shown to regulate other glycosyltransferases and may be susceptible to methylation.<sup>46</sup>

*FUT1* transcription has also been studied in other cell lines and cancer. In general, *FUT1* transcription occurs from exon 1A in epithelial cells, whereas in vascular endothelium transcription starts at exon 3.<sup>45</sup> In colon carcinoma, there is the upregulation of *FUT1* mRNA and Le<sup>y</sup> expression due to Elk-1, which promotes transcription from the E1A start site.<sup>47</sup> In ovarian cancer lines, *FUT1* is upregulated by c-jun, a member of the AP-1 transcription factor activator protein complex.<sup>48</sup>

## Transfusion and transplantation

### Transfusion

Anti-A, anti-B, and anti-A,B are clinically significant IgM antibodies, capable of fixing complement and causing intravascular hemolysis.<sup>11</sup> In contrast, anti-H is typically a low-titer, clinically insignificant cold autoantibody in most individuals. On occasion, group A<sub>1</sub> and B individuals possess an allo-anti-HI that can react with group O red cells at 37 °C. In contrast, the allo-anti-H observed in Bombay and *para*-Bombay individuals is always significant, and requires the transfusion of rare H-negative red cells. In cold autoimmune hemolytic anemia, autoantibodies with I and ABH activity are not uncommon (e.g., auto-HI and auto-sialyl-A).

For transfusion, red cells and plasma must be ABO compatible with the recipient to avoid acute hemolytic transfusion reactions.<sup>11</sup> Patients whose ABO type is unknown or cannot be resolved due to typing difficulties should receive group O red cells. In emergent situations requiring plasma transfusion support, patients without a valid ABO type should receive group AB or group A plasma. Due to the scarcity of group AB plasma, thawed group A plasma is increasingly used as the initial plasma replacement in the military and large trauma centers, with no discernable increase in adverse events.<sup>49</sup>

ABO compatibility is not required for platelet and cryoprecipitate transfusion, although most transfusion services attempt to dispense ABO type-specific and ABO-compatible components, if

possible. Transfusion of ABO-incompatible platelets can adversely affect the posttransfusion increment, especially in group O recipients.<sup>50</sup> Two studies have shown that A<sub>2</sub> platelets are group O compatible and are as effective as type-specific platelets when transfused to either group A or O patients.<sup>51,52</sup>

### Hemolytic disease of the fetus and newborn (HDFN)

ABO major incompatibility between the mother and fetus is not uncommon given the incidence of blood groups O and A in the population. Severe HDFN due to ABO incompatibility, however, is relatively rare, with a reported incidence of 0.04%.<sup>53</sup> One reason is the inability of IgM antibodies to cross the placenta. In addition, A/B antigens are poorly expressed on fetal red cells and may not efficiently support complement fixation by maternal immunoglobulin G (IgG) antibodies. HDFN due to ABO incompatibility is most commonly observed in group O mothers, especially with a history of a prior, non-O pregnancy and immune-stimulated anti-A/B IgG antibodies.

### Transplantation

#### Solid organ transplantation

In general, organs selected for allotransplantation are ABO compatible with the recipient's isoagglutinin profile. Transplants of ABO-incompatible organs are at risk for acute and hyperacute rejection due to immune recognition of A/B antigens on endothelium and epithelial tissues of the transplanted organ. To improve the availability of organs, group A organ donors are now screened to identify non-A<sub>1</sub> (A<sub>2</sub>, A<sub>weak</sub>) donors.<sup>54</sup> Because of the relative absence of A-antigen on endothelium and epithelium, these organs can be transplanted successfully into group O and B recipients.<sup>54</sup>

Protocols have been developed to permit transplantation of other types of ABO incompatible organs in selected patient populations. Due to their naive immune state, infants and neonates can be transplanted with ABO incompatible hearts with good outcomes, minimal risk of acute rejection, and a decreased incidence of HLA antibodies.<sup>55</sup> Unlike adults, neonates lack preformed ABO antibodies and memory B cells, eliminating the need for splenectomy and aggressive immunosuppression. Over time, infants who received ABO-incompatible transplant appear to develop a broad donor-specific tolerance to ABO and other antigens.<sup>55</sup> Likewise, there is evidence of equivalent outcomes for ABO-incompatible liver transplantation in infants and young pediatric patients.<sup>56</sup>

Unlike infants, ABO incompatibility is a significant barrier in adult transplantation. Several protocols have been developed for ABO-incompatible kidney transplantation. Recipients often undergo a series of presurgery plasma exchanges, coupled with immune suppression that may include steroids, ATG, rituximab, basiliximab, daclizumab, eculizimab, or alemtuzumab.<sup>57</sup> Nonetheless, ABO-incompatible kidney transplants are associated with a higher rate of postoperative bleeding, infection, graft loss and death.<sup>58</sup> ABO-incompatible liver transplantation is usually reserved for emergent situations in which an ABO-compatible organ is unavailable. Historically, ABO-incompatible liver transplants were associated with high rates (50%) of graft failure and retransplantation. More recent studies using aggressive immunosuppression have reported improved graft survival, although the risks of venous thrombosis and graft loss are still higher than with ABO-compatible transplants.<sup>54,56</sup>

#### Stem cell transplantation

Unlike solid organ transplantation, human progenitor or stem cell transplants are frequently ABO incompatible. It is estimated that 30–50% of all allotransplants are ABO mismatched.<sup>59</sup> ABO

incompatibility has no impact on white cell engraftment but can lead to hemolysis, delayed red cell engraftment, pure red cell aplasia, and increased transfusion requirements.<sup>59</sup> There are also reports linking ABO mismatched transplants with an increased incidence of venous occlusive disease, severe graft-versus-host disease, and transplant-related mortality.

Several variables influence the impact of ABO incompatibility in stem cell transplantation, including the type of stem cell product, whether the donor is ABO major or minor incompatible, the conditioning regimen (myeloablative versus reduced conditioning), and the type of post-transplant immunosuppression.<sup>59,60</sup> In general, ABO major-incompatible marrow transplants have more significant delays in red cell engraftment and pure red cell aplasia than peripheral blood stem cell transplants.<sup>60</sup> Loss of recipient ABO antibodies and donor red cell engraftment is often faster in matched unrelated donors, especially in the presence of graft-versus-host disease and increased transfusion requirements.<sup>60</sup> It is hypothesized that the loss of ABO antibodies may reflect a graft-versus-plasma cell effect as well as the impact of passive infusion of soluble ABH antigens, which could both adsorb recipient ABO antibodies and promote selective B-cell depletion.<sup>15,60,61</sup> Finally, ABO minor-incompatible transplants may be complicated by hemolysis due to donor lymphocyte syndrome.<sup>59</sup>

## Biological role

### ABO, cancer, and possible biological role

The biological role of ABH antigens is still unclear; however, the influence of ABH antigens in cancer biology may provide some insight. It is well known that the loss of A and B expression in lung, esophageal, and bladder cancers is associated with a poorer prognosis.<sup>62–65</sup> In immortalized colon carcinoma cell lines, the absence of A or B is associated with increased chemotactic and haptotactic cell migration in vitro, which can be blocked by transfecting cells with GTA or GTB transferases.<sup>66</sup> Because integrins are sensitive to changes in glycosylation, it is hypothesized that A/B antigens on  $\alpha$ - and  $\beta$ -integrins sterically interfere with heterodimer formation, integrin-mediated adhesion, and/or cell signaling.<sup>66,67</sup> In addition, ABH is a terminal glycan modification and competes with sialyltransferase for reactive nonreducing  $\beta$ -galactosyl residues on glycoproteins. As a result, ABH has the potential to modulate cellular interactions with galactins, siglecs (sialic acid-binding lectin glycoproteins), cadherins, and growth factors.<sup>68</sup>

ABH may also influence cell sensitivity to apoptosis and immune regulation. Increases in H-antigen and  $\alpha$ 1,2-fucosyltransferase activity are associated with decreased overall survival in colon cancer.<sup>69</sup> Furthermore, the induction of H-activity can increase resistance to apoptosis by heat shock and serum deprivation.<sup>65</sup> Finally, H-antigen neoexpression may make tumors less antigenic, allowing tumors to escape immune surveillance.<sup>65</sup>

There are associations between specific blood types and the incidence of cancer. The incidence of gastric and pancreatic cancer is higher among group A individuals, where A > B, AB > O.<sup>70,71</sup> When examined by A subtypes, the risk of pancreatic cancer is higher among A<sub>1</sub> individuals whereas no increase is observed for A<sub>2</sub>.<sup>71</sup> In lung cancer, blood group O has a higher mortality.<sup>62</sup> There is some evidence that many of these associations reflect differences in the upregulation of inflammatory mediators.<sup>62,69</sup>

### ABO and coagulation

There is a strong association between non-group O blood types and thrombosis risk. Two recent systematic reviews and meta-analysis

calculated that non-group O individuals have a twofold higher risk of thrombotic events than group O people.<sup>72</sup> In European studies, non-group O was the single most important population risk factor for venous thrombosis, including both deep venous thrombosis and pulmonary embolism. Non-group O blood types also have an increased risk of coronary heart disease, increased LDL, and total cholesterol.<sup>72</sup>

The observed linkage between ABO and thrombotic events appears to be multifactorial. GWA studies have found associations between ABO and serum levels of ICAM1, TNF $\alpha$ , P-selectin, E-selectin, LDL, and cholesterol levels.<sup>72</sup> More importantly, there is a clear correlation between vWF levels and ABO type, where AB > A, B > A<sub>2</sub> > O > O<sub>h</sub> (Bombay).<sup>72</sup> The lower vWF levels in group O individuals reflect accelerated vWF clearance relative to non-O individuals. Group O vWF is also more susceptible to proteolysis by ADAMTS13. In addition, there is evidence of accelerated vWF uptake by macrophages in those with blood group O.

## ABO and malaria

ABO is considered a major host susceptibility factor in malaria.<sup>73,74</sup> In fact, there is evidence that selection pressure by *Plasmodium falciparum* may underlie the high prevalence of blood group O in malaria-endemic populations located along the equator.<sup>73</sup> Why blood group O provides a survival and reproductive advantage against malaria is multifactorial and a subject of ongoing intensive investigation. Studies in Africa suggest that blood group O may protect against placental malaria through higher fetal birth weights and hemoglobin levels.<sup>74</sup> One critical mechanism appears to be protection against red cell resetting by some *P. falciparum* species.<sup>75</sup> The ability to rosette red cells is a major virulence factor associated with severe malaria, microvascular ischemia, thrombosis, and cerebral malaria.<sup>73–75</sup> The increased thrombotic risk in non-group O may also contribute to excess morbidity and mortality in malaria-endemic regions (see above). Finally, group O may provide an advantage in parasite clearance. Infected group O red cells appear more susceptible to oxidative damage as evidenced by the carbonylation of cytoskeletal proteins, hemichrome formation, and Band 3 clustering.<sup>76,77</sup> These senescent changes may facilitate phagocytosis and clearance of infected red cells, thereby decreasing the level and severity of parasitemia.<sup>78</sup>

## ABO, cholera, and other enteric infections

Group O is a host risk factor for the development of severe cholera by *Vibrio* strains carrying the El Tor and Bengal cholera toxin subtypes.<sup>74</sup> These two strains have been shown to bind difucosylated H antigen (Le<sup>Y</sup>) but not ABH-modified structures (BLE<sup>Y</sup>).<sup>79</sup> It is hypothesized that Le<sup>Y</sup> captures and concentrates cholera toxin, bringing it within close proximity to ganglioside GM1, the physiologic toxin receptor on small intestine epithelium. The result is increased cellular toxicity and secretory diarrhea in group O individuals. ABO has no impact on classical O1 cholera toxin infections.

Several other enteric pathogens also recognize ABH epitopes on intestinal epithelium. *Campylobacter jejuni* recognizes type 2 chain H and Le<sup>Y</sup> antigens on jejunal epithelium.<sup>80</sup> Norovirus and rotavirus, two highly infectious nonenveloped viruses, recognize type 1 ABH and/or Lewis antigens in the upper small intestine.<sup>73</sup> Norovirus G1 strains prefer type 1 H antigens, whereas GII strains are more promiscuous, recognizing a host of fucosylated structures.<sup>74,81</sup> Individuals lacking type 1 antigens (nonsecretors) are resistant to most norovirus infections.<sup>82,83</sup> Most human rotavirus strains (P4, P6, and P8)

also recognize type 1 H and Le<sup>b</sup>;<sup>84</sup> however, a few strains belonging to the PIII family (P9, P14, P25) have evolved to recognize the GalNAc $\alpha$ 1 → 3 Gal epitope present on group A antigen.<sup>85</sup>

### ABO and respiratory infections

ABO type may affect the course of COVID-19 (SARS-CoV-2 infection). Several large GWAS studies have identified the ABO locus as a host risk factor.<sup>86</sup> Although clinical studies have specifically linked group A with increased susceptibility, other studies have shown little or no correlation between ABO and COVID-19.<sup>87</sup> The primary receptor for COVID-19 is the ACE2 receptor; however, there is evidence that the viral spike protein also has lectin properties that might be important for host cell invasion.<sup>88</sup> Using recombinant COVID-19 spike protein, the Stowell laboratory showed a clear preference of spike protein for binding type 1 chain A antigen.<sup>88</sup> In addition, the COVID-19 spike protein is heavily glycosylated and able to express host ABO antigens, and ABO antibodies may protect against infection (see below).<sup>87,89</sup> The course of COVID-19 disease may also be impacted by the known association between ABO and thrombosis (see above).

### ABH antibodies and innate immunity

ABH antibodies, particularly anti-B, may play a role in the innate host defense against bacterial infection. As discussed in this chapter, ABH-like glycoconjugates are found on the LPS of many enteric bacteria.<sup>12</sup> In animal and in vitro studies, anti-B have been shown to bind bacteria with complement activation and increased phagocytosis.<sup>74</sup> Clinically, the absence of anti-B in group B and AB individuals has been linked to increased incidence of infection by *Escherichia coli* and *Salmonella*.<sup>90,91</sup>

ABH antibodies are also implicated in the susceptibility to infection by enveloped viruses and schistosomiasis.<sup>74</sup> HIV, SARS-CoV-1, and COVID-19 (SARS-CoV-2) viruses express host ABH antigens on viral glycoproteins and the lipid envelope.<sup>88,89,92,93</sup> Infection by HIV and SARS-CoV-1 can be blocked by monoclonal and/or human ABH antibodies in vitro, suggesting that ABO incompatibility at the time of primary viral exposure could offer some protection.<sup>92,93</sup> This was observed during a SARS-CoV-1 virus outbreak involving a single index patient in China.<sup>94</sup> Most infections (68%) occurred among non-group O health-care workers, whereas group O individuals were relatively resistant (OR = 0.18).

Due to widespread community spread during the Covid-19 pandemic, there is no clear data that ABO antibodies were beneficial in blocking infection or preventing severe disease in exposed individuals.<sup>87,89,95</sup> However, more recent data suggest that anti-Tn antibodies, a naturally occurring antibody directed against terminal GalNAc, may play a role. Anti-Tn antibodies were significantly

lower in COVID-19 patients, with the lowest levels observed in group A patients.<sup>96</sup> The Tn antigen is widely expressed on tracheal and bronchial epithelium regardless of ABO type.

Finally, group B and O individuals may have an increased resistance to schistosoma infections due to anti-A.<sup>74</sup> Anti-A is hypothesized to bind and block GalNAc epitopes on female flukes necessary for fluke chemoattraction and mating.<sup>97</sup>

### Lewis blood group

The Lewis blood group system was discovered shortly after World War II (1946–1948) and currently contains six antigens.<sup>20</sup> Serologically, Lewis status is defined by the expression of two main antigens, Le<sup>a</sup> (LE1) and Le<sup>b</sup> (LE2). Four additional antigens—Le<sup>ab</sup> (LE3), Le<sup>bH</sup> (LE4), ALe<sup>b</sup> (LE5), and BLe<sup>b</sup> (LE6)—reflect the biosynthetic and immunologic interaction of Le<sup>a</sup>, Le<sup>b</sup>, and ABO antigens. Unlike ABH, Lewis antigens are not of erythroid origin but are passively adsorbed onto red cells from a pool of Lewis-active GSL present in plasma. Adsorbed Lewis antigen is also found on platelets, lymphocytes, and endothelium. Cells synthesizing Lewis antigens are typically of endodermal origin and include gastrointestinal, urogenital, and respiratory epithelium. Soluble Lewis antigen can be found in plasma, saliva, breast milk, and urine.

### Serology

There are four possible Lewis phenotypes, although only three are commonly encountered in adults (Table 9.4): Le(a+b−), Le(a+b+), and Le(a−b−). The Le(a+b+) is unusual but can be observed in neonates and up to 20% of Japanese, Chinese, and Polynesians.<sup>20</sup> There are racial and geographic differences in the distribution of Lewis phenotypes and alleles. Because Lewis antigens are adsorbed and not synthesized on red cells, Lewis phenotype is sensitive to changes in red cell turnover and plasma volume.<sup>32</sup> Lewis antigens can be transiently decreased in chronic hemolysis. Pregnancy, cirrhosis, and chronic renal failure can lead to decreased Lewis expression due to an expanded blood volume and changes in circulating lipid concentration.

### Synthesis and biochemistry

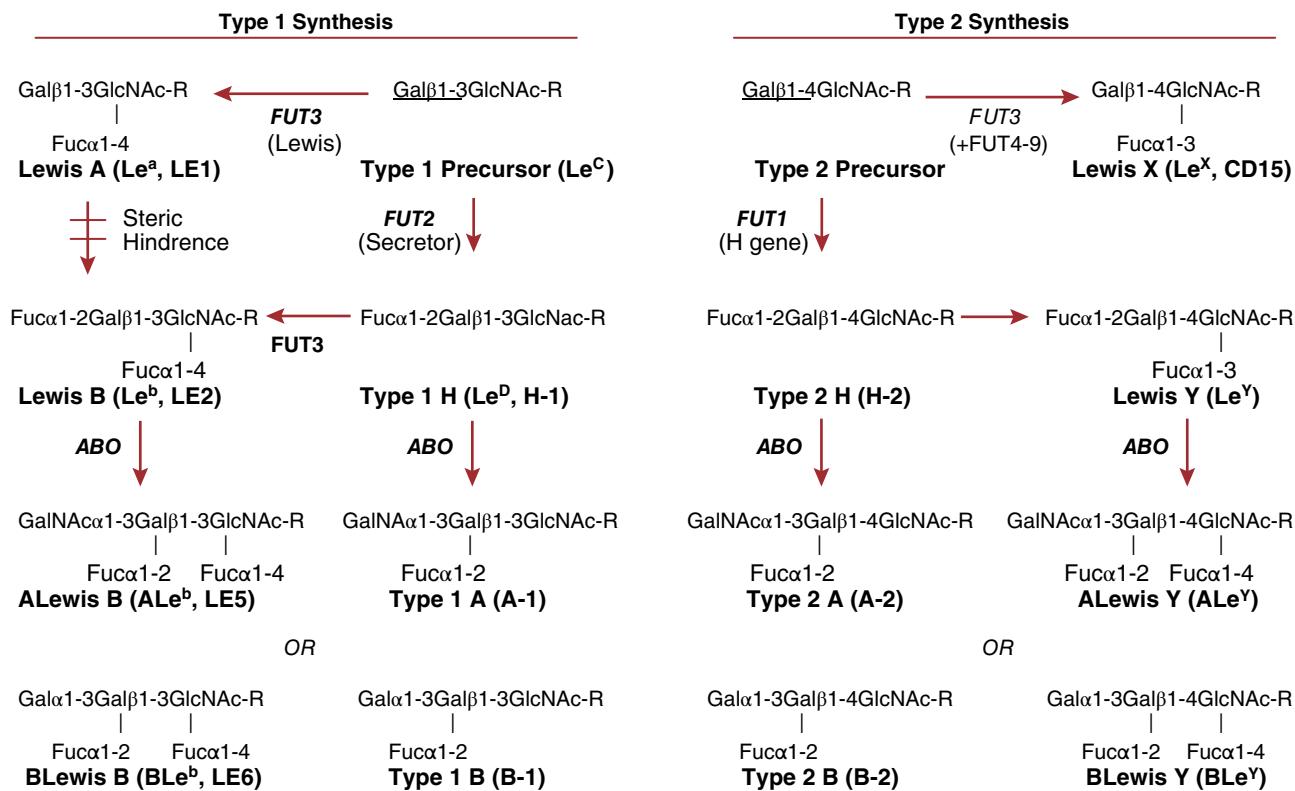
Le<sup>a</sup> and Le<sup>b</sup> are fucosylated type 1 chain antigens and reflect the interaction of two autosomal-dominant fucosyltransferases: Lewis (FUT3) and Secretor (FUT2). As discussed earlier in this chapter, FUT2 is an H-type  $\alpha$ 1,2-fucosyltransferase-specific for type 1 chain substrates, whereas FUT3 is considered an  $\alpha$ 1,3/1,4-fucosyltransferase, able to utilize both type 1 and type 2 chain substrates. As a result, FUT3 can synthesize type I Lewis antigens as well as type II chain Le<sup>X</sup> and Le<sup>Y</sup> antigens (Figure 9.2). This is

**Table 9.4** Lewis Serology and Genetics

Lewis Type	Incidence		Genes*			Secreted Antigens†		
	White	Black	FUT2	FUT3	ABH Secretor†	Le <sup>a</sup>	Le <sup>b</sup>	ABO
Le (a+b−)	22%	23%	se/se	Le	N	+	0	0
Le (a+b+)	72%	55%	Se	Le	Y	+	+	+
Le (a+b <sup>w</sup> )	Rare	Rare	Se <sup>w</sup>	Le	Y	+	↓	↓
Le (a−b−)	6%	22%	Se	le/le	Y	0	0	+
			se/se	le/le	N	0	0	0

\* Inherited at least one functional allele of FUT3/Lewis (Le) and FUT2/Secretor (Se). Lewis null and nonsecretors are homozygous for FUT3 (le/le) and FUT2 (se/se) amorph alleles, respectively.

† Presence of secreted ABH substances in saliva and body fluids.



**Figure 9.2** Biosynthetic pathways for Lewis, and type 1 and type 2 chain ABO antigens.

observed in gastrointestinal mucosa, where deep glandular epithelial stem cells express FUT3 and type 2 chain precursor with synthesis of Le<sup>x</sup> and Le<sup>y</sup>, but switch to type 1 chain synthesis upon further differentiation, with expression of Le<sup>a</sup> and Le<sup>b</sup> structures on villus epithelium.<sup>1,74</sup>

Synthesis of Le<sup>a</sup> and Le<sup>b</sup> proceeds from type 1 chain precursor, historically known as Le<sup>C</sup>, along two different pathways. Le<sup>a</sup> is a monofucosylated antigen and requires only FUT3, whereas Le<sup>b</sup>, a difucosylated antigen, requires the action of both FUT3 and FUT2 for its synthesis. To synthesize Le<sup>a</sup>, FUT3 transfers an α1 → 4 fucose to the subterminal *n*-acetylglucosamine (GlcNAc) of type 1 precursor. To synthesize Le<sup>b</sup>, type 1 precursor is initially fucosylated by FUT2 to form type 1 H (Le<sup>D</sup>), which then serves as a substrate for FUT3 to form Le<sup>b</sup>. Le<sup>b</sup> cannot be synthesized directly from Le<sup>a</sup> due to steric blocking by the subterminal fucose. Once formed, Le<sup>b</sup> can be further modified by ABO to form ALe<sup>b</sup> (LE5) and BLe<sup>b</sup> (LE6). In A<sub>1</sub> individuals, ALe<sup>b</sup> is the predominant Lewis- and A-active GSL in plasma.<sup>98</sup> Because Le<sup>b</sup> requires FUT2 for synthesis, it is accompanied by the synthesis and secretion of type 1 chain ABH antigens.

It is now clear how FUT2 and FUT3 determine Lewis phenotype (Table 9.3). The Le(a+b-) phenotype reflects inheritance of at least one functional FUT3 gene and homozygosity for FUT2-null alleles (*se/se*). As a result, Le(a+b-) individuals also fail to express type 1 chain ABH and are considered ABH “nonsecretors.” Only type 1 chain precursors (Le<sup>C</sup>) and Le<sup>a</sup> are found in saliva and secretions.

The Le(a+b+) phenotype has inherited at least one functional FUT3/Lewis and FUT2/*Se* allele with secretion of Le<sup>a</sup>, Le<sup>b</sup>, and type 1 ABH antigens. The apparent absence of Le<sup>a</sup> on red cells is deceptive because both Le<sup>a</sup> and Le<sup>b</sup> are synthesized. It appears that FUT2 outcompetes FUT3 for type 1 precursor, leading to predominantly Le<sup>b</sup> synthesis.

Decreases in FUT2 activity account for the Le(a+b+) phenotype. In neonates, it is believed that there is a developmental delay in FUT2 expression in gastrointestinal mucosa. In contrast, mutations leading to weak FUT2 activity underlie the Le(a+b+w) phenotype commonly encountered in Japanese, Chinese, and Polynesians.<sup>20</sup> Weak FUT2 activity permits FUT3 to successfully compete for type 1 precursor, tipping the balance toward Le<sup>a</sup> synthesis.

The Le(a-b-) phenotype is a FUT3/Lewis-null phenotype. Le(a-b-) may be either ABH nonsecretor or secretors, depending on the inheritance of FUT2. Le(a-b-) does not secrete Lewis-active substances.

### Molecular biology of Lewis and secretor genes

#### Lewis gene (FUT3)

FUT3 is located on chromosome 19p13.3 as a tandem cluster of three homologous glycosyltransferases: FUT5–FUT3–FUT6.<sup>20</sup> All three genes belong to the GT10 family of α1,3-fucosyltransferase, which is widely conserved in vertebrates and bacteria.<sup>43</sup> The gene consists of three exons, although only exon 3 encodes the active enzyme. The translated protein is a 361-amino-acid, type II glycoprotein with five cysteines and two N-glycan sites.<sup>99</sup> Detailed analysis indicates that the globular catalytic domain starts around residue 86 and extends through the carboxy-terminus of the enzyme. FUT3 shares two highly conserved α-fucosyltransferase motifs in the GDP-nucleotide donor (motif I, amino acid 152–171) and acceptor domains (motif II, amino acids 239–272) of the enzyme, respectively.<sup>43</sup> Motif I includes an N-glycan site and the DxD motif that interacts with divalent cations (Mn<sup>2+</sup>). The region immediately adjacent to motif I has been identified as critical for transferring fucose to type 1 substrates (amino acids 105–151).<sup>100</sup> This region contains two highly conserved cysteines (Cys<sup>81</sup>, Cys<sup>91</sup>) that are

believed to form disulfide bridges with the formation of a small loop structure necessary for enzyme activity.<sup>101</sup> An additional conserved disulfide bond pair is located toward the carboxy-terminus (Cys<sup>338</sup>, C<sup>341</sup>). The enzyme is believed to reside in the *trans*-Golgi.

*FUT3* is tissue-restricted and correlates fairly well with *FUT2* expression.<sup>42</sup> The strongest *FUT3* expression is observed in trachea, intestine, bladder, and lower female reproductive tract.<sup>42</sup> Depending on tissue, up to four different mRNA transcripts can be produced due to differential splicing.<sup>102</sup> A basal promoter in the 5' UTR that is active in intestinal mucosa was located –636 to –674 bp upstream of exon 1 and contains AP-1 and c/EBPβ transcription binding sites. The 5' UTR also contains multiple c-Myc sites that can upregulate *FUT3* and an enhancer region (–674 to –854).<sup>102,103</sup> Gene suppression can occur by methylation and a negative regulatory region located between –855 and –1220.<sup>102,104</sup>

There are nearly 50 *FUT3* null (*le*) alleles associated with a Le(a–b–) phenotype.<sup>20,105</sup> A majority of alleles (89%) have at least two mutations, and many alleles contain four or more SNPs. Several null alleles show distinct geographic and ethnic distributions. In whites and Pakistanis, alleles containing T202C (Trp68Arg) and C314T (Thr105Met) are common (*le*<sup>202,314</sup>), whereas *le*<sup>59</sup>, *le*<sup>59,508</sup>, and *le*<sup>59,1067</sup> predominate in Japan, China, Korea, Northern India, Asia, and Brazilian Amazon.<sup>74,105</sup> In African and US black populations, several additional mutations have been identified, including G13A (Gly5Ser), G484A (Asp162Asn), G667A (Gly223Arg), and A808G (Val270Met). In blacks, the most common alleles are *le*<sup>13,484,667</sup>, *le*<sup>484,667</sup>, and *le*<sup>59,308</sup>.

Some mutations have an apparently greater effect on Lewis-GSL expression, leading to Lewis-type discrepancies between saliva, tissues, and red cells. A mutation in the transmembrane domain (Leu20Arg; *le*<sup>59</sup>) is associated with marked loss in Lewis-GSL synthesis but retains Lewis expression on intestinal glycoproteins.<sup>106,107</sup> It is believed this mutation leads to mislocalization and trafficking of the enzyme within the Golgi.<sup>98</sup> *FUT3* also displays gene dosage with reduced enzyme activity in heterozygous individuals (*Le/le*).<sup>108</sup> Heterozygous individuals can be red cell type as Le (a–b–) but retain Lewis expression on gastrointestinal and other tissues.<sup>99,100</sup>

### Secretor gene (*FUT2*)

Like *FUT1*, *FUT2*/Se is a galactoside-2-α-L-fucosyltransferase, catalyzing the addition of an α1 → 2 linked fucose to a terminal galactose. Unlike *FUT1*, which is specific for type 2 chain precursors, *FUT2* recognizes type 1 chain substrate with the synthesis of type 1 chain ABH and Le<sup>b</sup> antigens. *FUT2* is primarily responsible for the synthesis of type 3 and type 4 ABH antigens, which also share a terminal Galβ1 → 3HexNAc acceptor.<sup>9,10</sup> *FUT2* resides on chromosome 19q13.3 as part of a 100-kb gene cluster that includes *FUT1* and *Sec1*, an inactive *FUT2*-like pseudogene.<sup>44</sup> The *FUT2* gene contains two exons, with exon 2 encoding the active enzyme.<sup>20,44</sup> The translated protein is 343 amino acids long and shares 68% sequence homology with *FUT1*.<sup>44</sup> It shares three motifs common to eukaryotic and prokaryotic α1,2-fucosyltransferases at amino acids 184–204 (motif I), 226–239 (motif II), and 278–288 (motif III).<sup>43</sup> The *Sec1*-*FUT2*-*FUT1* gene cluster is susceptible to mutation and genetic recombination due to the high homology of the three genes and their proximity to several *Alu* sequences, including nine *Alu* repeats in exon 1.<sup>101</sup>

*FUT2* is highly expressed in trachea, parotid, salivary gland, gastric and intestinal mucosa, uroepithelium (bladder and kidney), and female reproductive tract (vagina, cervix, and ovary). Little or no *FUT2* mRNA is observed in placenta, bone marrow, or spleen

which uses *FUT1* almost exclusively.<sup>42</sup> Intestinal *FUT2* expression can be induced by commensal bacteria that utilize fucose as a nutrient<sup>109</sup> and could theoretically account for the developmental delay in Le<sup>b</sup> expression observed in newborns. Babies slowly acquire *FUT2*-inducing bacteria through ingestion of breast milk and other foods, only achieving a normal “adult-type” intestinal microbiome by 1 year of age.<sup>110</sup>

The minimal basal promoter for *FUT2* is located at 109 to 56 and includes several GC-rich regions. Multiple transcription factor binding sites are present, including those for CDX2 (8 sites), GATA, Sp1, AP2 (5 sites), and E2A.<sup>103,111</sup> CDX2 binding appears critical for *FUT2* expression in human intestinal cell lines.<sup>103</sup> Animal studies suggest that demethylation and Sp1 may also play a role.<sup>112</sup> An SNP located at approximately 190 is associated with decreased transcription in colon cell lines and is unique to African populations.<sup>113</sup> Finally, *FUT2* mRNA has a long 3' UTR that can form a large stem-loop structure, which could affect mRNA stability and translation. It also contains a binding site for microRNA (miRNA-15b) which can downregulate *FUT2* transcription.<sup>109,114</sup>

Individuals homozygous for two *FUT2*-null alleles (*se/se*) are considered nonsecretors and express only Le<sup>a</sup> and/or type 1 precursor substance (Le<sup>C</sup>) in their secretions and tissues (Table 9.3). ISBT currently lists 27 *FUT2* weak and null alleles, many with distinct geographic and ethnic distributions ([www.isbtweb.org](http://www.isbtweb.org)). Most null alleles are the result of nonsense mutations leading to the synthesis of a truncated protein.<sup>20</sup> The most common null mutation is *se*<sup>428</sup> (Trp143stop and *FUT2*'01N.02), an ancient mutation that arose nearly three million years ago. The *se*<sup>428</sup> allele is the most frequent nonsecretor allele in whites and is common among Africans, Iranians, and Turks. As a consequence, it is frequently included in genetic studies (G428A: rs601338). Unusual *se* alleles due to *Alu*-mediated deletion and recombination have also been identified in India and Asia.<sup>20</sup> Six missense mutations involve highly conserved amino acids often within or adjacent to conserved motifs.

*FUT2* variants with weak activity (*Se<sup>W</sup>*) are common in Asia and tend to be missense mutations. In China and neighboring Asian countries, *Se*<sup>385</sup> (Ile129Phe; *FUT2*'01W.02) is the predominate *Se<sup>W</sup>* allele.<sup>115</sup> *Se<sup>W</sup>/Se<sup>W</sup>* and *Se<sup>W/se</sup>* individuals can type as Le(a+b<sup>W</sup>), Le(a+b–), or Le(a–b–). Individuals with a *Se<sup>W/se</sup>* genotype are particularly at risk for phenotyping as nonsecretors.<sup>115</sup>

### Blood transfusion and transplantation

Lewis antibodies are generally low-titer, IgM saline agglutinins and are clinically insignificant.<sup>11</sup> Lewis antibodies can display ABO reactivity, reacting stronger with group O (anti-Le<sup>bH</sup>), A (anti-ALe<sup>b</sup>), or B (anti-BLe<sup>b</sup>) red cells. With rare exceptions, they are not associated with HDFN or hemolytic transfusion reactions. There is a single small report linking a Le(a–b–) phenotype with an increased risk of rejection in kidney transplantation.<sup>116</sup>

### Biological roles

#### Cancer

Sialyl-Le<sup>a</sup> (sLe<sup>a</sup>; CA19.9) is a receptor for E-selectin, an important adhesin in cancer biology.<sup>117</sup> Epithelial tumors with high sLe<sup>a</sup> (or sLe<sup>c</sup>; CEA) expression have increased metastatic potential and a poorer clinical prognosis. Upregulation of *FUT3*, coupled with downregulation of *FUT2*, is an early step in the epithelial–mesenchymal transition during malignant transformation.<sup>103</sup> In cancer cell lines, *FUT3* silencing by siRNA will inhibit E-selectin binding and adhesion to endothelial cells, as well as reduces cellular proliferation.<sup>117</sup>

Conversely, the upregulation of *FUT2* is observed in breast, lung, and hepatocellular carcinoma.<sup>114,118–120</sup> In hepatocellular carcinoma, the downregulation of miRNA-15b, which normally suppresses *FUT2* expression, is attributed to increased *FUT2* and globo-H synthesis (see P blood group).<sup>114</sup> Finally, the synthesis of cancer markers CA19.9 and CEA is affected by genetic mutations in *FUT3* and *FUT2* genes, and should be considered when interpreting and setting normal ranges for these cancer markers.<sup>120</sup>

### Atherosclerosis

A Le(a–b–) phenotype has been linked to a twofold increased risk of atherosclerotic disease and coronary death.<sup>121</sup> Although the basis for the observation is unknown, Le(a–b–) individuals did demonstrate lower triglyceride levels, which could potentially alter plasma Lewis-GSL concentrations. Molecular typing of 1735 individuals in the Framingham offspring study subsequently showed a 57% increased risk of atherothrombotic disease in confirmed *le/le* individuals.<sup>122</sup> Of three common *FUT3* mutations studied, the *le*<sup>59</sup> mutation had the strongest association with atherosclerotic disease. A Danish study also linked the Le(a–b–) phenotype with an increased risk for stroke.<sup>123</sup>

### Necrotizing enterocolitis and other inflammatory bowel disorders

Necrotizing enterocolitis occurs in 7–10% of premature infants and carries a high mortality rate (20–30%).<sup>124</sup> It is characterized by bowel inflammation and necrosis with evidence of a disturbed intestinal microbiota. Recently, a correlation between NEC, salivary H antigen, and *FUT2* genotype was identified.<sup>125</sup> Infants with low salivary H had a significantly higher risk of death from NEC and sepsis ( $p < 0.001$ ). When examined by genotype, survival was highest among *Se/Se* homozygotes (98%) and *Se/se* heterozygotes (95%) when compared to *se/se* nonsecretors (87%).

From animal and human studies, there is evidence that fucosylation protects against infectious and inflammatory insults to the gastrointestinal tract. In adults, there is a direct spatial relationship between bacterial density and fucosylation, with highest density of bacteria and fucosylation in the colon and rectum.<sup>126,127</sup> As noted earlier, commensal bacteria that utilize fucose as a nutrient can specifically induce *FUT2* in intestinal tissue—a demonstration of a mutually beneficial symbiotic relationship.<sup>110</sup> In mice, inflammatory insults rapidly upregulate *FUT2*, leading to the shedding of fucosylated glycoproteins that promote commensal bacterial growth, while suppressing the expression of bacterial virulence genes.<sup>128</sup> In addition, fucosylation protects against tissue injury with more rapid tissue recovery.<sup>127</sup> In contrast, nonsecretor animals are susceptible to chronic colitis, diarrhea, and weight loss.<sup>128,129</sup> Not surprisingly, the nonsecretor phenotype has also been linked with Crohn's disease and is implicated in intestinal graft-versus-host disease, inflammatory bowel disorder, and altered microflora.<sup>130,131</sup> The nonsecretor phenotype, however, does not appear to alter the composition of the normal gut microbiome.<sup>132</sup>

### *Helicobacter pylori* and other infections

*H. pylori* is a flagellated, Gram-negative pathogen of the stomach associated with chronic gastritis, gastroduodenal ulcers, and gastric cancer.<sup>74</sup> A major colonization and virulence factor is BabA, a 78 kD lectin that recognizes type 1 H and Le<sup>b</sup> structures. Surveys of BabA-positive strains from around the world show that most are “generalists,” capable of binding Le<sup>b</sup>, ALe<sup>b</sup>, BLe<sup>b</sup>, and type 1 H antigen. Le<sup>b</sup>, however, is the preferred ligand in binding assays.<sup>133</sup> The ability to

recognize ABH-modified-Le<sup>b</sup> structures may explain the lack of clear correlation between ABO type and *H. pylori* infection despite older studies showing a linkage between group O and duodenal ulcers.<sup>74</sup> The correlation between *H. pylori* and secretor status is equally unclear, with some studies showing higher colonization rates and worse inflammation among nonsecretors.<sup>134,135</sup>

There is some evidence to suggest that a nonsecretor phenotype may increase the susceptibility to cholera toxin and the related enterotoxigenic *E. coli*, which secretes a cholera-like toxin. Two studies have found an increased incidence of nonsecretors among cholera patients.<sup>136,137</sup> In the largest study of 522 subjects, infected symptomatic individuals were twice as likely to be Le(a+b–) nonsecretors.<sup>137</sup> Likewise, a nonsecretor phenotype may increase the risk of *E. coli* infection in young children. Le(a+b–) children were more likely to have symptomatic infections (71% vs. 29%), especially by strains expressing the CFA I adhesin.<sup>138</sup> Both Lewis and secretor affect susceptibility to *Shigella flexneri*, where Le(a+b–) infants had the highest rates and Le(a+b+) had the lowest rates of infection, respectively.<sup>139</sup> Using the HT-29 colon cell line, the authors showed that fucosylation decreases *S. flexneri* invasion but had no effect on cell-to-cell spread.

Secretor status and fucosylation also contribute to genitourinary and respiratory tract infections.<sup>74</sup> Secretor status is protective against recurrent urinary tract infections by P-fimbriated *E. coli* (see P blood group, below). Secretor may also protect against recurrent vaginitis by *C. albicans*. Three small studies have shown an increased risk for Candida vaginitis among nonsecretors.<sup>74</sup> In animal studies, *FUT2*-null mice had significantly worse *C. albicans* infection than the wild-type *FUT2*+ controls. *C. albicans* strains can bind H-active antigens on cervical mucins, preventing the colonization of vaginal epithelial cells.<sup>140,141</sup> In the respiratory tract, several large pediatric GWAS studies have identified links between secretor and *S. pneumoniae* infection, otitis media, severe asthma, and *P. aeruginosa* bronchiectasis.<sup>142–144</sup>

Finally, secretor status is considered a host susceptibility factor in several viral infections. As noted earlier, norovirus and rotaviruses recognize type 1 ABH structures. In most outbreaks, nonsecretors are relatively resistant to infection.<sup>82,83</sup> Nonsecretor may also provide mild protection against heterosexual HIV transmission, with HIV infection rates 14–30% lower than *Se+* individuals.<sup>145,146</sup> One older study also showed a higher rate of influenza infection among *Se+*.<sup>147</sup>

### I blood group

The I blood group (ISBT 027) was initially described in 1956 in a patient with a potent cold agglutinin and episodes of profound hemolysis characteristic of cold agglutinin disease.<sup>148</sup> Serologic studies showed a panagglutinin reactive with more than 22,000 donors, family members, and several animal species. After extensive testing, five compatible donors were identified, leading investigators to designate her antibody as an anti-I in order to “emphasize the high degree of individuality” of rare I-negative (i+) donors.<sup>148</sup> Dr. Lawrence Marsh later confirmed the inverse serologic relationship between I and i antigens after identification of an anti-i. This antibody was reactive with *i<sub>adult</sub>* and cord red cells but not adult, I+ red cells.<sup>149</sup>

### I/i expression

The I antigen is a widely distributed, developmentally regulated antigen. At birth, cord red cells are I–, presumably as protection

against HDFN due to ABO incompatibility (see later). After three months of age, I antigen progressively increases, accompanied by decreased i antigen, with a normal adult-type I+I- phenotype by 18–24 months.<sup>149–151</sup> Quantitative estimates of I antigen on adult red cells can vary from 32,000 to 130,000/cell due to differences in the fine specificity of anti-I antibodies used for these studies.<sup>150</sup> Estimates of i antigen on untreated cord and adult red cells are 25,000–75,000/cord and 1000–30,000/adult cell, respectively.<sup>150</sup> The i antigen is elevated on reticulocytes and can be increased on mature red cells as a sign of stressed erythropoiesis, dyserythropoiesis, and disordered Golgi trafficking.<sup>150</sup> Conditions associated with elevated i antigen include erythroleukemia, megaloblastic anemia, chronic hemolytic disorders, and hereditary erythroblastic multinuclearity with a positive acidified serum lysis test (HEMPAS).

### I-null ( $i_{adult}$ )

An I-i+ phenotype is a characteristic of  $i_{adult}$  red cells, a rare autosomal-recessive phenotype due to mutations in the I gene (*GCNT2*, *IGnT*) with an estimated incidence of 1:4400 to 1:17,000.<sup>151,152</sup> Heterozygous family members can have an intermediate I+i+ phenotype consistent with gene dosage. Individuals with  $i_{adult}$  can make an allo-anti-I reactive with normal adult red cells.

There are two clinical  $i_{adult}$  phenotypes based on the presence or absence of congenital cataracts.<sup>152,153</sup> In non-Asians, there is an isolated deficiency of I antigen on red cells only. Conversely, many Asian  $i_{adult}$  kindreds show a global loss of I antigen on all tissues, including human lens, accompanied by congenital cataracts. In China, *GCNT2* mutations account for 4–6% of all childhood cataract cases.<sup>154</sup>

### Biochemistry

I and i antigens are biosynthetically and structurally related oligosaccharides, composed of repeating units of *n*-acetyllactosamine (LacNAc,  $[Gal\beta \rightarrow 4GlcNAc]_n$ ). The minimum i antigen epitope is defined as a linear, unbranched type 2 chain oligosaccharide bearing at least two successive LacNAc units (Figure 9.3B).<sup>155</sup> The i antigen is sensitive to the enzyme  $\beta$  endo-galactosidase.

The I antigen is a branched, polygalactosamine derivative of i antigen. The I gene (*GCNT2*, *IGnT*) encodes a  $\beta 1 \rightarrow 6$  *n*-acetylgalactosaminyltransferase that transfers a  $\beta 1 \rightarrow 6$  GlcNAc to a subterminal  $\beta 1 \rightarrow 3$  Gal.<sup>152,153</sup> Extensive studies with purified oligosaccharides indicate that the enzyme recognizes a four-sugar donor acceptor composed of two successive LacNAc units.<sup>156</sup> This is consistent with isolated erythroid glycans, which show at least two LacNAc motifs between branch points.<sup>8</sup> The enzyme can form branches on both distal and centrally placed  $\beta 1 \rightarrow 3$  Gal residues, but will not recognize LacNAc residues modified by fucose or neuraminic acid.<sup>156</sup> As a result, sialylation and fucosylation (ABH,  $Le^X$ , and  $Le^Y$ ) are regulators of I and i antigens, blocking both polygalactosamine elongation and branching. Anti-I antibodies can recognize a variety of glycans but require a  $[-GlcNAc\beta 1 \rightarrow 6 (GlcNAc\beta 1 \rightarrow 3) Gal\beta 1 -]$  as part of the immune epitope (Figure 9.3B).<sup>150,155</sup>

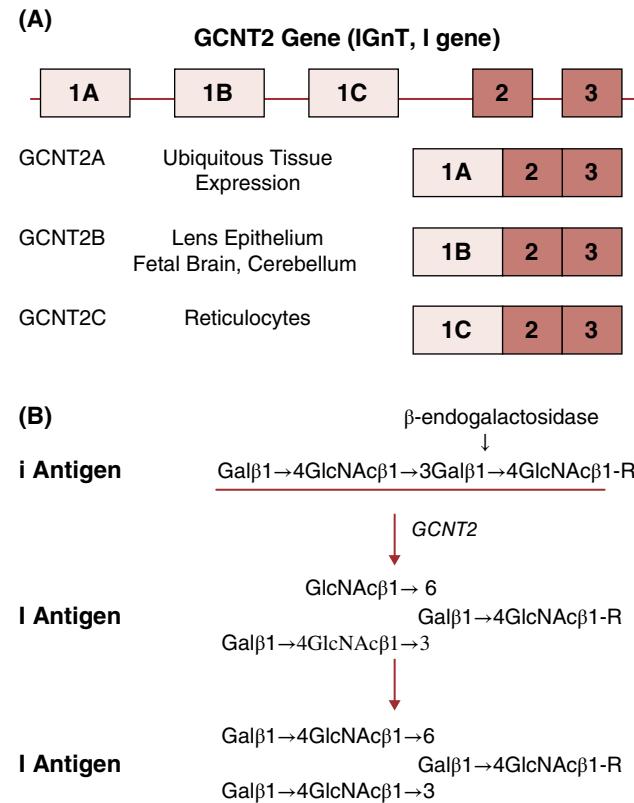
### Molecular biology and genetics

The I gene, *GCNT2* (*IGnT*), is located in an *Alu*-rich region on chromosome 6p24.2.<sup>157</sup> The gene consists of seven exons: five coding exons responsible for the active enzyme (E1-E3) and two upstream noncoding exons. *GCNT2* possesses three tissue-specific variants of exon 1 (E1A, E1B, and E1C; Figure 9.3A), resulting in three different isoforms of the enzyme depending on which exon 1 is utilized.<sup>152,153</sup> *GCNT2A* (*IGnTA* and *IGnT2*) is expressed by most

humans tissues and is derived from exon E1A. *GCNT2B* uses exon E1B and is the only mRNA transcript identified in human lens. *GCNT2C* (*IGnTC*) is an erythroid-specific enzyme that is responsible for I antigen expression on red cells. *GCNT2* is highly homologous to *C2GnT*, a  $\beta 1 \rightarrow 6$  *n*-acetylglucosaminyltransferase responsible for I-type structures on core 2 and core 3 O-glycans.<sup>158</sup>

The enzyme is 400–402 amino acids long and, unlike most glycosyltransferases, does not require a metal ion for enzyme activity.<sup>153</sup> The enzyme retains nine highly conserved cysteines necessary for enzyme folding and five N-glycans but lacks the classic DXD motif found in metal-dependent transferases. Exon 1 encodes 77% of the translated protein, including the transmembrane domain, stem region, and UDP-nucleotide binding domain (UDP-GlcNAc).<sup>150,153</sup> The acceptor domain is primarily encoded by exons E2 and E3. Due to sequence differences in exon 1, the three *GCNT2* isoforms share only 66–73% homology and subtly differ in enzyme activity. In enzyme assays, the erythroid-specific *GCNT2C* isoform shows nearly twofold higher enzyme activity.<sup>153</sup>

*GCNT2C* is transcriptionally regulated in red cells.<sup>159–161</sup> Studies have identified a promoter sequence approximately 250 base pairs (−318 to −251) upstream of the E1C translation initiation site that contains transcription factor binding sites for Oct-2, Sp1, and C/EBP $\alpha$ . All three transcription factors bind the *GCNT2C* promoter region; however, only C/EBP $\alpha$  is absolutely critical for *GCNT2C* transcription. Interestingly, C/EBP $\alpha$  binding and transcriptional activation require dephosphorylation of a specific serine (Ser-21) on C/EBP $\alpha$ , via ERK and SHP2 pathways.<sup>159,161</sup> Phosphoserine



**Figure 9.3** (A) Structure of the I gene (*GCNT2*) and three mRNA isoforms. (B) Synthesis of I antigen from i-active polygalactosamine precursor.

residues are known to regulate C/EBP $\alpha$  function in early hematopoiesis and myeloid differentiation.<sup>159</sup>

*GCNT2C* is also regulated via methylation and microRNA (miRNA) interactions.<sup>161</sup> In early erythroblasts, *GCNT2* is hypermethylated with transcriptional silencing. Upon differentiation, there is demethylation of a critical CpG within the proximal promoter that permits *GCNT2* transcription. In colon, CpG residues within exon E1B are key to *GCNT2* transcription and enzyme expression.<sup>162</sup> Finally, the miRNAs Let-7 and 199 are implicated in downregulating *GCNT2* transcription in red cells and colon, respectively.<sup>161</sup> Two miRNA-199 binding sites were identified in the 3' UTR, although only the site proximal to the poly-A tail appears to play a role in suppressing *GCNT2* expression.<sup>163</sup>

### The molecular basis for *i<sub>adult</sub>*

Mutations in *GCNT2* are responsible for the *i<sub>adult</sub>* phenotype.<sup>20,150,161</sup> Individuals with mutations in E1C, which is responsible for the erythroid-specific *GCNT2C*, show an isolated loss of I antigen expression in red cells only; I expression is retained in other tissues via *GCNT2A* and *GCNT2B*.<sup>152</sup> These individuals are not at an increased risk for congenital cataracts (Table 9.5). Three previously identified *i<sub>adult</sub>* alleles have been reclassified as causing very weak I expression.<sup>161</sup>

The *i<sub>adult</sub>* with congenital cataracts is the result of mutations affecting all three *GCNT2* isoforms, leading to a tissue-wide loss of all enzyme activity.<sup>153</sup> To date, seven-point mutations have been identified in E2 and E3, which are shared by all three enzyme isoforms. In four unrelated kindreds, large deletions (70–93 kb) encompassing most of *GCNT2* (exons E1B, E1C, E2, and E3) were identified. Because *GCNT2* resides in an *Alu*-rich region, it may be particularly susceptible to *Alu*-mediated deletion and genomic rearrangements.<sup>157</sup>

It is presumed that the absence of branched N-glycans in human lens underlies the development of congenital cataracts. This has not been substantiated in a *GCNT2*-null mouse model.<sup>164</sup> *GCNT2*-null mice had no apparent increase in either the incidence or onset of cataracts. This conundrum may reflect species-specific differences in glycan expression. Indirect evidence for the latter is the early development of cataracts in *A3GALT*-null mice, which are unable to synthesize oligosaccharides containing Gal $\alpha$ 1 → 3Gal (linear B) epitopes.<sup>165</sup>

### Transfusion

Anti-I is a common, low-titer, IgM cold agglutinin in normal human sera.<sup>150</sup> Anti-I can demonstrate significant crossreactivity with i epitopes and can show a preference for polylactosamines bearing sialic acid or ABH epitopes. Anti-i, by contrast, is less frequent. Anti-i is commonly associated with infectious mononucleosis, Burkitt's lymphoma, and cirrhosis.<sup>11,150</sup> Many examples of anti-i are sialoagglutinins with a preference for linear structures bearing a terminal sialic acid.<sup>150</sup>

An increase in anti-I/i titers and/or thermal amplitude is not uncommon following infection and in certain lymphoproliferative disorders. B cell malignancies, in particular, can secrete high-titer, monoclonal autoantibodies capable of inducing hemolysis and cold agglutinin disease. In cold agglutinin disease, monoclonal antibodies classically bear the V<sub>H</sub>4–34 IgM heavy gene, which inherently recognizes lactosamine due to the terminal FR1 domain of the molecule.<sup>166</sup> Cold agglutinin disease also displays light chain preferences with clonal rearrangements of the IGKV3-20 gene and CDR3 regions, which may contribute to fine differences in antigen specificity (anti-I vs anti-i).<sup>167,168</sup> In contrast, anti-I/i coincident with infection are polyclonal and reflect a global increase in IgM antibody.<sup>150</sup>

In general, antibodies against anti-I/i are clinically insignificant.<sup>11</sup> However, blood typing difficulties and hemolysis can be observed in patients with high-titer antibodies, particularly if there is strong reactivity above 30–32 °C. These patients may require the use of a blood warmer for transfusion. There are rare cases of hemolytic transfusion reactions with warm-acting anti-IH following transfusion of group O or A<sub>2</sub> red cells.<sup>169</sup>

### Biological roles

#### Hemolytic disease of the fetus and newborn

There is a theory that the absence of I antigen on cord red cells is protective against HDFN due to ABO incompatibility. There is a parallel increase in both I and A/B antigens in the first few months of life, consistent with increasing erythroid *GCNT2C* activity.<sup>150</sup> Supporting evidence for the key role of branching in ABH expression can be found on the bi-antennary N-glycan of Band 3/Diego. On cord cells, the N-glycan is relatively simple and unbranched, with few ABH epitopes on the long, linear α1,6 mannose arm.<sup>150,170</sup> In contrast, adult red cells display a massive N-glycan with extensive β1–6 branching along both α1,6 and α1,3 arms, allowing display of multiple ABH epitopes per N-glycan.<sup>7,150</sup>

**Table 9.5** *GCNT2* Mutations Associated with *i<sub>adult</sub>* Phenotype

Exon	Mutation		Phenotype		
	Nucleotide	Amino Acid	I RBC	<i>i<sub>adult</sub></i> RBC	Cataracts
E1C	243T > A	N81L	Weak	–	–
E1C	505G > A	A169T	Weak	–	–
E1C	683G > A	R228Q	Weak	–	–
E1C	651delA	V244X	+	–	–
E2	935G > A	G312D	+	+	–
E2	984G > A	W328X	+	+	–
E2	1006G > A	G336R	+	+	–
E3	1046A > G	Y349C	(+)	+	–
E3	1049G > A	G350E	+	+	–
E3	1154G > A	R385H	+	+	–
Deletion	D98 kb	E1A, E1B	(+)	–	–
Deletion	Δ70 kb	E1B- > E3	+	+	–
Deletion	Δ93 kb	E1B- > E3	+	+	–
Deletion	D189 kb	E1A- > E3	+	+	–

Nucleotide and amino acids are based on *GCNT2C*, which is 402 amino acids long. Note that many publications have listed mutations based on *GCNT2A*, which is 400 amino acids long. (+) is a presumed red cell phenotype based on genetic sequence.

## Galectins

Galectins are a family of highly conserved galactose binding proteins that recognize lactosamine via a 130 amino-acid-long carbohydrate binding domain.<sup>171</sup> Galectin families differ in the size and location of the lactosamine epitope (disaccharide or repeating lactosamine tetrasaccharide; terminal or internal), and their ability to tolerate branching, sialylation, and fucosylation. In general, glycoproteins bearing several large, complex, branched N-glycans with long extended polylactosamines are the preferred receptors for galectins.<sup>161,171</sup>

Galectin binding to N-glycans is believed to create an extracellular lattice structure several angstroms above the lipid membrane.<sup>171</sup> As such, galectins are integral for protein–protein interactions and the composition and function of membrane microdomains. In B-cells, Gal-9 binding to i-active glycans on CD45 have been shown to modulate CD45–CD22 interaction and B cell signaling.<sup>172</sup> Gal-3 binding to i-active glycans on melanoma contribute to tumor growth and metastasis.<sup>173</sup>

## Embryonic and hematopoietic development

I/i antigens are differentially expressed during embryonic development and are considered oncofetal antigens.<sup>150,161</sup> GCNT2 is one of the 90 proteins identified as markers of human embryonic stem cells.<sup>161</sup> In human hematopoiesis, GCNT2 mRNA is weakly expressed by the earliest pluripotent progenitors but is lost with leukocyte differentiation.<sup>161</sup> Aberrant GCNT2 expression is found in mycosis fungoides and adult T-cell leukemia/lymphoma.<sup>174,175</sup> Interestingly, lymphoid and myeloid leukemia cells that express I antigen appear more susceptible to killing by NK cells, possibly through altered interaction with death ligands.<sup>176</sup>

The i antigen was identified as a marker of human mesenchymal stem cells and might serve as a ligand for galactin-3.<sup>177</sup> Galectin-3 is abundantly expressed and secreted by macrophages and is implicated in giant cell formation, dendritic cells, fibrosis, and chronic inflammation.<sup>178</sup> Galactin-3 is hypothesized to play a role in the immunosuppressive properties of mesenchymal stem cells. Galactin-3 also binds and directly activates human neutrophils, promoting neutrophil recruitment, adhesion, and respiratory burst.<sup>177</sup>

## Breast, prostate, and colon cancer

GCNT2 is overexpressed in highly metastatic and basal-like breast cancer cells and was recently identified as a possible disease modifier in BRCA2 breast cancer.<sup>179</sup> A GWA study identified a polymorphism (rs9348512) located on chromosome 6p24, within 1 MB of GCNT2. Homozygosity for the minor “A” allele was associated with a 15% decreased risk of developing breast cancer and correlated with GCNT2 mRNA levels. Furthermore, GCNT2 overexpression promoted breast cancer cell migration, adhesion, and invasion.<sup>173,180</sup> Similar findings have been found in prostate cancer, where high GCNT2 mRNA is linked to cell invasion and extracapsular disease.<sup>173,181</sup> In breast cancer, GCNT2 upregulation is dependent on tumor growth factor  $\beta$ /Smad signaling and may facilitate the mesenchymal–epithelial transition (MET)—an early critical step in epithelial cancers.<sup>163</sup> GCNT2 upregulation has also been linked to MET, tumor depth, and lymphatic spread in colon cancer.<sup>161,162</sup>

## Melanoma

Unlike epithelial cancers, GCNT2 is downregulated in melanoma and is a biomarker for metastatic disease.<sup>173,182</sup> A study of N-glycans on melanoma shows predominantly simple, i-active polylactosamines. Transfection of melanoma cell lines with GCNT2 led to a

decrease in cell signaling, cell activation, and tumor growth.<sup>182</sup> The mechanism behind GCNT2 downregulation is unknown. Rare cases of familial melanoma have been linked to large chromosomal deletions and GCNT2 loss.<sup>161</sup>

## P blood group system

The system historically known as the *P blood group* was initially discovered in 1927 by Landsteiner and Levine after immunizing rabbits with human red cells.<sup>183</sup> The resulting antibody recognized the P<sub>1</sub> antigen, which is variably expressed on most, but not all, human red cells. The P<sup>k</sup> and P antigens were added to the system after the discovery of rare null phenotypes. The historical P system has now expanded into three blood group systems and six serologic antigens, which are assigned based on the last glycosyltransferase necessary for their synthesis.<sup>184</sup> The P1PK system (ISBT 003) encompasses P<sup>k</sup>, P<sub>1</sub>, and NOR antigens, which all share a terminal  $\alpha 1 \rightarrow 4$ Gal epitope. GLOB (ISBT 028) contains the P and PX2 antigens, which terminate in a  $\beta 1 \rightarrow 3$ GALNAc. FORS (ISBT 031) contains a single antigen, Forssman—a xenoantigen expressed by many animals and rare A<sub>par</sub> red cells. *Luke antigen on erythrocytes* (LKE) is a high-incidence antigen that is still classified under the GLOB collection 209. Other related antigens include galactosygloboside, globo-H, and globo-A antigens. Globo-A is a target for anti-A<sub>1</sub>, whereas globo-H, galactosygloboside, and LKE are oncofetal antigens found on stem cells and many epithelial cells.<sup>185</sup>

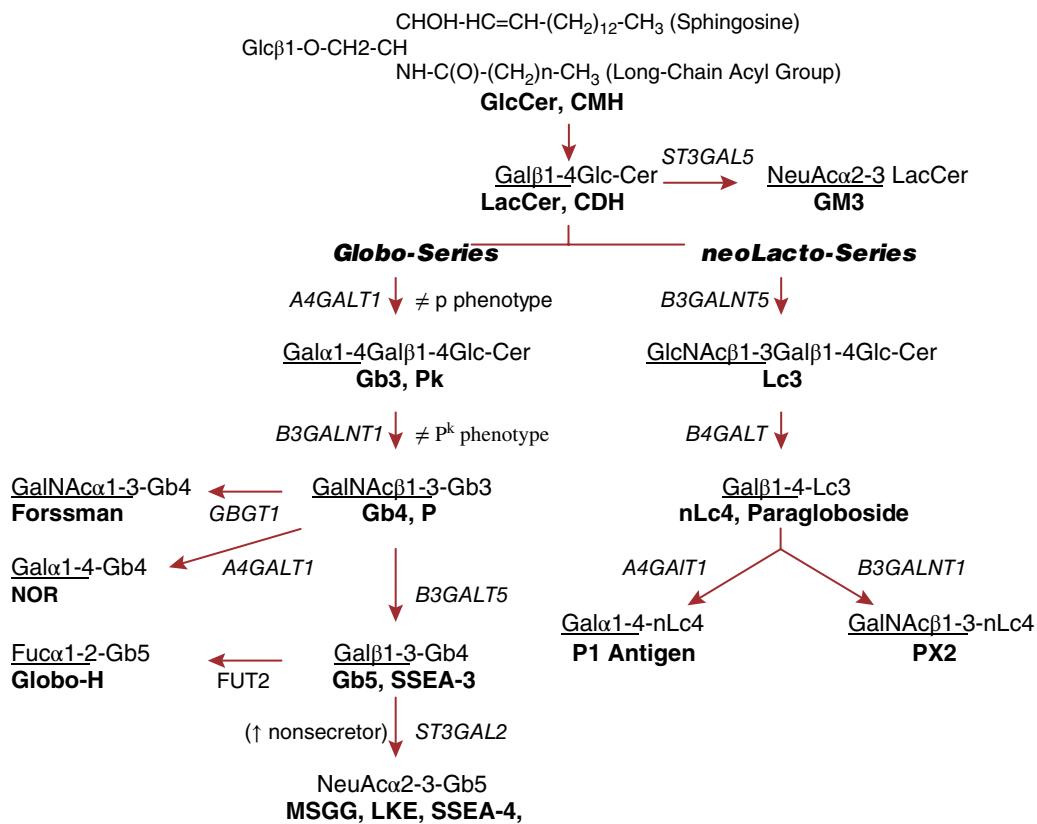
## Biochemistry and synthesis

Unlike ABH, Ii, and Lewis antigens, the antigens of the P system are almost exclusively expressed on glycosphingolipids (GSLs). Chemically, GSLs consist of a carbohydrate head covalently linked to a ceramide lipid tail (*N*-acylsphingosine), which anchors the molecule in the membrane. Most members of the P-system belong to the globoseries of GSLs and share a Gal $\alpha 1 \rightarrow 4$ Gal $\beta 1 \rightarrow 4$ Glc-Ceramide or Gb3 core (Table 9.6). Exceptions are P<sub>1</sub> and PX2, which are type 2 chain, neolacto GSLs derived from paragloboside. A trace amount of P<sub>1</sub> can also be found on red cell N-glycans.<sup>186</sup>

**Table 9.6** GSL Structures Related to Globo, P1, and PX2 Synthesis

GSL	Alias	Structure
GlcCer	CMH	Glc-Cer
LacCer	CDH	Gal $\beta 1$ -4Glc-Cer
GM3		NeuAc $\alpha 2$ -3Gal $\beta 1$ -4Glc-Cer
<b>Globo GSL</b>		
Gb3	P <sup>k</sup> , CD77	Gal $\alpha 1$ -4Gal $\beta 1$ -4Glc-Cer
Gb4	P, globoside	GalNAc $\beta 1$ -3Gal $\alpha 1$ -4Gal $\beta 1$ -4Glc-Cer
$\alpha$ Gal-Gb4	NOR1	Gal $\alpha 1$ -4GalNAc $\beta 1$ -3Gal $\alpha 1$ -4Gal $\beta 1$ -4Glc-Cer
Forssman	Forssman	GalNAc $\alpha 1$ -3GalNAc $\beta 1$ -3Gal $\alpha 1$ -4Gal $\beta 1$ -4Glc-Cer
Gb5	SSEA3	Gal $\beta 1$ -3GalNAc $\beta 1$ -3Gal $\alpha 1$ -4Gal $\beta 1$ -4Glc-Cer
Fuc-Gb5	Globo H, SSEA5	Fuc $\alpha 1$ -2Gal $\beta 1$ -3GalNAc $\beta 1$ -3Gal $\alpha 1$ -4Gal $\beta 1$ -4Glc-Cer
MSGG	LKE, SSEA4	NeuAc $\alpha 2$ -3Gal $\beta 1$ -3GalNAc $\beta 1$ -3Gal $\alpha 1$ -4Gal $\beta 1$ -4Glc-Cer
$\alpha$ Gal-Gb5	“Band 0.03”	Gal $\alpha 1$ -4Gal $\beta 1$ -3GalNAc $\beta 1$ -3Gal $\alpha 1$ -4Gal $\beta 1$ -4Glc-Cer
<b>Neolacto GSL</b>		
Lc3		GlcNAc $\beta 1$ -3Gal $\beta 1$ -4Glc-Cer
nLc4	Paragloboside	Gal $\beta 1$ -4GlcNAc $\beta 1$ -3Gal $\beta 1$ -4Glc-Cer
$\alpha$ Gal-nLc4	P1	Gal $\alpha 1$ -4Gal $\beta 1$ -4GlcNAc $\beta 1$ -3Gal $\beta 1$ -4Glc-Cer
GalNc-nLc4	PX2	GalNAc $\beta 1$ -3Gal $\beta 1$ -4GlcNAc $\beta 1$ -3Gal $\beta 1$ -4Glc-Cer
NeuAc-nLc4	SPG	NeuAc $\alpha 2$ -3Gal $\beta 1$ -4GlcNAc $\beta 1$ -3Gal $\beta 1$ -4Glc-Cer

Cer, ceramide; Fuc, fucose; Gal, galactose; GALNAc, *n*-acetylgalactosamine; Glc, glucose; GlcNAc, *n*-acetylglucosamine; NeuAc, *n*-acetylneuraminc acid.



**Figure 9.4** Synthesis of P blood group and related glycosphingolipid antigens.

Lactosylceramide (LacCer) is the precursor of both globo- and type 2 chain GSLs (Figure 9.4). Globo-family GSLs proceed from the addition of an  $\alpha 1 \rightarrow 4$ Gal to LacCer by A4GALT1 to form globotriaosylceramide (Gb3, P<sup>k</sup>), the first globo-family GSL. Gb3/P<sup>k</sup> may then serve as a substrate for B3GALNT1, which adds a terminal  $\beta 1 \rightarrow 3$ GalNAc to form globoside (Gb4, P antigen). In animals and rare A<sub>Pae</sub> cells, globoside/P antigen is further modified by the addition of an  $\alpha 1 \rightarrow 3$ GalNAc to form Forssman antigen.<sup>184</sup> The rare NOR antigen shares features of both P<sup>k</sup> and P antigens, with an  $\alpha 1 \rightarrow 4$ Gal terminus on a Gb4/P antigen core.<sup>187</sup>

Synthesis of LKE and extended globo-family antigens requires B3GALT5, which adds a terminal  $\beta 1 \rightarrow 3$ Gal to form galactosylgloboside or Gb5.<sup>185</sup> B3GALT5 is a rate-limiting enzyme for both Gb5 and type 1 chain synthesis and shows developmental and tissue-specific expression.<sup>188</sup> Once formed, Gb5 can serve as a substrate for H-active fucosyltransferases to form globo-H.<sup>9,10</sup> In A<sub>1</sub> individuals, globo-H is converted to globo-A: expression of globo-A is believed responsible for antigenic differences between A<sub>1</sub> and A<sub>2</sub> red cells.<sup>16</sup>

Gb5 can also be sialylated by the  $\alpha 2,3$ sialyltransferase ST3GAL2 to form LKE antigen (MSGG and SSEA-4).<sup>185</sup> In genitourinary tissue, there is competition between FUT2 and ST3GAL2 for Gb5, leading to decreased LKE/MSGG synthesis on epithelial cells of secretors (Se<sup>+</sup>).<sup>189</sup> In some tissues, including red cells, LKE is further sialylated to form disialogalactosylgloboside (DSGG).<sup>190</sup> In human platelets, a minor GSL with a Gb5 core and terminal  $\alpha 1 \rightarrow 4$ Gal was identified in 10–20% of donors.<sup>191</sup>

A4GALT1, the enzyme responsible for P<sup>k</sup> synthesis, is also capable of recognizing paragloboside to form P<sub>1</sub> antigen. Unlike P<sup>k</sup>, P<sub>1</sub> is a minor red cell GSL with variable expression. Paragloboside can also serve as an alternate substrate for B3GALNT1 to form PX2, a

P-active GSL recognized by some allo-anti-P.<sup>184</sup> Synthesis of PX2 is limited to rare p null phenotypes, which lack Gb3/P<sup>k</sup> (see the “Serology” section).

### Serology

**P<sub>1</sub>/P<sub>2</sub>:** The classic P system contains two main phenotypes, P<sub>1</sub> and P<sub>2</sub>. P<sub>1</sub> and P<sub>2</sub> have normal expression of globo-series antigens and only differ in the expression of P<sub>1</sub> antigen (Table 9.7). P<sub>1</sub> is the predominant phenotype in most populations and can vary in strength between individuals. The P<sub>2</sub> (P<sub>1</sub>-negative) phenotype ranges from 6% in Blacks to 75–80% in some Asian populations (China and Vietnam).<sup>20,74</sup> The P<sub>1</sub> antigen is absent on p cells (see below). P<sub>2</sub> individuals can make a naturally occurring anti-P<sub>1</sub>.

**P<sup>k</sup>:** P<sup>k</sup> is a rare autosomal-recessive phenotype due to homozygous inheritance of null B3GALNT1 alleles. P<sup>k</sup> can occur on either a P<sub>1</sub> or P<sub>2</sub> background, giving rise to P<sub>1</sub><sup>k</sup> or P<sub>2</sub><sup>k</sup> phenotype, respectively (Table 9.7). P<sup>k</sup> red cells lack Gb4/P and all subsequent globo-series GSLs (LKE, globo-H), accompanied by increased expression of P<sup>k</sup>/Gb3, LacCer, and type 2 chain GSLs (paragloboside and sialylparagloboside).<sup>184,192</sup> P<sup>k</sup> variants, with increased P<sup>k</sup>/Gb3 and decreased Gb4/P, have been described and may reflect decreased B3GALNT1 activity.<sup>192</sup> P<sup>k</sup> individuals make a potent, clinically significant allo-anti-P that is reactive with all red cells except rare P<sup>k</sup> and p cells. Anti-P can contain reactivity against PX2 as well as other globo-GSLs (Gb5 and LKE).<sup>184,192</sup>

**p null:** The p phenotype is a true globo-null phenotype due to homozygosity for amorph A4GALT1 alleles.<sup>184</sup> Red cells and other tissues lack P<sub>1</sub> and all globo-GSL (Figure 9.4). Red cells show a compensatory increase in type 2 chain GSLs, including paragloboside and PX2. Heterozygous individuals (A4GALT1+/-) show normal

**Table 9.7** P Blood Group Serology

Type	Incidence	GT Genes*		Antigens on RBC†						Antibodies
		A4GALT1	B3GALNT1	P <sup>k</sup>	P	LKE	P <sub>1</sub>	Other		
P <sub>1</sub>	80% <sup>‡</sup>	+	+	+	+	+	+			
P <sub>2</sub>	20%	+	+	+	+	+	0			
LKE <sup>S</sup>	80–90%	+	+	+	+	+	+/0			
LKE <sup>W</sup>	10–20%	+	+	+	+	↓	+/0			
LKE <sup>N</sup>	1–2%	+	+	+	+	tr <sup>§</sup>	+/0			
p <sup>k</sup>	Rare	+	0	+	0	0	+			
p <sup>k</sup>	Rare	+	0	+	0	0	0			
p	Rare	0	+	0	0	0	0	PX2		
NOR	Rare	+	+	+	+	na	+/0	NOR		
Fors	Rare	+	+	+	+	na	+/0	Fors		

\* Inheritance of at least one functional glycosyltransferase (GT) gene (+).

† Incidence in US whites.

‡ Trace antigen, not detected by hemagglutination.

globo-GSL expression with no evidence of gene dosage. Sera from p individuals contain a mix of naturally occurring IgM agglutinins crossreactive with P<sub>1</sub>, P, and P<sup>k</sup> cells (allo-anti-PP<sub>1</sub>P<sup>k</sup>). In solid phase testing, anti-P<sup>k</sup> and anti-P are the predominant specificities.<sup>192</sup>

**LKE:** LKE, also known as Luke, is a minor, high-incidence ganglioside on red cells. There are three recognized LKE phenotypes: LKE-strong positive (LKE<sup>S</sup>), LKE-weak positive (LKE<sup>W</sup>), and LKE-negative (LKE<sup>N</sup>). LKE<sup>N</sup> red cells often show a P<sup>k</sup> variant-like phenotype, with increased P<sup>k</sup>/Gb3 and decreased Gb4 expression.<sup>193</sup> LKE<sup>N</sup> can also occur in weak P red cells, which have marked decrease in Gb3 and Gb4 expression.<sup>192</sup> In P<sub>1</sub> and P<sub>2</sub> individuals, LKE<sup>N</sup> is associated with trace LKE/MSGG (10% normal) on red cells: true LKE-null is only observed in p and P<sup>k</sup> phenotypes.<sup>192</sup> LKE/MSGG is also known as stage-specific embryonic antigen-4 (SSEA4) and is a marker of embryonic, mesenchymal, and cancer stem cells.<sup>9,185,194</sup>

**NOR:** NOR is a rare polyagglutinable phenotype that appears to have arisen in a Polish kindred.<sup>184,187</sup> NOR red cells express unusual penta- and hepatoglycosylglobo-GSLs that bear a Galα1 → 4GalNAc terminus (Table 9.6). Normal human sera contain two classes of naturally occurring antibodies reactive with NOR, with slightly different specificities: type 1, anti-P<sup>k</sup>+NOR and type 2, anti-NOR. NOR is not recognized by anti-P<sub>1</sub>.

**Forssman:** Forssman is a very rare polyagglutinable human phenotype with weak antigen expression, even in Fors+ individuals.<sup>195</sup> Nearly all human sera contain low levels of naturally occurring anti-Forssman. Because the Forssman antigen is commonly expressed by many animal species, Forssman antibodies are major barriers to xenotransplantation.<sup>196</sup> Anti-Forssman can interfere with the monospot test, which reacts sera against sheep or horse red cells, and was used as a rapid screen for patients with suspected infectious mononucleosis. Since both Forssman and A antigen share a common terminal GalNAc, human anti-A can crossreact with Forssman antigen.<sup>197</sup>

## Genetics and molecular biology

### P1PK synthase (A4GALT1)

A4GALT1 encodes an α1,4 galactosyltransferase responsible for P<sup>k</sup>, P<sub>1</sub>, and NOR antigen synthesis.<sup>184</sup> A4GALT1 resides on chromosome 22q13 and is organized into three exons, with the active enzyme localized within exon 3. A4GALT1 belongs to the GT32 family of glycosyltransferases and shares 43% homology with A4GNT, an α1,4 galactosyltransferase involved in gastric mucin

synthesis.<sup>198</sup> A4GALT1 mRNA is ubiquitously expressed in most human tissues.<sup>42</sup>

The predominant enzyme in P<sub>1</sub> and P<sub>2</sub> individuals is a 353-amino-acid type II glycoprotein containing two N-glycosylation sites, five cysteine residues, and a classic DXD motif.<sup>198</sup> There is evidence that A4GALT1 resides in the *trans*-Golgi network, possibly in a heterodimeric complex with LacCer synthase.<sup>199</sup> Synthesis of Gb3 and other globo-GSLs can be suppressed by competing glycosylation pathways, altered Golgi trafficking, and lysosomal degradation.<sup>199–202</sup>

The basis of the P<sub>1</sub>/P<sub>2</sub> phenotype took considerable effort to uncover. Originally, the P<sub>2</sub> phenotype was linked to polymorphisms in the promoter region (−551insC and −160A).<sup>203</sup> Subsequently, Thursson *et al.* identified a minor A4GALT1 mRNA transcript utilizing an alternate exon (exon 2a) that contained a SNP (42C > T) in European P<sub>2</sub> individuals.<sup>204</sup> A later, large systematic study identified one SNP (3084G > T; rs4741348) with P<sub>2</sub> and decreased A4GALT1 transcription.<sup>205</sup> The site was subsequently shown to bind the RUNX1 transcription factor.<sup>206</sup> A4GALT1 does show evidence of gene dosage, where A4GALT1 levels are highest in P<sup>1</sup>/P<sup>1</sup> > P<sup>1</sup>/P<sup>2</sup> > P<sup>2</sup>/P<sup>2</sup>.<sup>204,205</sup>

The NOR phenotype is the result of a missense mutation (Gln211>Glu) in the catalytic domain of the enzyme.<sup>207</sup> A comparison of A4GALT1 sequences from humans, gorilla, pig, cattle, rat, and mouse indicates that Q211 is a conserved amino acid. It is hypothesized that the mutation broadens acceptor substrate specificity, allowing the enzyme to recognize either a β-Gal or β-GalNAc acceptor.<sup>207</sup> The p phenotype can arise from a variety of missense, frameshift, nonsense, and deletion mutations. Over 30 amorph A4GALT1 alleles are described to date.<sup>20,184</sup>

### P synthase (B3GALNT1)

B3GALNT1 is located on chromosome 3q26 and encodes a β1 → 3 n-acetylgalactosaminyltransferase responsible for P and PX2 antigen synthesis.<sup>20,184</sup> Originally named B3GALT3, B3GALNT1 is a member of the GT31 family of glycosyltransferases and is ubiquitously expressed in most human tissues.<sup>42,208</sup> The gene contains five exons with the open reading frame located in exon 5. The translated enzyme is 331 amino acids with three β1,3 galactosyltransferase motifs, five potential N-glycans, and six cysteines, including four cysteines conserved among other β1,3 galactosyltransferases.<sup>208</sup> Fourteen null alleles associated with the P<sup>k</sup> phenotype are reported to date.<sup>20</sup>

### Forssman synthase (GBGT1)

*GBGT1* encodes an  $\alpha 1 \rightarrow 3$ galactosaminyltransferase belonging to the GT6 family of  $\alpha 1,3$ -Gal/GalNAc transferases and is responsible for Forssman synthesis.<sup>195,209</sup> Members of the GT6 family arose from a common ancestral gene and includes the *ABO* gene, isogloboside synthase (*A3GALT2*), and the enzyme necessary for linear B synthesis (*GGTA1*).

*GBGT1* is located on chromosome 9q34 as a pseudogene.<sup>209</sup> Like the *ABO* gene, the open reading frame is spread over several exons. Surprisingly, *GBGT1* mRNA can be detected in a wide host of human tissues despite the absence of Forssman expression. The absence of Forssman antigen in humans and apes is the consequence of two inactivating mutations (Gly230Ser; Gln296Arg) that arose late in primate evolution. Using recombinant enzyme constructs, Yamamoto and colleagues were able to restore enzyme activity in human *GBGT1*, where Gly230+Gln296 > Gly230 > Gln296.<sup>209</sup> Gly230 is homologous to Gly235 in the human *ABO* gene, which is crucial for UDP-GalNAc and H-antigen acceptor recognition. Forssman expression on rare A<sub>pa</sub>e red cells is due to an Arg296 > Gln mutation that restores partial enzyme activity. It is hypothesized that de novo Forssman expression reported in some cancers could reflect a similar process.<sup>209</sup>

### Transfusion medicine

P<sub>2</sub> individuals can make a naturally occurring anti-P<sub>1</sub>, a low-titered, IgM agglutinin.<sup>11</sup> Anti-P<sub>1</sub> is generally clinically insignificant, although rare acute hemolytic transfusion reactions have been reported. High-titer anti-P<sub>1</sub> can be observed in bird fanciers and certain Helminth infections due to the presence of P<sup>k</sup>- and P<sub>1</sub>-like substances in bird droppings and parasites.<sup>11,74</sup> Although there is no evidence that anti-P<sub>1</sub> is advantageous against Helminth infections, it is interesting that the highest incidence of P<sub>2</sub> occurs in regions with a high incidence of *Echinococcus* infection.<sup>74</sup>

Unlike anti-P<sub>1</sub>, the allo-anti-PP<sub>1</sub>P<sup>k</sup> and allo-anti-P present in p and P<sup>k</sup> individuals are always clinically significant antibodies, capable of causing acute hemolytic transfusion reactions.<sup>11</sup> Patients require rare antigen-negative units for transfusion. Females are also at risk for recurrent miscarriages, even at relatively low antibody titers.<sup>210,211</sup> Miscarriage tends to occur in the first trimester due to P and P<sup>k</sup> antigen expression on the fetal placenta. Early intervention has been used with variable success including plasma exchange, IVIG, steroids, and aspirin.<sup>210</sup>

A hemolytic auto-anti-P is the most common antibody specificity observed in paroxysmal cold hemoglobinuria (PCH). Patients typically present with acute hemolysis, a positive DAT (IgG-negative, C3-positive) and a negative antibody screen. The antibody is a biphasic, low affinity IgG hemolysin that binds red cells and fixes complement at cool temperatures but dissociates at 37°C. The Donath-Landsteiner test is required to confirm PCH. PCH patients can be safely transfused with P+ units through a blood warmer.<sup>212</sup>

Anti-LKE is rare, with only six known cases.<sup>192</sup> Most examples of anti-LKE were low-titer, IgM antibodies and clinically insignificant. There is one report of a hemolytic transfusion reaction associated with a high-titer anti-LKE in a patient with newly diagnosed lymphoma.<sup>192</sup>

### Biological role

#### Development and cancer

Globo-GSLs are expressed on embryonic stem cells, where they may play a role in development. Two extended globo-GSLs, Gb5 (SSEA-3) and LKE/MSGG (SSEA-4), are markers of embryonic

stem cells, embryonic carcinoma, and teratocarcinomas.<sup>213</sup> In vitro studies with human embryonal carcinoma cell lines have shown homotypic cell-cell adhesion and signaling mediated by Gb4-Gb5 interactions.<sup>214</sup> In murine embryos, LKE/MSGG-enriched lipid rafts participate in cytokinesis, localizing along the cleavage furrows in cooperation with actin and E-cadherin.<sup>215</sup> In mice, the deletion of *B3GALNT1*, with loss of Gb4, Gb5, and MSGG, is embryonic lethal.<sup>216</sup>

Several globo-GSLs are increased in malignancy and serve as cancer markers. Gb3 is elevated in Burkitt's lymphoma and is a marker of apoptotic germinal center B cells.<sup>184</sup> Globo-H, Gb5, and MSGG are expressed by breast cancer and cancer stem cells.<sup>9,217</sup> In breast cancer, Gb5- and MSGG-enriched lipid rafts contribute to tumor invasion and resistance to apoptosis through their interaction with integrins, cSrc, FAK, AKT, and RIP.<sup>217,219</sup> A MSGG-binding protein FKBP4 was recently identified in breast cancer cells.<sup>218</sup> In vitro studies have shown that de novo MSGG synthesis is associated with a decreased adhesion and epithelia phenotype consistent with a mesenchymal–epithelial transition (MET, see I blood group).<sup>220</sup> Finally, MSGG and/or disialosylgalactosylgloboside (DSGG) are elevated on renal cell carcinoma.<sup>190,221</sup> DSGG binds siglec-7 and is associated with pulmonary metastasis and inhibition of NK cells.<sup>221,222</sup>

#### Immune response

Gb3 is expressed on B-cells and may serve as a stimulatory glycolipid antigen for iNKT cells.<sup>223</sup> In addition, Gb4 was identified as an endogenous ligand for toll-like receptors (TLRs), a member of the innate immune system expressed on monocytes, spleen, and phagocytic cells.<sup>224</sup> In Gram-negative sepsis, the TLR4-MD2 complex binds bacterial LPS via the lipid A tail, triggering an intense inflammatory response. In *A4GALT1*-knockout mice, exogenous Gb4 administration can reduce LPS-induced inflammation and mortality by binding TLR4-MD2 and blocking LPS binding. In wild-type mice, LPS upregulates *A4GALT1* transcription with upregulation of Gb4 synthesis, coupled with the recruitment of TLR4-MD2 into Gb4-enriched lipid rafts.

### Parvovirus B19 and HIV

P antigen is a cofactor for parvovirus B19 on red cells, the causative agent of erythema infectiosum or fifth disease.<sup>74</sup> B19 is an erythrovirus, preferentially infecting marrow erythroblasts, resulting in reticulocytopenia, anemia, and, on occasion, aplastic crisis and pure red cell aplasia. Erythroblasts may be particularly susceptible to B19 due to their high concentration of Gb4.<sup>74</sup> P<sup>k</sup> and p individuals, who lack P antigen, are resistant to B19 infection. Current research suggests that infection requires a two-step process, in which B19 initially binds Gb4 or related glycolipid, leading to a conformational change in the viral capsid.<sup>225,226</sup> The modified B19 can then bind a secondary receptor that is likely localized within a lipid raft and undergoes clathrin-mediated endocytosis.<sup>225</sup> Although P antigen is not mandatory for viral binding, P antigen appears to be required for productive viral infection.<sup>226</sup>

GSL-enriched lipid rafts play an important role in HIV fusion, endocytosis, and HIV budding.<sup>227</sup> In human lymphoid cells, there is an inverse relationship between Gb3 and HIV infection. Lymphocytes from p individuals are hypersusceptible to HIV in vitro, whereas lymphocytes from P<sup>k</sup> donors, or patients with Fabry disease, are HIV resistant.<sup>228,229</sup> Other investigators, on the other hand, have identified Gb3 as a coreceptor, facilitating the interaction between HIV, CD4, and CXCR4.<sup>230</sup>

### **Shigella, E. coli, and Streptococcus suis**

*Shigella dysenteriae* and enterohemorrhagic *E. coli* express shiga toxins (Stx) that recognize GSLs bearing a terminal Gal $\alpha$ 1 → 4Gal epitope, including P<sub>1</sub>, Gb<sub>3</sub>, and “band 0.03.”<sup>74</sup> Gb3, however, is the physiologic receptor in infection and development of hemolytic uremic syndrome (HUS). Gb3-enriched lipid rafts are necessary for toxin binding, Stx-mediated cell signaling, and endocytosis. Six studies have examined the correlation between P<sub>1</sub>/P<sub>2</sub> phenotype, HUS, and clinical outcomes, with mixed results.<sup>74</sup> A study from the *E. coli* O157:H7 network, however, found a fourfold increased risk of HUS in P<sub>1</sub><sup>strong</sup> individuals.<sup>231</sup> Gb3 is also a receptor for *S. suis*, a pathogen of pigs that can cause life-threatening infections in humans.<sup>74</sup>

Globo-GSLs are also receptors for P-fimbriated, uropathogenic *E. coli* strains. P-fimbriae recognize all globo-GSLs, although LKE/MSGG is the preferred receptor in solid phase testing. The ability of FUT2 to decrease MSGG synthesis is likely responsible for the 3.4–26-fold higher incidence of UTI in nonsecretors.<sup>232,233</sup> The impact of the P<sub>1</sub>/P<sub>2</sub> phenotype on *E. coli* UTI is less clear. Early pediatric studies showed a slight increase in UTI and recurrent pyelonephritis in P<sub>1</sub> children.<sup>74</sup> In contrast, studies in adults have found little or no association between UTI and P<sub>1</sub> phenotype.<sup>232</sup>

### **Sd<sup>a</sup> (SID)**

The Sd<sup>a</sup> antigen or SID was named after a longtime volunteer blood donor (Sidney) at the London Lister Institute.<sup>234</sup> First described in 1967, it was officially designated as a blood group by ISBT in 2019. Sd<sup>a</sup> is widely expressed on human tissues including red cells, stomach, colon, and kidney, where Sd<sup>a</sup> is most commonly associated with the Tamm–Horsfall (uromodulin) protein along the distal convoluted tubules and collecting ducts.<sup>235</sup> In the intestine, there is an inverse expression of Sd<sup>a</sup> and ABH, with Sd<sup>a</sup> predominant in the descending colon.<sup>236</sup> Sd<sup>a</sup> is also found in fluids such as saliva, serum, milk, meconium, and urine.<sup>237</sup> Sd<sup>a</sup> is also expressed on many animal tissues and fluids. Although Sd<sup>a</sup> is not considered a clinically significant antigen for transfusion, Sd<sup>a</sup> is currently recognized as an important carbohydrate antigen in cancer and xenotransplantation.

### **Serology and transfusion**

Sd<sup>a</sup> is a high incidence red cell antigen with variable expression and autosomal dominant inheritance.<sup>32</sup> Accurate Sd<sup>a</sup> typing requires both RBC and urine since nearly half of individuals with Sd(a–) red cells still express Sd<sup>a</sup> on other tissues and fluids, like urine.<sup>235</sup> In many ways, Sd<sup>a</sup> phenotyping is similar to determining “secretor status,” in which individuals can type as Le(a–b–) but still secrete ABO antigens in urine (see the Lewis blood group, earlier).

The majority (90%) of donors express weak Sd<sup>a</sup> (+ to 1+; Sda+), with <1% of donors typing as Sd<sup>a</sup> strong (2+, Sda++).<sup>235</sup> CAD, a very rare polyagglutinable phenotype, is also strongly Sd<sup>a</sup>-positive (sometimes referred to as “super Sd<sup>a</sup>”).<sup>32</sup> Approximately 9% of donors are negative for Sd<sup>a</sup> on red cells; however, nearly half of these donors are found to secrete Sd<sup>a</sup> in urine. As a result, only 4% of donors are considered true Sd(a–).<sup>235</sup> Sd<sup>a</sup> is absent on cord cells but is present by 10 weeks of age. Sd<sup>a</sup> is reported on i<sub>adult</sub> cells.<sup>32</sup>

Anti-Sd<sup>a</sup> is a naturally occurring IgM antibody, reactive at room temperature.<sup>32</sup> The reaction gives a characteristic mixed field appearance, with small red cell agglutinates in a field of unagglutinated cells. This mixed field agglutination can give a “shimmering appearance” similar to that described for some Lutheran antibodies

(e.g., anti-Lu<sup>a</sup>). Antibody inhibition is used to confirm Sd<sup>a</sup> specificity by incubating samples with guinea pig urine, which is a particularly rich source of Sd<sup>a</sup> substance. Pooled, concentrated human urine can also be used but is less inhibitory than guinea pig urine.<sup>235</sup> Sd<sup>a</sup> inhibitory substance is not available commercially and requires local collection and processing of urine.

Anti-Sd<sup>a</sup> is identified in ~1% normal donors when tested against common Sd(a+) red cells.<sup>32</sup> A higher frequency is observed when testing donors against Sd(a++) strong red cells, and nearly all donors will react to rare polyagglutinable CAD cells.<sup>32</sup> Anti-Sd<sup>a</sup> is not clinically significant and is not associated with HDFN, transfusion reactions, or shortened red cell survival.

### **Biochemistry and synthesis**

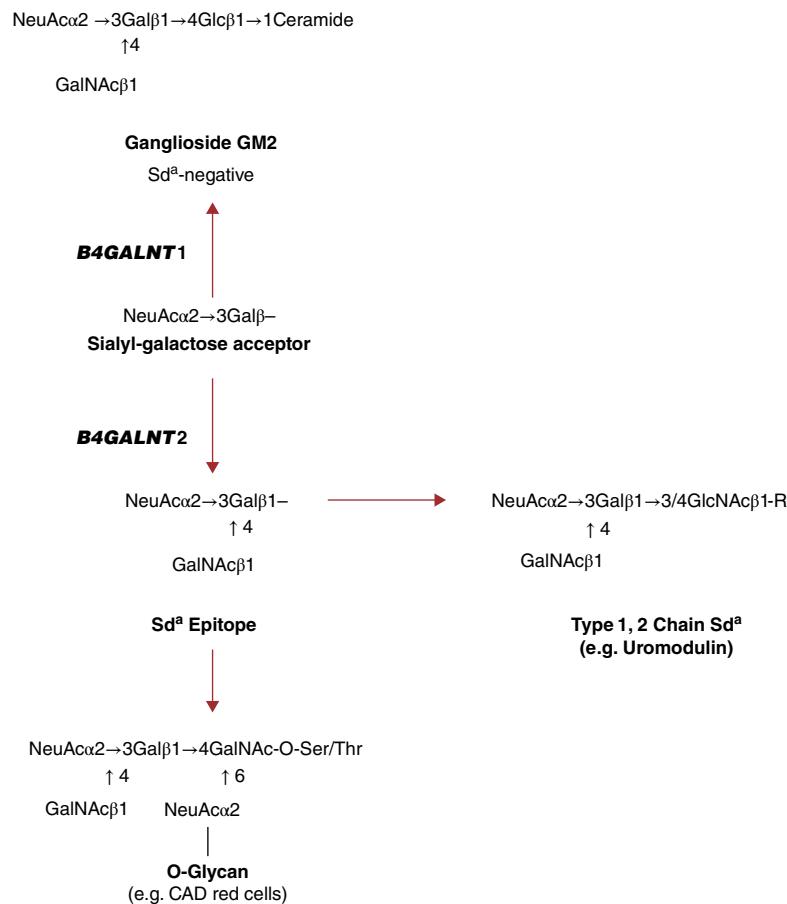
Sd<sup>a</sup> is a terminal, sialylated trisaccharide epitope characterized by an immunodominant β1-4GalNAc. Sd<sup>a</sup> synthesis is dependent on the N-acetyl-galactosaminyltransferase B4GALNT2, which recognizes the sialylated disaccharide NeuAcα2 → 3Gal-R.<sup>235</sup> The enzyme adds a GalNAc, in a β1 → 4 linkage, to the subterminal Gal, resulting in the trisaccharide GalNAcβ1 → 4(NeuAcα2 → 3)Gal-R. Studies have shown a strict requirement for the terminal sialic acid (NeuAcα2 → 3Gal) for enzyme activity.

As shown in Figure 9.5, the Sd<sup>a</sup> trisaccharide can be expressed by an array of different glycan scaffolds (glycolipids, N- and O-glycans) and chain types (type 1, type 2, branched; O-glycan cores 1–4). The nature of the Sd<sup>a</sup>-active antigen on red cells is still not clear. In the rare polyagglutinable phenotype CAD, Sd<sup>a</sup>-type structures were identified on glycophorin A and trace type 2 chain gangliosides; however, these same antigens were not identified in Sd<sup>a+</sup>, CAD-red cells.<sup>32,235</sup> Like Lewis antigens, Sd<sup>a</sup> may represent an adsorbed glycolipid antigen based on some serologic findings including (1) resistance to enzymes; (2) absence on cord cells; (3) presence in plasma, saliva, and urine; (4) decrease/loss during pregnancy; and (5) rich expression in gastrointestinal tissue, which are also the source for Lewis glycolipids. Unfortunately, attempts to convert Sd(a–) red cells to Sd(a+) by incubating cells in Sd(a+) plasma were unsuccessful.<sup>32</sup> Ganglioside GM2 (figure 1), which shares an Sd<sup>a</sup>-type terminus, is not expressed by red cells and is not recognized by human anti-Sd<sup>a</sup>.<sup>234</sup>

### **Molecular biology**

The gene for B4GALNT2 is on chromosome 17q21.33 and shows 39% homology to B4GALNT1, the glycosyltransferase responsible for ganglioside GM2 synthesis.<sup>235</sup> The B4GALNT2 gene is large with 11 exons, including two forms of exon 1 (E1S and E1L), leading to two major enzyme isoforms that differ only in the length of the cytoplasmic N-terminal domain.<sup>235</sup> Exon E1S encodes a 506 amino acid enzyme whereas E1L is responsible for a minor 566 amino acid enzyme with an unusually long 66 amino acid cytoplasmic tail. The E1S short enzyme has higher activity than the less common longer isoform. Both isoforms are located in the trans-Golgi; however, the long isoform is also found in cytoplasmic vesicles and plasma membrane.<sup>238</sup>

Transcriptional regulation of B4GALNT2 is complex and still under investigation. B4GALNT2 possesses several CpG islands and can be upregulated by demethylating agents like 5-azacytidine.<sup>235,239</sup> Methylation immediately 5' of exon E1s has been linked to decreased synthesis of the major short form of the enzyme.<sup>237</sup> Paradoxically, hypermethylation of a CpG located between exons 6 and 7 is associated with high B4GALNT2 mRNA in human colon cancer.<sup>238</sup> There is indirect evidence for the miRNA-204-5p in



**Figure 9.5** Synthesis of Sda-active glycans by B4GALNT2. Ganglioside GM2, which is synthesized by B4GALNT1 and shares a similar epitope, is not recognized by anti-Sda.

downregulating colon *B4GALNT2* expression.<sup>239</sup> It is believed that specific transcription factors also play a role in *B4GALNT2* expression.<sup>239</sup>

Although the role of B4GALNT4 in Sd<sup>a</sup> synthesis has been known for over 20 years, it was only recently that mutations in *B4GALNT4* were identified as the etiology of the Sd(a-) phenotype.<sup>240</sup> Both missense (Arg523Trp; Gln436Arg) and splice site mutations were identified in four Sd(a-) individuals, with most being compound heterozygotes. No mutations were identified in a fifth Sd(a-) person and may represent mutations in regulatory regions. Weak *B4GALNT2* mRNA expression is reported in bone marrow preparations but not in erythroid cells. The latter adds to evidence that Sd<sup>a</sup>, like Lewis antigens, may not be of erythroid origin.

### Biological role

#### Sd<sup>a</sup> and cancer

Sd<sup>a</sup>/B4GALNT2 have been identified as good prognostic markers in gastric and colon cancers.<sup>239,241,242</sup> In these cancers, *B4GALNT2* expression is depressed relative to normal tissue.<sup>241,242</sup> Colon cancer patients with the highest residual *B4GALNT2* expression had better long-term survival than patients with little or no *B4GALNT2* expression.<sup>239</sup> In transfected cancer cell lines, *B4GALNT2* expression led to a loss of “stemness” as measured by aldehyde dehydrogenase activity and distinct changes in the cell transcriptome.<sup>239,241</sup> Likewise, *B4GALNT2* expression led to decreased clonogenic and metastatic potential in vivo and in vitro, respectively.<sup>241,242</sup> *B4GALNT2*

suppresses expression of sLe<sup>X</sup>, a selectin receptor and adhesion factor that is known to play a role in many malignancies.<sup>242</sup> *B4GALNT2* utilizes and competes for the same NeuAc $\alpha$ 2 → 3Gal-R acceptor used for the synthesis of sLe<sup>X</sup> (and sLe<sup>a</sup>, type 1 chain).<sup>242</sup>

#### Sd<sup>a</sup> and xenotransplantation

Sd<sup>a</sup> is also expressed by other species including pigs, cows, ferrets, sheep, and mice.<sup>234,243</sup> The most extensive work exploring Sd<sup>a</sup> and xenotransplantation has been in transgenic pigs, where Sd<sup>a</sup> has been identified as a major, non- $\alpha$ Gal xenoantigen on endothelium and other tissues. Despite the fact that most people are Sd(a+) and only 1% possess a weak anti-Sd<sup>a</sup>, essentially all people will react with porcine Sd<sup>a</sup> antigen.<sup>242</sup> Furthermore, the transfection and expression of porcine *B4GALNT2* into human HEK93 kidney cell lines are associated with increased human antibody binding and a 20-fold increase in antibody-mediated cytotoxicity.<sup>243</sup> Conversely, knocking out *B4GALNT2* in transgenic pigs leads to a marked decrease in human antibody recognition.

The pig *B4GALT4* gene is 76% homologous with human *B4GALNT4*, but the chemical nature of the Sd<sup>a</sup> antigen expressed on porcine cells is not known.<sup>234</sup> It is assumed that porcine Sd<sup>a</sup> antigen is presented on alternate carbohydrate structures that affects antibody recognition, analogous to anti-A1 and type 4 globo-A (see ABO). At least one investigator has suggested that the porcine Sd<sup>a</sup> antigen may resemble the CAD antigen on human RBC, a rare polyagglutinable red cell phenotype that is reactive with all human serum.<sup>234</sup>

## Sd<sup>a</sup> and infection

Influenza infection initially requires colonization of nonciliated cells of the tracheobronchial tree via a sialic-acid-specific viral hemagglutinin.<sup>74</sup> Neuraminic acid in  $\alpha$ 2,6 linkage (NeuAc $\alpha$ 2 → 6) is rich in the upper respiratory tract whereas sialic acid in  $\alpha$ 2,3 linkage (NeuAc $\alpha$ 2 → 3) is enriched in the lower respiratory tract. Although most human influenza strains prefer NeuAc $\alpha$ 2 → 6 epitopes, viral strains recognizing NeuAc $\alpha$ 2 → 3 do arise from epizootic transmission and are often associated with pneumonia and severe infections (e.g., H5N1). Parainfluenza virus, a cause of croup, also recognizes NeuAc $\alpha$ 2 → 3 epitopes.<sup>74</sup> B4GALNT2, which modifies NeuAc $\alpha$ 2 → 3 epitopes, was found to significantly decrease viral binding and infection by  $\alpha$ 2,3NeuAc-specific influenza strains.<sup>244,245</sup> Although B4GALNT2 may act as a host resistance factor in animal species, B4GALNT2 is not expressed by normal lung epithelium.

B4GALNT2 appears to moderate bacterial colonization in mice. The absence of B4GALNT2 activity leads to altered murine microbiota.<sup>246</sup> As a result, B4GALNT2 may increase susceptibility to *Helicobacter* species and *Salmonella*.<sup>246,247</sup>

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## CHAPTER 10

# Rh and LW blood group antigens

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## Summary

Although the Rh and LW antigens are carried on entirely different proteins, they are incorporated together in this chapter based on a historic serologic connection and evidence that they are physically associated within the red cell membrane.

## Rh blood group system

### History and nomenclature

The Rh system is second only to the ABO system in importance in transfusion medicine because Rh antigens, especially D, are highly immunogenic, and antibodies to these antigens cause hemolytic disease of the fetus and newborn (HDFN) and hemolytic transfusion reactions (HTRs). HDFN was first described by a French midwife in 1609 in a set of twins, one of whom was hydropic and stillborn, whereas the other was jaundiced and died of kernicterus.<sup>1</sup> That a wide range of observed clinical scenarios involving red cell hemolysis were related—from severely hydropic stillborn fetuses to infants with mild or significant levels of jaundice and kernicterus—was not realized until 1932.<sup>2</sup> The cause of red cell hemolysis remained elusive until 1939, when Levine and Stetson described a woman who delivered a stillborn fetus and also suffered a severe hemolytic reaction when transfused with blood from her husband. Levine and Stetson correctly surmised that the mother had been immunized by a fetal red cell antigen inherited from the father and suggested that the cause of what was termed “erythroblastosis fetalis” was maternal antibody in the fetal circulation.<sup>3</sup> They did not give the target blood group antigen a name. Meanwhile Landsteiner and Wiener, to discover new blood groups, injected rabbits and guinea pigs with rhesus monkey red cells. The antiserum they obtained agglutinated not only rhesus monkey red cells but also the red cells of 85% of subjects, whom they called “Rh positive”; the remaining 15% of individuals tested were termed “Rh negative.”<sup>4</sup> The “anti-Rhesus” serum seemed to be reacting similarly to the maternal antibody in serologic testing; hence, the blood group system responsible for HDFN came to be known as “Rh.” The anti-Rhesus serum, in fact, was detecting the LW antigen (subsequently named for Landsteiner and Wiener), which is present in greater amounts on Rh-positive than on Rh-negative red cells.<sup>5</sup> Years of

debate followed concerning whether the human antibodies and the Rhesus antibodies were detecting the same antigen. Interestingly, this debate extended long after serologic reactivity largely confirmed they were reacting with different structures. Landsteiner and Wiener never accepted the LW terminology because doing so would have implied that they had not discovered the cause of HDFN.<sup>6</sup>

It was soon obvious that Rh was not a simple, single-antigen system. In 1941, Fisher named the C and c antigens (A and B had been used for ABO) and used the next letters of the alphabet, D and E, to define antigens recognized by additional antibodies. In 1945, the e antigen was identified.<sup>5</sup>

It is helpful to appreciate that the often-confusing nomenclature used to describe Rh antigens results from the difference in opinion that existed concerning the number of genes that were involved in the expression of the antigens. The Fisher–Race nomenclature reflect their opinion that three closely linked genes (C/c, E/e, and D) were responsible, whereas the Wiener nomenclature (Rh–Hr) was based on his belief that a single gene encoded one “agglutinogen” that carried several blood group factors. Even though neither theory was correct (there are two genes—RHD and RHCE—correctly proposed in 1986 by Tippett<sup>7</sup>), both nomenclatures are still in use. For written communication, the Fisher–Race designation (DCE) for haplotypes is preferred; when speaking about the Rh system, a modified version of Wiener’s nomenclature is generally used (Table 10.1). “R” indicates that D is present while use of a lowercase “r” (or “little r”) indicates that it is not. The C or c and E or e Rh antigens carried *in cis* with D are represented by subscripts: 1 for Ce ( $R_1$ , DCe), 2 for cE ( $R_2$ , DcE), 0 for ce ( $R_0$ , Dce), and Z for CE ( $R_z$ , DCE). The antigens present in the absence of D are represented by superscript symbols: “prime” for Ce (r’), “double-prime” for cE (r’’), and “y” for CE (r’’). The “R” versus “r” terminology allows one to convey the Rh antigens present on one chromosomal haplotype in a single term, e.g., a “ $R_0$ ” haplotype encodes D, c, and e, but not C or E.

The major Rh antigens are D, C, c, E, and e, but the Rh system is more complex because of the number of additional antigens that have been reported (Table 10.2). These include compound antigens *in cis* (e.g., f [ce], Ce, and CE), low-incidence antigens arising from hybrid proteins (e.g., D<sup>w</sup>, Go<sup>a</sup>, and Evans), and antigens arising from various point mutations in the RHCE protein (e.g., C<sup>w</sup>, C<sup>x</sup>, and VS).

**Table 10.1** Nomenclature and Prevalence of Rh Haplotypes

Haplotype (Fisher-Race)	Haplotype (Modified Wiener)	Occurrence (%) <sup>a</sup>		
		Whites	Blacks	Asians
DCe	R <sub>1</sub>	42	17	70
DcE	R <sub>2</sub>	14	11	21
Dce	R <sub>0</sub>	4	44	3
DCE	R <sub>z</sub>	<0.01	<0.01	1
ce	r	37	26	3
Ce	r'	2	2	2
CE	r''	1	<0.01	<0.01
CE	r'	<0.01	<0.01	<0.01

**Table 10.2** International Society for Blood Transfusion (ISBT) Numerical Terminology and Symbols for Rh Antigens

Numeric	Symbol	Numeric	Symbol	Numeric	Symbol
RH1	D	RH27	cE	RH48	JAL
RH2	C	RH28	hr <sup>H</sup>	RH49	STEM
RH3	E	RH29	"total" <sup>b</sup>	RH50	FPTT
RH4	c	RH30	Go <sup>a</sup>	RH51	MAR
RH5	e	RH31	hr <sup>B</sup>	RH52	BARC
RH6	ce or f	RH32	Rh32	RH53	JAHK
RH7	Ce	RH33	Rh33	RH54	DAK
RH8	C <sup>w</sup>	RH34	hr <sup>B</sup>	RH55	LOCR
RH9	C <sup>x</sup>	RH35	Rh35	RH56	CENR
RH10	V <sup>t</sup>	RH36	Be <sup>a</sup>	RH57	CEST
RH11	E <sup>w</sup>	RH37	Evans	RH58	CELO
RH12	G	RH39	C-like	RH59	CEAG
RH17	hr <sup>*</sup>	RH40	Tar	RH60	PARG
RH18	Hr <sup>0</sup>	RH41	Ce-like	RH61	CEVF
RH19	hr <sup>s</sup>	RH42	Ce <sup>s</sup>	RH62	CEWA
RH20	VS <sup>t</sup>	RH43	Crawford		
RH21	C <sup>g</sup>	RH44	Nou		
RH22	CE	RH45	Riv		
RH23	D <sup>w</sup>	RH46	Sec <sup>s</sup>		
RH26	c-like	RH47	Dav		

Note: Rh13 through 16, 24, and 25 are obsolete.

\* High-prevalence antigen. The antibody is made by D-/D-- (lacking RHCE) antigens) and similar phenotypes.

<sup>b</sup> Low-prevalence antigens, except in Blacks: 30% of Blacks are V positive and up to 40% are VS positive.

<sup>a</sup> High-frequency antigen. The antibody is made by rare individuals lacking all RH antigens.

<sup>s</sup> High-frequency antigen. The antibody is made by rare D+ Black individuals.

Table 10.2 also includes the numeric designations for Rh antigens.<sup>8</sup> With a few exceptions (RH17 and RH29), the numeric designations are not widely used in the clinical laboratory in the US but are more often used in other countries.

### Genes and their expressed proteins

Two genes designated *RHD* and *RHCE* encode the Rh proteins.<sup>10</sup> Each has 10 coding exons. The sequence of the exons are 97% identical and result from a gene duplication on chromosome 1p34–36.<sup>11</sup> Rh-positive individuals have both genes, whereas most Rh-negative individuals have only the *RHCE* gene (below). RH genes are noted in italics.

RhD and RhCE are 417-amino-acid transmembrane spanning proteins that atypically lack glycosylation. One protein carries the D antigen, and the other carries various combinations of the CE antigens (ce, cE, Ce, or CE). RhD differs from RhCE by 32–35 amino acids depending on which form of RhCE is present (Figure 10.1).<sup>12–15</sup> The Rh proteins migrate in sodium dodecyl sulfate polyacrylamide gels with an approximate  $M_r$  of 30–32 kD and hence were

sometimes referred to as the *Rh30* proteins. They are predicted to span the membrane 12 times and are covalently linked to fatty acids (palmitate) in the lipid bilayer (Figure 10.1).<sup>16</sup>

### Association with RhAG

RhAG is a 409-amino-acid glycosylated protein encoded by the *RHAG* gene on chromosome 6. The protein's name (Rh-associated glycoprotein) derives from its properties. It shares 37% amino acid identity with RhD and RhCE proteins, has the same predicted membrane topology, but has one N-glycan chain that carries ABO and Ii specificities.<sup>17</sup> RhAG is not as polymorphic as RhCE and RhD, and only four antigens, forming a separate blood group system, RHAG, have been identified.<sup>18</sup> RhAG, which is important for targeting the Rh proteins to the membrane during erythroid maturation, co-immunoprecipitates with RhD and RhCE.<sup>19</sup> Based on the conformation of their protein homologs (see Rh protein family and function), RhD and RhCE are predicted to form a trimer with RhAG. The exact composition of the trimer is unknown, but most likely 2 RhAG monomers associate with 1 RhD or RhCE monomer.<sup>20</sup>

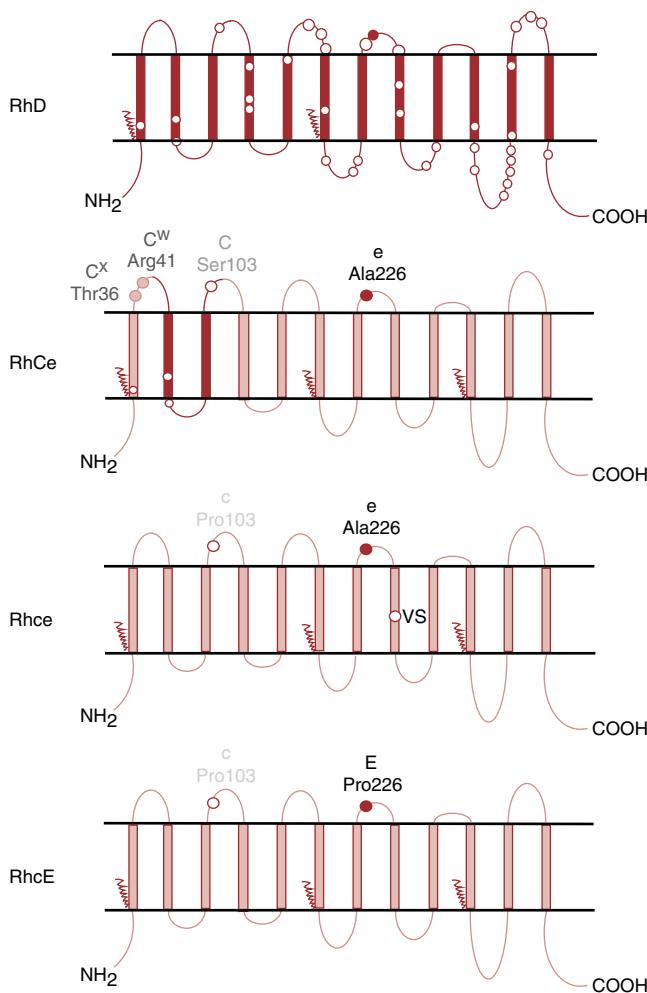
### Basis for antigen expression

#### D antigen

"Rh-positive" and "Rh-negative" refer to the presence or absence of RhD, respectively. The Rh-negative phenotype occurs in 15–17% of Whites but is not as common in other ethnic populations and is very rare in Asians.<sup>5,21</sup> The absence of D on the red cells of people of European ancestry was caused by a complete deletion of the *RHD* gene<sup>22</sup> which occurred on a Dce (R<sub>0</sub>) haplotype because the allele most often carried with the deletion is *RHCE'ce*. Deletion of the *RHD* gene is associated with being "Rh-negative" in all populations, but inactive or silenced *RHD* is also a cause of D-negative phenotypes in Asians or Africans. D-negative phenotypes in Asians occur with a frequency of <1%,<sup>5</sup> and most carry mutations in *RHD* genes associated with *RHCE'Ce*, indicating that they probably originated on a DCe (R<sub>1</sub>) haplotype. Only 3–7% of South African Blacks are D-negative, but 66% have *RHD* genes that contain a 37-bp internal duplication, which results in a premature stop codon,<sup>23</sup> and 15% result from a hybrid *RHD\*DIIIa-CE-D* gene that does not encode D epitopes.<sup>24</sup> This is important when designing polymerase chain reaction (PCR)-based methods to predict the D status of a fetus and the possibility of HDFN. The population being tested, and the different molecular events responsible for D-negative phenotypes (i.e., gene deletion or gene mutation) must be considered. Even among D-negative Whites, cases of an *RHD* gene that is not expressed because of mutation or nucleotide insertion or deletion (indels) have been reported.<sup>25</sup>

#### Weak D

An estimated 1–2% of individuals have reduced expression of the D antigen, which is characterized by weaker than expected reactivity with anti-D typing reagents or, alternatively, as failure of such red cells to agglutinate directly with anti-D requiring the use of an indirect antiglobulin test for detection. The genetic basis of weak expression of D is heterogeneous but is primarily associated with the presence of point mutations often predicted to be intracellular or in the transmembrane regions of RhD rather than on the outer surface of the red cell (Figure 10.2).<sup>26</sup> These mutations appear to affect the efficiency of insertion and the quantity of protein in the membrane without altering the expression of D epitopes. This explains why most individuals with a weak D phenotype can safely



**Figure 10.1** Predicted membrane topology of RhD and the major RhCE proteins. The amino and carboxy termini are cytoplasmic, and the proteins are predicted to transverse the membrane 12 times. The locations of the amino acid residues that differ between D and CE are represented by open circles on RhD, only five of which are predicted to be strictly extracellular, by the most recent estimations.<sup>20</sup> The C/c (Ser103Pro) polymorphism located on the second extracellular loop and the e/E (Ala226Pro) polymorphism on the fourth extracellular loop are shown. The shared region of RhD and RhCe responsible for expression of the G antigen is shown in dark gray. Amino acid changes responsible for C<sup>x</sup> and C<sup>w</sup> are located on the first extracellular loop; and the amino acid change responsible for the VS antigen, common in Blacks, is in the eighth transmembrane domain of Rhce. The zigzag lines represent covalent linkage to fatty acid in the lipid bilayer.

receive D-positive blood and do not make anti-D. There are over 200 different mutations known to cause weak D antigen expression, many of which have been named to reflect this (weak D type 1, weak D type 2, etc.).

For transfusion recipients, testing for weak expression of D antigen is not required. If they mistakenly test as D-negative, they can receive Rh negative units without untoward effects. However, this approach will increase the use of D-negative blood products, which are always in short supply. There is benefit to confirming that patients have the weak D phenotype. Observational studies indicate that individuals with weak D types 1, 2, and 3, which are the most

common weak D types in Whites, are unlikely to make anti-D and are not at risk for clinically significant anti-D. These patients can safely receive D-positive blood, and females are not candidates for Rh immune globulin.<sup>27</sup> When weaker than expected reactivity with anti-D is seen when typing females of childbearing potential, *RHD* genotyping should be considered to guide transfusion therapy, conserve D-negative donor units, and determine candidates for Rh immune globulin. A very weak form of D expression (D<sub>e</sub>), which cannot be detected by routine serology methods but can be demonstrated by adsorbing and eluting anti-D, is relatively common in Asians (10–30% of apparent D-negative).

In contrast, from the blood donation perspective, it is important that donor center procedures detect and label weak D RBC units as D-positive because they can stimulate the formation of anti-D in D-negative recipients.<sup>28,29</sup>

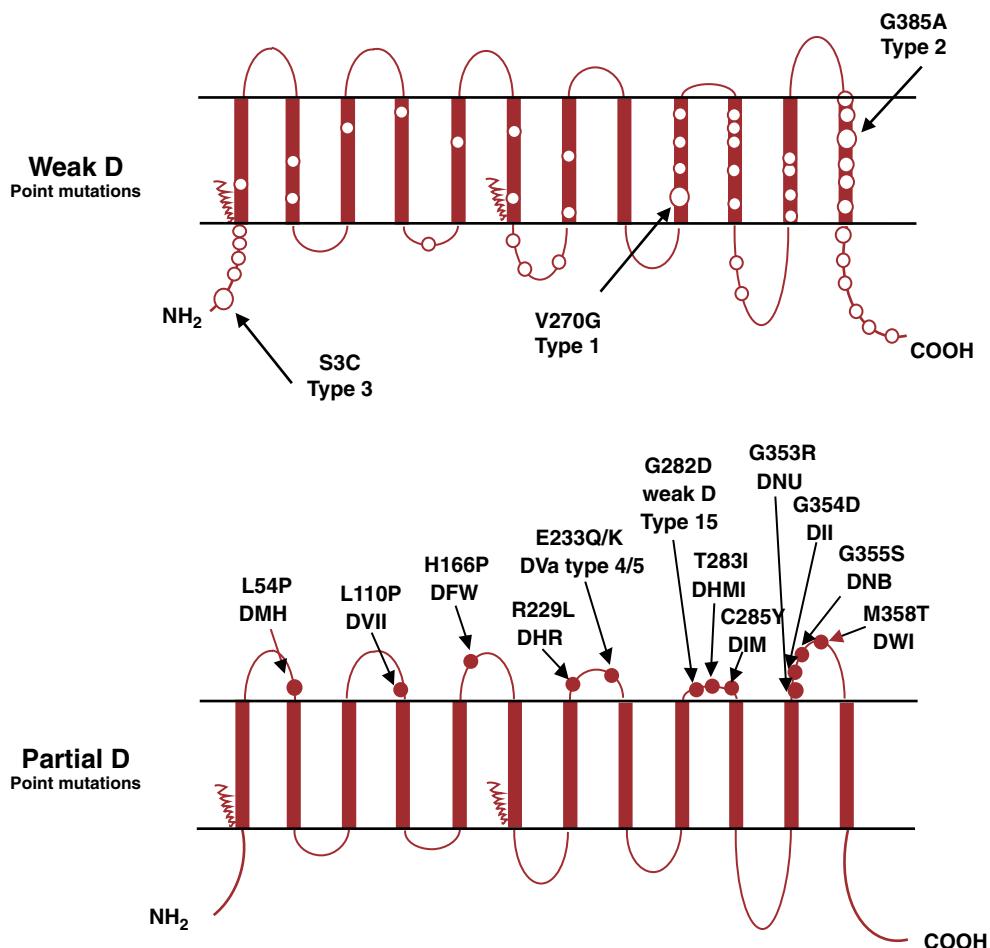
#### Partial D

“Partial D” refers to RBCs that type as Rh-positive with anti-D reagents but lack some D epitopes. This is why some apparently Rh-positive individuals produce anti-D when exposed to D antigen. At the genetic level, portions of *RHD* are replaced by corresponding portions of *RHCE* in many cases (Figure 10.3).<sup>25,30</sup> In addition to lacking D epitopes, the novel sequences of amino acids and the conformational changes that result from segments of RhD joined to segments of RhCE can generate new antigens (e.g., BARC, D<sup>w</sup>, FPTT, DAK, Go<sup>a</sup>, Evans, and Rh32) (Figure 10.3 and Table 10.2). The replacements are the result of gene conversion, the hallmark being that the donor gene is unchanged. Gene conversions can involve single or multiple exons, whereas others involve short stretches of amino acids (Figure 10.3). Loss or alteration of D epitopes resulting in “partial D” phenotypes can also result from single-amino-acid changes (e.g., DII, DVII, and DNB), most often predicted to be located on the extracellular loops of the protein, whereas those which are predicted to be cytoplasmic or transmembrane are more likely to cause weak D phenotypes (above). Lastly, any changes that modify interprotein interactions in the Rh complex can alter D epitopes causing a partial D phenotype.<sup>20</sup>

From a clinical standpoint, individuals with partial D antigens are at risk for anti-D. Ideally, females of childbearing potential with partial D should receive D-negative blood and, if they become pregnant, are candidates for Rh immune globulin. Some RBCs with partial D react weaker than expected with anti-D reagents, but they cannot be distinguished by serologic methods from weak D RBCs. *RHD* genotyping can guide clinical decision making for Rh immune globulin prophylaxis and transfusion.<sup>27</sup> In practice, many individuals with partial D phenotypes will type strongly D-positive and will be recognized only after they have made anti-D following a transfusion with D-positive cells or pregnancy with a D-positive fetus. Routine *RHD* genotyping is now recommended to overcome the limitations of serologic D typing for these individuals.<sup>27,31</sup>

#### C/c and E/e antigens

*RHCE* gene(s) encode four different proteins: Rhce, -Ce, -cE, and -CE (Figure 10.1).<sup>14</sup> The corresponding alleles are noted *RHCE\**c<sub>e</sub>, *RHCE\**C<sub>e</sub>, *RHCE\**c<sub>E</sub>, and *RHCE\**C<sub>E</sub>, respectively. C and c antigens differ by four amino acids: p.Cys16Trp encoded by exon 1, and p.Ile60Leu, p.Ser68Asn, and p.Ser103Pro encoded by exon 2 (Figure 10.1, open circles on RhCe). Of those four amino acids, only the residue at p.103 is predicted to be extracellular and is located on the second loop. All the amino acids encoded by exon 2 of *RHCE\**C<sub>e</sub> are identical to those encoded by exon 2 of *RHD*



**Figure 10.2** Predicted location of point mutations that cause the most common weak D phenotypes. Weak D types 1, 2, and 3 reflect amino acid changes predicted to be intracellular or in the transmembrane regions of RhD. Many other mutations have been shown to cause a weak D phenotype.

(Figure 10.1, dark color on RhCe). *RHCE*<sup>cE</sup> arose from the transfer of exon 2 from *RHD* into *RHCE*<sup>ce</sup>.

The sharing of exon 2 encoded amino acids by RhD, RhCe, and RhCE accounts for the expression of the G antigen on red cells that are D or C positive. Individuals who lack D and C can make anti-G, which can be distinguished from a combination of anti-D and anti-C by absorption and elution studies.<sup>6</sup> The presence of anti-G can explain why a D-negative person who was transfused with D-negative, but C-positive RBCs (or exposed to such cells through pregnancy), can appear to have made anti-D. The identification of anti-G is generally of academic interest only because transfusion requirements for such an individual would be the same (i.e., RBCs that are both D and C negative). However, in the case of a pregnancy, it is important to identify individuals with anti-G only, as Rh immune globulin prophylaxis would be indicated.

E and e differ by one amino acid, Pro226Ala, predicted to reside on the fourth extracellular loop of the protein (Figure 10.1, solid circle). The E antigen arose from a single point mutation that occurred in exon 5 of *RHCE*<sup>ce</sup> giving rise to *RHCE*<sup>cE</sup>.

#### Silenced CE

In several rare RBC phenotypes, designated D--, Dc-, and DC<sup>w-</sup>, some or all CE antigens are silenced. Genetic rearrangements analogous to the partial D rearrangements described in the "Partial D"

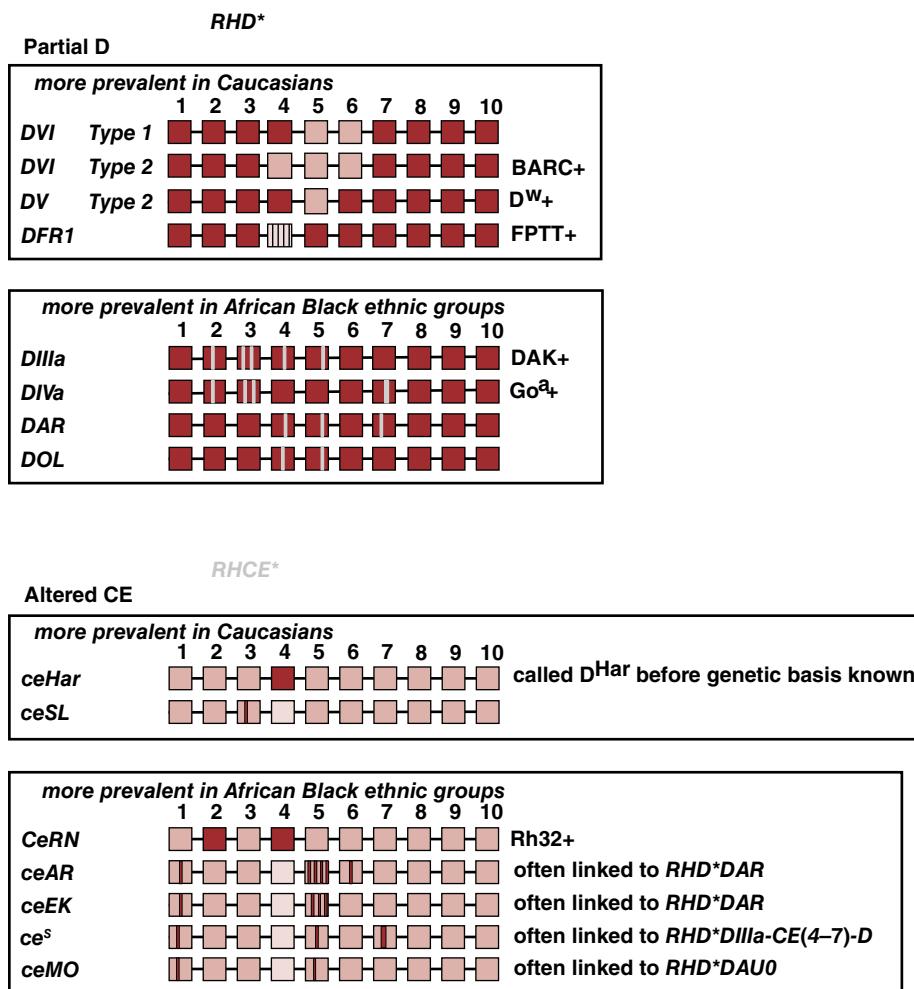
section above are sometimes responsible, but in this case they represent the replacement of portions of *RHCE* by *RHD*. The additional *RHD* sequences in *RHCE* along with a normal *RHD* lead to enhanced D antigen expression and account for the reduced or missing CE antigens. Point mutations, insertions, and deletions can also cause a premature stop codon or frameshift and silence CE antigens. Individuals with such altered CE phenotypes can make anti-Rh17 when immunized. Clinically, significant anti-Rh17 reacts with all cells that have RhCE proteins, and the only compatible RBCs are those with the D-- phenotype (or those expressing the same Rhce variant).

#### Altered CE

Over 150 *RHCE* alleles have been described to date, many of which encode weak or partial Cc or Ee antigens not immediately apparent by serology, as described above for *RHD*.

C<sup>w</sup> and C<sup>x</sup> are low-incidence antigens that result from single amino-acid changes (p.Gln41Arg and p.Ala36Thr, respectively) located on the first extracellular loop of RhCE (Figure 10.1, light colored circles).<sup>32</sup> These antigens are more common in Finns (4%) and are most often present on RhCe.<sup>33</sup>

V and VS antigens, which are expressed on RBCs of more than 30% of Blacks, result from a p.Leu245Val substitution in the predicted eighth transmembrane segment of Rhce (Figure 10.1).<sup>34</sup>



**Figure 10.3** Gene conversion events between *RHCE* and *RHD* produce chimeric Rh proteins and new low prevalence antigens or loss of high prevalence antigens (shown on right side). Dark gray (*RHD*) and light gray (*RHCE*) boxes represent the 10 exons that encode Rh proteins. Gene conversion events involve amino acid changes (vertical bars) or whole exons (filled boxes). Replacement of portions of *RHD* by *RHCE* (upper set of panels), with or without additional point mutations, causes many of the partial D phenotypes with the highest risk of anti-D formation and hemolytic complications, some of which are shown here. Two alleles with altered CE antigen expression (third panel) which may interfere with D antigen typing in the absence of normal D are shown here. Replacement of portions of *RHCE* by *RHD* (last panel) causes altered RhCE expression. Some of the *RHCE* alleles with the highest risk of antibody formation and hemolytic complications are shown here. Many more conversion events or point mutations not shown here can alter antigen expression with a risk of antibody formation.

The V-VS+ phenotype results from a p.Gly336Cys change on the 245Val background.<sup>35</sup> V+ and VS+ are associated with weak and altered expression of e antigen, indicating that p.Leu245Val causes a local conformation change on the fourth extracellular loop where the e-specific amino acid resides.

Most individuals have e-positive RBCs, but the e antigen is second in complexity to D antigen because variant or altered expression has frequently been observed.<sup>36</sup> The e antigen is altered most often on RBCs from African Blacks, and some of the more common alleles encoding altered expression are shown (Figure 10.3). Extensive discussion is beyond the scope of this chapter, but these alleles are prevalent in patients with sickle cell disease (SCD),<sup>37,38</sup> who not infrequently produce anti-e following transfusion despite having an e-positive red cell phenotype. RH genotyping of these patients can help to determine if the anti-e is an allo- or auto-antibody and to inform selection of blood for transfusion.<sup>39</sup>

E antigen variants are not common and include categories EI, EII, and EIII, which result from a point mutation (EI) or gene conversion and replacement of RhCE amino acids with RhD residues (EII and EIII) with concurrent loss of some E epitope expression. Category EIV red cells, which have an amino acid substitution in an intracellular domain, do not appear to lack E epitopes but have reduced E expression.<sup>39</sup>

Variants of c antigen are infrequent. The very rare RH:-26 results from a p.Gly96Ser transmembrane amino acid change that abolishes Rh26 and weakens c expression.<sup>40</sup> The lack of anti-c associated with c antigen variants in humans compared to the other Rh antigens, and the preservation of expression of c on the RBCs of non-human primates, suggests that the two proline residues involved (p.P102 and p.P103) form a stable structure that is resistant to perturbations and changes in Rhce.<sup>41</sup>

In summary, point mutations and genetic exchange, mainly involving gene conversion events between *RHD* and *RHCE*, are primarily responsible for the large number of Rh antigens. Additional complexity results because many of the Rh epitopes are highly conformational, and single-amino-acid changes in one part of the protein, including changes within the transmembrane regions, can affect the expression of cell-surface-exposed antigen epitopes on other parts of these proteins.

### Rh membrane complex and Rh<sub>null</sub> phenotype

The Rh proteins exist in the red cell membrane as complexes with several other proteins, with Rh and RhAG serving as the core of the complex.<sup>42</sup> That several other proteins interact with the Rh core complex is based on observations of Rh<sub>null</sub> RBCs, which lack expression of all Rh antigens. Individuals suffer from a compensated hemolytic anemia, with variable degrees of spherocytosis,

stomatocytosis, and increased red cell osmotic fragility.<sup>43</sup> The Rh<sub>null</sub> phenotype is rare and occurs on two different genetic backgrounds that disrupt the RhAG/Rh trimer association in the membrane. In the more common “regulator” type of Rh<sub>null</sub>, the absence of Rh antigens is caused by the mutation of *RHAG* with absence or defective RhAG protein.<sup>44</sup> The “amorph” type of Rh<sub>null</sub> is due to absence of *RHD* along with mutation/silencing of *RHCE*. RBCs with no RhD or RhCE proteins have reduced amounts (~20%) of RhAG.

Rh<sub>null</sub> cells also have reduced glycophorin B (GPB), a sialoglycoprotein that carries S, s, and U antigens. GPB appears to aid RhAG trafficking to the membrane because the RhAG protein in GPB-deficient cells has increased glycosylation reflecting longer dwell time in the endoplasmic reticulum. Rh<sub>null</sub> cells also lack LW (discussed in more detail below), a glycoprotein of unknown function that belongs to the family of intercellular adhesion molecules (ICAM-4). Band 3 (the anion exchanger) enhances the expression of the Rh antigens in transfected cells, suggesting that band 3 may also be associated with the Rh core complex.<sup>45</sup> The Rh core complex is linked to the membrane skeleton through interactions between CD47 and protein 4.2<sup>46</sup> and through an Rh/RhAG–ankyrin cytoskeleton connection.<sup>47</sup> Rh<sub>null</sub> RBCs also have reduced expression of CD47, an integrin-associated protein (IAP) that has wide tissue distribution, binds β<sub>3</sub> integrins, and is required for integrin-regulated Ca<sup>2+</sup> entry into endothelial cells. Its function on the red cells is unknown; however, it has been suggested that CD47 acts as a “do not eat me” signal and has become an experimental target for the treatment of hematologic malignancies and solid tumors.<sup>48</sup> Anti-CD47 therapies can interfere with pretransfusion testing.<sup>49</sup>

## Rh family and function

### MEP/Amt/Rh superfamily and ammonia transport

The Rh proteins reveal the power of comparative genomics and proteomics, in which sequence analysis and homology modeling can give important insight into mammalian protein function.<sup>20,50,51</sup> The Rh blood group proteins are well-known to blood bank professionals because of their importance in blood transfusion. However, the mammalian family of Rh proteins also includes RhAG in erythrocytes and the related proteins, RhBG and RhCG, in other tissues (kidney, liver, etc.). Homologous proteins can also be found in many other organisms, including bacteria, yeast (MEP), and plants (AMT),<sup>52</sup> where they function as ammonia transporters. For example, in the bacterial transporters, charged H<sup>+</sup> and neutral NH<sub>3</sub> are carried separately across the membrane after NH<sub>4</sub><sup>+</sup> deprotonation.<sup>53</sup> RhAG, RhBG, and RhCG have also been shown to transport ammonia across the plasma membrane in an electroneutral process that is driven by the NH<sub>4</sub><sup>+</sup> concentration and the transmembrane H<sup>+</sup> gradient.<sup>54,55</sup>

### Clinical relevance of Rh protein expression in RBCs

On the other hand, RhCE and RhD, when expressed in heterologous systems, do not transport ammonia<sup>56</sup> as these Rh proteins lack the highly conserved histidine residues located in the membrane pores that are critical for ammonia transport. Phylogenetic analysis indicates that RhCE and RhD proteins diverge the most from their homologs.<sup>57</sup> One hypothesis is that their main function is structural with the formation of the Rh trimer with RhAG. RhD and RhCE are subject to a low selection pressure as protein folding will be conserved for all alterations described except Rh<sub>null</sub> cells. Through the formation of Rh trimers, RhD/CE proteins maintain the RBC biconcave shape by interacting with the cytoskeleton.<sup>47</sup> In addition

to ammonia transport mentioned above,<sup>54</sup> RhAG is important for ion balance in RBCs.<sup>58,59</sup> Hereditary overhydrated stomatocytosis (OHSt), an autosomal dominant macrocytic hemolytic anemia, is caused by a p.Phe65Ser mutation in RhAG. OHSt red cells exhibit cation leak with elevated Na<sup>+</sup> and reduced K<sup>+</sup> content, and loss of ammonia conductance.<sup>58</sup>

## RH genotyping

### RHD zygosity

Serologic testing for red cell expression of D, C/c, and E/e can predict the likelihood that a sample is homozygous (D/D) or hemizygous (D/-) for *RHD*, but genotyping enables zygosity to be determined by assaying for the presence of a recessive D-negative allele. In prenatal practice, paternal *RHD* zygosity testing is important to predict the fetal D status when the mother has anti-D. Several different genetic events cause a D-negative phenotype, and multiple assays must be performed to accurately determine zygosity.<sup>60</sup> If the father is *RHD* homozygous, the fetus will be D-positive, and monitoring of the pregnancy will be required. If the father is hemizygous or heterozygous with one inactive or silenced *RHD*, there is a 50% possibility the fetus will be D-positive, and the D type of the fetus can be determined by fetal genotyping.

### Fetal typing

Genotyping is important in the prenatal setting to determine whether the fetus has inherited the paternal antigen to which the mother has a clinically significant antibody.<sup>61</sup> Fetal DNA can be isolated from cells obtained by amniocentesis; however, the discovery that cell-free, fetal-derived DNA is present in maternal plasma or serum by approximately five weeks gestation allows maternal plasma to be used as a source for fetal DNA testing.<sup>62</sup> Fetal DNA in maternal plasma is derived from apoptotic syncytiotrophoblasts, increases in concentration with gestational age, and is rapidly cleared following delivery.<sup>63</sup> There are technical challenges in that the small quantity of cell-free fetal DNA present relative to maternal DNA requires positive controls for the isolation of sufficient fetal DNA to validate negative results.

Isolation of fetal DNA from maternal plasma for *RHD* testing has become routine in several European countries to determine the fetal RhD phenotype and assess which patients are candidates for administration of Rh immune globulin.<sup>64</sup> This has been most successful for D typing because the D-negative phenotype in most samples is caused by the lack of *RHD*. Testing for the presence or absence of a gene is less demanding than testing for a single gene polymorphism or SNP. For the same reasons as *RHD* zygosity testing, diverse populations pose technical challenges.<sup>65</sup> Testing the maternal plasma for the presence of a fetal *RHD* gene eliminates the unnecessary administration of antepartum Rh immune globulin (RhIG) to the approximately 40% of D-negative women who are carrying a D-negative fetus. This approach is cost effective for some healthcare systems and reduces exposure to RhIG that is not entirely risk free as it is a human blood product.<sup>66</sup> Noninvasive testing for other fetal RBC antigens, including K and c, and platelet antigens (HPA), has also been reported.<sup>67,68</sup> Fetal typing from maternal plasma is not readily available in the United States as the cost of testing and liability concerns require widespread adoption nationally as a standard of practice to be feasible.

### Distinction between weak D and partial D

As indicated above, altered expression of D antigen is not uncommon. Weak D phenotypes have amino acid changes that primarily

affect the quantity of RhD in the membrane. Partial D phenotypes have amino acid changes that alter D epitopes or are hybrid proteins with portions of RhD joined to portions of RhCE. The distinction between weak D and partial D phenotypes is of clinical importance because the latter are at risk for anti-D. Routine serologic typing reagents cannot distinguish between these red cells; however, *RHD* genotyping strategies can discriminate between weak D and partial D and should be performed whenever the D type is in question.<sup>27</sup>

#### **Detecting patients at risk for production of Rh antibodies to high incidence antigens**

Alloimmunization is a serious complication of chronic transfusion, particularly in patients with SCD requiring long-term transfusion support. Many transfusion programs attempt to prevent or reduce the risk and incidence of alloantibody production in patients with SCD by transfusing RBC units that are antigens matched for D, C, E, and K. Although this approach reduces the incidence of alloimmunization, its effectiveness can be reduced because variant *RHD* and *RHCE* genes are common in African Blacks and individuals of mixed ethnic backgrounds. The prevalence of *RH* alleles that encode altered D, C, and e antigens in this patient group explains why some SCD patients become immunized to Rh despite Rh antigen matching for D, C, and E.<sup>38,69</sup> These antibodies often have complex Rh specificities, and it can be difficult or impossible to find compatible units after alloimmunization.

RH genotyping provides an effective approach to identify those patients who are homozygous for variant *RH* alleles and at risk for production of alloantibodies to high-incidence Rh antigens, as well as to identify compatible donors for transfusion. In some countries where the transfusion system is organized at the national level and in some US programs, all SCD patients are genotyped once for the RH variants to identify which of the patient's antigen(s) present a risk for alloantibody formation.<sup>70</sup> A challenging future approach will be to match SCD recipients and donor units according to RH alleles. This is expected to further reduce alloimmunization and is currently used to transfuse some patients with complex clinically significant RH antibodies.<sup>71</sup>

### **Immune response to Rh**

#### **Medical aspects**

Human red cells can express combinations of more than 400 different blood group antigens. Typing patient and donor cells for every known antigen with the intention of providing perfectly matched blood would not be practical or feasible. Fortunately, such extensive testing is not required for several reasons, the most important of which is that exposure to most foreign red cell antigens through transfusion does not lead to the production of clinically significant antibodies. RhD is one notable exception. As many as 50% of D-negative patients on deliberate immunization with D-positive red cells may develop high-titer, polyreactive, IgG antibodies that may persist for the rest of their lives even if they are never exposed to the antigen again.<sup>72</sup>

These antibodies can cause HTRs and can cross the placenta, causing HDFN when present in a D-negative female carrying a D-positive fetus. Although other Rh antibodies to C, c, E, and e can cause HTRs and HDFN, these antigens are much less immunogenic than D (~1% rate of sensitization).

In practice, D-positive patients can be transfused with either D-positive or D-negative RBCs—the absence of D on transfused cells will cause no harm—but it is deemed prudent to reserve units of D-negative blood (~15% of donor units) for D-negative

individuals. In cases of trauma and/or massive transfusion in which the patient's D status is unknown, efforts are made to provide D-negative blood for females of childbearing potential until the appropriate testing can be completed. When D-negative blood is in short supply, it may be necessary to transfuse D-negative patients with D-positive units.

Unlike ABO blood group antigens, which are expressed by all transfused blood cells including platelets, the D antigen is present only on red cells. Theoretically, the selection of platelet units for transfusion should be independent of the D status of the donor. However, a platelet transfusion may introduce as much as 5 mL of donor red cells, which if they are D-positive may be sufficient to immunize a D-negative patient. Therefore, the standard of care is to avoid transfusing D-negative females of childbearing potential with platelet units derived from D-positive donors. If such units are unavoidable, the administration of RhIG can be considered. A standard 300-µg dose of RhIG, which may inhibit the immunizing potential of up to 15 mL of D-positive red cells, would neutralize the effects of D-positive red cells from several mismatched platelet transfusions. If mismatched platelet transfusions are repeatedly given over time and their content of D-positive red cells is not expected to exceed the volume of red cells for which a standard dose of RhIG is indicated, then a single dose of the drug should be sufficient for at least 2–4 weeks of prophylaxis given the three-week half-life of IgG.<sup>73</sup> With respect to the transfusion of plasma products, the D status of the donor is not an issue because plasma products do not contain cellular or soluble material capable of inducing anti-D.

#### **Serologic aspects**

The immune response to Rh, like that to other peptide antigens, is typically thymus-dependent, requiring T-cell help. Upon exposure to a foreign Rh antigen, an IgM response may develop, but this is quickly followed by the production of IgG antibodies. Consequently, nearly all examples of Rh antibodies are IgG (primarily IgG1 and IgG3), which bind optimally to red cells at 37 °C and require the addition of an antoglobulin reagent to produce hemagglutination during in vitro testing. Although IgG1 and IgG3 classically initiate complement activation, most anti-Rh-containing sera do not do so. The usual explanation for this cites the relatively low copy number of Rh antigens per red cell, which results in Rh molecules situated too far apart on the cell surface to permit the simultaneous binding of C1q by multiple Rh IgG antibodies, as well as the cytoskeletal linkage and absence of capping of the Rh complex. Therefore, hemolysis from the transfusion of Rh-incompatible RBCs is generally extravascular resulting from the phagocytosis of IgG-coated erythrocytes by cells of the reticuloendothelial system.

Thanks to the use of RhIg in pregnancy, anti-D antibodies have become less common in patients. The Rh antibodies most found in the sera of alloimmunized individuals are now anti-E > anti-c > anti-e > anti-C.<sup>74</sup>

In approximately 50% of cases of warm-type autoimmune hemolytic anemia (WAIHA), autoantibodies are believed to be directed to Rh antigens by virtue of their “pan-reactivity” with all red cell phenotypes except  $Rh_{null}$  cells. However, direct binding of autoantibodies to putative epitopes common to D and CE polypeptides or to other components of the Rh membrane complex (RhAG, CD47, etc.) has yet to be demonstrated in WAIHA. The difficulties in approaching this problem are largely technical in nature and relate to both the inability to produce workable quantities of pure patient autoantibody in vitro (i.e., clone the autoantibody-producing B

lymphocytes) and the inability to purify Rh proteins in a way that retains their native, conformationally dependent epitopes.

### Molecular aspects of antibodies

The characterization of Rh antibodies on a molecular level, particularly that of anti-D, has been the focus of much study not only because of their clinical significance, but also because of the need to develop suitable *in vitro* methods for their production.<sup>75</sup> Ironically, because of better transfusion practice and the use of RhIG, alloimmunization of D-negative individuals is significantly less common (as are sera donors willing to be purposely hyperimmunized) so that supplies of Rh antibodies for use as typing reagents and for the preparation of RhIG are dwindling. To better understand the molecular makeup of Rh antibodies, investigations have focused on analyzing their variable regions to determine whether there are commonly shared genetic and/or structural features among Rh antibodies made by different individuals.

The repertoire of heavy chain variable regions in IgG anti-D was shown to be restricted to  $V_H$ 3–33 and highly related genes.<sup>76–79</sup> The use of molecular approaches such as site-directed mutagenesis,<sup>80</sup> complementarity-determining region (CDR) sequence randomization,<sup>81</sup> and heavy-chain/light-chain “shuffling”<sup>82</sup> has demonstrated the genetic relatedness among anti-D molecules directed against different D epitopes as well as among antibodies with D and E specificity. These studies and others<sup>83,84</sup> have supported the hypothesis that a restricted “Rh footprint” for D alloantibodies and a process termed “epitope migration”<sup>85</sup> play a role in the molding of the anti-Rh immune repertoire.<sup>86</sup>

Although the precise significance of immunoglobulin germline gene restriction by Rh antibodies is not fully understood, it may have practical significance for the preparation of anti-D for both therapeutic and diagnostic use. Although IgM anti-D monoclonal antibodies are well suited for antigen typing because they may serve as direct agglutinins, the fact that they are most often encoded by  $V_H$ 4–34 (the germline gene to which cold agglutinins are also restricted)<sup>87,88</sup> may explain why many IgM monoclonal anti-D typing reagents falsely agglutinate D-negative cells when used at cooler than recommended temperatures. This phenomenon may also explain the body of literature claiming that the D antigen was present on numerous nonerythroid cells. Using IgM anti-D, these investigators may have been detecting antigens of the I/i blood group system.

There have been many unsuccessful attempts to develop recombinant formulations of RhIG that would function as effectively as naturally derived anti-D, which have highlighted the role that Fc glycosylation may play in inducing anti-D immune prophylaxis.<sup>63,75,85,86,89</sup> Small, recent clinical trials with one recombinant monoclonal anti-D have shown promising results that need to be confirmed.<sup>90</sup>

## LW blood group system

### History and nomenclature

The LW antigens are the original so-called “Rhesus” antigens shared by humans and the rhesus monkey. As discussed earlier, the confusion occurred because LW antigens are more abundant on D-positive than on D-negative red cells. When the situation was clarified, the term *Rh* remained associated with the human antigen, so the antigen shared by humans and the rhesus monkey was renamed *LW* in honor of Landsteiner and Wiener (an unwelcome honor, as mentioned above).<sup>6</sup>

The confusion can be understood today in the transfusion service when weak examples of anti-LW<sup>a</sup> often appear initially to be anti-D. The LW system has undergone additional terminology revisions. The historical terminology of LW<sub>1</sub>, LW<sub>2</sub>, LW<sub>3</sub>, LW<sub>4</sub>, and LW<sub>0</sub> used to describe phenotypes was based on both the LW and the D status of the red cells but is now obsolete.<sup>6</sup> The phenotypes are now designated as LW(a+b−), LW(a−b+), LW(a+b+), and the rare LW(a−b−); the antigens are designated LW<sup>a</sup>, LW<sup>b</sup>, and LW<sup>ab</sup>.

### Genes and their expressed proteins

LW is encoded by a single gene located on chromosome 19. The 42-kD LW glycoprotein is a member of the family of Intracellular Adhesion Molecules (ICAMs) and has been renamed ICAM-4 (CD242). LW passes through the red cell membrane once, and the N-terminal extracellular region is organized into two immunoglobulin superfamily (IgSF) domains.<sup>91</sup>

### Basis for antigen expression

LW<sup>a</sup> is the common antigen, whereas LW<sup>b</sup> has an incidence of less than 1% in most Europeans.<sup>5</sup> The LW<sup>a</sup>/LW<sup>b</sup> polymorphism is caused by a single amino acid substitution, p.Gln70Arg, on the LW glycoprotein.<sup>92</sup> An increased frequency of the uncommon LW<sup>b</sup> antigen in Latvians and Lithuanians (6%), Estonians (4%), Finns (3%), and Poles (2%) suggests that the LW<sup>b</sup> mutation originated in the people of the Baltic region.<sup>93</sup> The LW<sup>ab</sup> antigen was originally defined by an alloantibody made by the only known (genetically verified) LW(a−b−) person, who lacks expression of all LW antigens.<sup>6</sup>

Rh antigen expression was not altered, and there was no clinical evidence of an associated pathology. The *LW* gene in this rare LW(a−b−) individual has a 10-bp deletion and a premature stop codon in the first exon.<sup>94</sup> Rh<sub>null</sub> red cells also lack LW antigens but do not have defective *LW* genes. Rh proteins appear to be required for LW to traffic to the membrane, and association with RhD is preferred.

LW antigens require divalent cations (e.g., Mg<sup>2+</sup>) for expression<sup>95</sup> and have intramolecular disulfide bonds that are sensitive to dithiothreitol (DTT) treatment.<sup>96</sup> This is helpful to differentiate anti-LW from anti-D because the D antigen is resistant to DTT. DTT-treated and untreated D-positive red cells can be prepared and tested for reaction with patient serum.<sup>97</sup> Also helpful in identifying anti-LW is the fact that LW antigens are expressed equally well on group O, D-positive and D-negative cord blood red cells.<sup>5</sup>

Transient loss of LW antigens has been described in pregnancy and patients with diseases, particularly Hodgkin’s disease, lymphoma, leukemia, sarcoma, and other forms of malignancy, in the absence of any overt associated RBC abnormality. Transient loss of LW antigens is associated with the production of autoanti-LW that can appear to be alloantibody.<sup>5</sup>

### LW function

The LW glycoprotein, ICAM-4, is a ligand with broad specificity for a number of  $\beta 1$ ,  $\beta 2$ ,  $\beta 3$ , and  $\beta 5$  integrins, including  $\alpha_L\beta_2$  (LFA-1),  $\alpha_M\beta_2$  (Mac-1),  $\alpha_4\beta_1$  (VLA-4),  $\alpha_v\beta_1$ ,  $\alpha_v\beta_5$ , as well as platelet integrin  $\alpha_{2b}\beta_3$ .<sup>91,98–101</sup> In addition, LW binds to the I domains of CD11a/CD18 and CD11b/CD18 on leukocytes.<sup>99,102</sup> The function of LW glycoprotein on mature red cells is not known, but LW may have a role in erythroblastic island formation through interaction with macrophage integrins.<sup>103,104</sup> LW appears to play a pathophysiologic role in the development of vaso-occlusion in SCD patients where it is mediated by endothelial cell integrins<sup>105,106</sup> and in the development of thrombosis mediated by activated integrins on platelets.<sup>101</sup>

## Summary

The genetic basis for the Rh antigens has been known for over 25 years. A gene deletion or silent *RHD* gene explains the absence of the D antigen in Rh-negative individuals. The large number of amino acid differences between the RhD and RhCE proteins explains why exposure in an individual lacking D often results in a vigorous immune response characterized by a very heterogeneous population of antibodies. The proximity of *RHD* and *RHCE*, duplicated genes on the same chromosome, has resulted in numerous exchanges by gene conversion between them. This has generated new polymorphisms and explains the many antigens observed in this blood group system. Rh antigen expression is affected not only by changes in extracellular amino acids but also by intracellular changes, highlighting the conformational nature of these blood group antigens and complicating attempts to map the epitopes to specific amino acid residues.

Serology cannot identify samples with altered RhD associated with risk for clinically significant anti-D, and *RHD* genotyping should be used to inform clinical decisions. *RH* genotyping can be used to determine paternal *RHD* zygosity to predict HDFN, and the fetus can be typed from amniocytes or from the maternal plasma. *RH* genotyping can also be used in patients facing long-term transfusion therapy to identify those who are homozygous for variant *RH* alleles and at risk to produce alloantibodies to high-incidence Rh antigens. When partnered with *RH* genotyping of donors, this approach promises to have a positive impact on transfusion therapy outcomes by reducing alloimmunization, especially in patients with SCD.

Questions remain concerning the function of the Rh proteins in erythrocytes. The discovery that Rh protein homologs also exist in the liver and kidney indicates that the Rh blood group antigens belong to a conserved family of proteins that function in ammonia

transport. Similarly, the function of LW on the mature red cell is not entirely clear, but its ability to interact as an adhesion molecule with a broad range of integrin-binding specificity suggests an important role in both normal red cell development as well as disease-associated processes such as vaso-occlusion and thrombosis.

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## CHAPTER 11

# Other protein blood groups

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Of the 43 currently known blood group systems, 8 are carbohydrate in nature and are discussed in Chapter 9. Antigens of the remaining 35 blood group systems are carried by (glyco)proteins. Three of these 35 systems—Rh (ISBT 004), RhAG (ISBT 030), and the closely associated LW (ISBT 016)—are discussed in detail in Chapter 10. The remaining blood group systems are summarized in Table 11.1 and are discussed here.

Many of the proteins carrying blood group antigens are functionally important, yet not all antibodies directed against these antigens represent an immunological barrier in transfusion medicine. Antibodies against the clinically important antigens can, however, result in a variety of complications ranging from mild to severe, delayed or acute. These adverse antigen–antibody interactions, deemed hemolytic transfusion reactions (HTRs), result in decreased survival/hemolysis of transfused antigen-positive red blood cells (RBCs). During pregnancy they can give rise to hemolytic disease of the fetus and newborn (HDFN) in a mother with antibodies directed at paternally inherited antigens on the fetal erythrocytes. Some antibodies are even implicated in difficulty to carry a fetus full term. Furthermore, the presence of antibodies may delay the availability of compatible units of RBCs for transfusion and, occasionally, make the transfusion of incompatible RBCs unavoidable, particularly in urgent settings or with antibodies to high-incidence antigens. This chapter addresses only those antigens that elicit formation of clinically significant antibodies while briefly commenting on the other blood group systems. Further details may be found in review articles<sup>1,2</sup> and specialized textbooks.<sup>3–5</sup>

### MNS blood group system (ISBT 002)

Antigens of the MNS blood group system are carried by glycoporphin A (GPA) and glycoporphin B (GPB). Both molecules are highly expressed in the erythrocyte membrane, with an estimated  $0.5\text{--}1.0 \times 10^6$  copies of GPA and  $0.1\text{--}0.3 \times 10^6$  copies of GPB per red cell.<sup>6</sup> GPA and GPB are encoded by homologous genes, GYPA and GYPB, at chromosome 4q28–q31. A third gene, GYPE, is also a member of this gene family but does not normally produce any protein. Both GPA and GPB are integral membrane proteins with a

single transmembrane  $\alpha$ -helical segment and with the N-termini located extracellularly (Figure 11.1). GPA was the first blood group-bearing protein whose primary structure was determined by amino acid sequencing.<sup>7</sup>

The M and N antigens are located on the N-terminus of GPA. Although two amino acid substitutions underlie M/N antigen identity (Ser20Leu and Gly24Glu), an important requirement for the recognition of these antigens by human antibodies is the presence of sialylated O-glycans attached to terminal serine and threonine residues. These glycans also provide the RBC with a net negative charge.

Although the genes share greater than 95% identity, GYPB encodes a shorter protein due to a single nucleotide variation (SNV) in intron 3 that otherwise mirrors the GYPA exon 3/intron 3 boundary. Since GYPB arose by gene duplication of GYPA, the first 26 amino acids of GPB are identical to those of GPA<sup>N</sup>, and GPB expresses an N-like antigen designated as 'N' (Figure 11.1).

The S, s, and U antigens are carried on GPB. S and s differ by one amino acid at position 48, with the S allele (*GYPB*\*03) encoding methionine and the s allele (*GYPB*\*04) encoding threonine. The U antigen is a high-prevalence antigen whose epitope is adjacent to the point where GPB enters the lipid bilayer (Figure 11.1).<sup>8</sup>

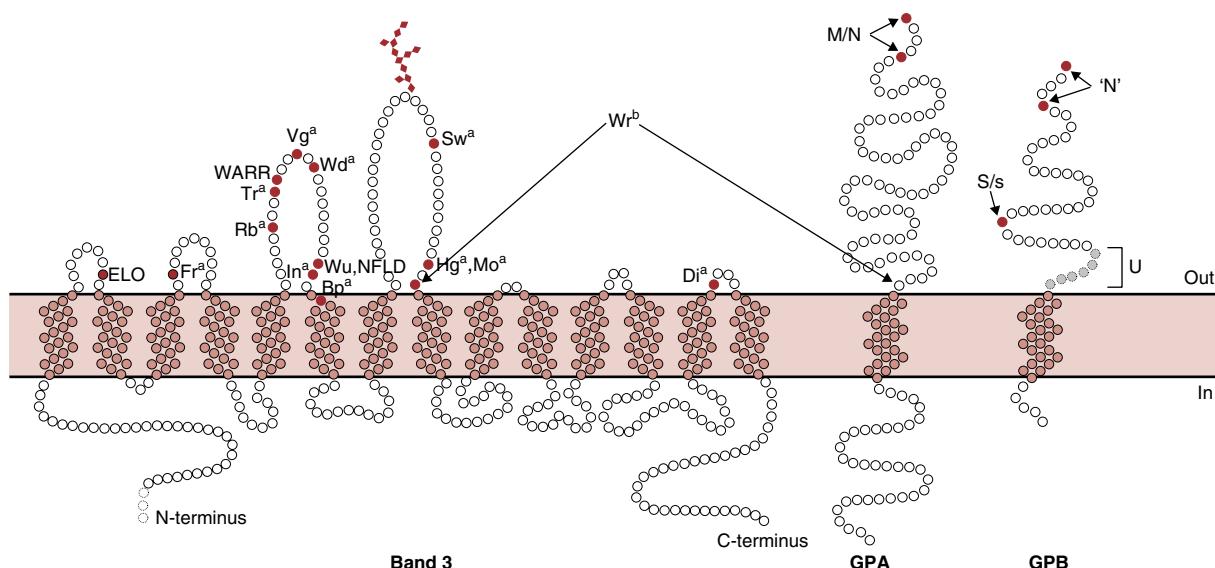
Homologous genes in close proximity to each other are often targets for unequal crossing over or gene conversion events. GYPA and GYPB are no exception and numerous recombination events have been described, resulting in hybrids of the two proteins.<sup>9</sup> This phenomenon is responsible for many low-prevalence MNS antigens carried by different hybrid proteins such as Mi<sup>a</sup>, V<sup>w</sup>, Hil, MUT, MINY, St<sup>a</sup>, and DANE. In addition to creating low-prevalence antigens, the expression of S and s antigens may also be altered on some hybrid proteins that can result in false-negative phenotype results.<sup>3,10</sup> Of note, while hybrids carrying the Mi<sup>a</sup> and Mur antigens are seldom encountered in European and African individuals, they are common in people from Southeast Asia. Targeted screening of Asian American blood donors at one large US blood center showed that 4.5% of donations were Mi(a+).<sup>11</sup>

GPA associates in the red cell membrane with Band 3 (SLC4A1), the RBC anion exchanger. The epitope of the Wr<sup>b</sup> antigen from the

**Table 11.1** Overview of the Protein Blood Group Systems other than Rh, RhAG, and LW

ISBT Number	System Name	Gene Name ISBT	Gene Name HGNC	Erythrocyte Membrane Component	Number of Antigens	Examples of Antigens
002	MNS	MNS	GYPA GYPB	Glycophorin A (CD235a), Glycophorin B (CD235b)	50	M, N, S, s, U
005	Lutheran	LU	LU	B-CAM (CD239)	27	Lu <sup>a</sup> , Lu <sup>b</sup>
006	Kell	KEL	KEL	Kell glycoprotein (CD258)	36	K, k, Js <sup>a</sup> , Js <sup>b</sup> , Kp <sup>a</sup> , Kp <sup>b</sup>
008	Duffy	FY	ACKR1	ACKR1 (CD234)	5	Fy <sup>a</sup> , Fy <sup>b</sup>
009	Kidd	JK	SLC14A1	Solute carrier 14A1	3	Jk <sup>a</sup> , Jk <sup>b</sup>
010	Diego	DI	SLC4A1	Solute carrier 4A1 (CD233)	23	Di <sup>a</sup> , Di <sup>b</sup> , Wr <sup>a</sup> , Wr <sup>b</sup>
011	Yt	YT	ACHE	Acetylcholinesterase	5	Yt <sup>a</sup> , Yt <sup>b</sup>
012	Xg	XG	XGCD99	Xg glycoprotein CD99	2	Xg <sup>a</sup> , CD99
013	Scianna	SC	ERMAP	Erythroblast membrane-associated protein	9	Sc1, Sc2, Sc3, Rd
014	Dombrock	DO	ART4	ADP-ribosyltransferase 4	10	Do <sup>a</sup> , Do <sup>b</sup>
015	Colton	CO	AQP1	Aquaporin-1 (AQP1)	4	Co <sup>a</sup> , Co <sup>b</sup>
017	Chido/Rodgers	CH/RG	C4A, C4B	Complement components C4A/C4B	9	Ch1, Ch2, Ch3, Rg1, Rg2
019	Kx	XK	XK	XK glycoprotein	1	Kx
020	Gerbich	GE	GYPC	Glycophorin C (CD236), Glycophorin D	13	Ge2, Ge3, Ge4
021	Cromer	CROM	CD55	Decay accelerating factor (CD55)	20	Cr <sup>a</sup>
022	Knops	KN	CR1	Complement receptor 1 (CD35)	12	Kn <sup>a</sup> , McC <sup>a</sup> , Sl <sup>a</sup>
023	Indian	IN	CD44	CD44	6	In <sup>a</sup> , In <sup>b</sup>
024	OK	OK	BSG	Basigin (CD147)	3	Ok <sup>a</sup>
025	RAPH	RAPH	MER2	CD151	1	MER2
026	JMH	JMH	SEMA7A	Semaphorin 7A (CD108)	8	JMH1
029	GIL	GIL	AQP3	Aquaporin 3	1	GIL
032	JR	ABCG2	ABCG2	ATP-binding cassette transporter G2	1	Jr <sup>a</sup>
033	LAN	ABC86	ABC86	ATP-binding cassette transporter B6	1	Lan
034	Vel	SMIM1	SMIM1	Small integral membrane protein 1	1	Vel
035	CD59	CD59	CD59	CD59	1	CD59.1
036	AUG	ENT1	SLC29A1	Equilibrative nucleoside transporter 1	4	At <sup>a</sup>
037	KANNO	KANNO	PRNP	Prion protein	1	KANNO1
039	CTL2	CTL2	SLC44A2	Choline transporter-like protein 2	2	Ver, Rif
040	PEL	PEL	ABCC4	ATP-binding cassette transporter C4	1	PEL
041	MAM	MAM	EMP3	Epithelial membrane protein 3	1	MAM
042	EMM	EMM	PIGG	Phosphatidylinositol glycan anchor	1	Emm
043	ABCC1	ABCC1	ABCC1	ATP-binding cassette transporter C1	1	WLF

ISBT, International Society of Blood Transfusion; HGNC, Human Gene Nomenclature Committee.



**Figure 11.1** Antigens of the Diego and MNS blood group systems in a schematic of band 3 and glycophorins A and B. The membrane domain of band 3 with 14 transmembrane segments is shown. Mutations underlying the Diego blood group antigens are located in the putative first, second, third, fourth, and seventh extracellular loops. Positions of the M and N antigens in GPA and the 'N', S, s, and U antigens in GPB are indicated. Arrows point to the sites in band 3 and GPA that are involved in the formation of the Wr<sup>b</sup> epitope and, therefore, have to come into close contact in the membrane.

Diego blood group system (see below) is formed by the association of GPA with Band 3. This further demonstrates the intimate association of GPA and Band 3 in the plasma membrane.<sup>12</sup>

### MNS in transfusion medicine

The most commonly encountered antibodies are directed against the M, N, S, and s antigens. Anti-M is a common antibody that may be found in the sera of persons who have not been exposed to human erythrocytes, which is sometimes termed “naturally occurring.” They are predominantly IgM with a thermal optimum below 30 °C; however, anti-M frequently contain an IgG component and occasionally are exclusively IgG. Nevertheless, anti-M is rarely clinically significant, and cases of hemolytic anti-M are usually IgG and react at 37 °C. Selection of blood for transfusion can be made based on a negative crossmatch irrespective of M antigen status. Similarly, anti-M is not considered to be an important antibody with regard to HDFN, although occasional cases have been described.<sup>13,14</sup> Anti-N is rare, most likely because of the immune tolerance induced by the ‘N’ antigen on GPB, and is usually a weak, cold-reactive antibody of no clinical significance. These should be distinguished from the strong and potentially clinically significant antibodies observed in persons of the rare phenotype M+N–S–s–U– who do not express GPB and for whom phenotypically similar, crossmatch-compatible blood should be provided.

In contrast to anti-M and anti-N, antibodies to S, s, and U usually occur after exposure to allogeneic erythrocytes and should be considered clinically significant because all are capable of causing HTRs and HDFN. The appropriate antigen-negative, crossmatch-compatible blood should be selected for transfusion for patients expressing these antibodies.

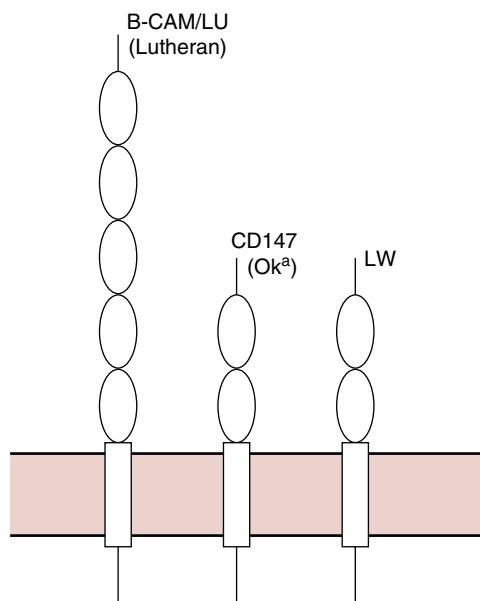
Lastly, severe HDFN due to rare antibodies to low-prevalence MNS antigens has also been reported and should be suspected in a strongly DAT-positive or symptomatic newborn where alloantibodies cannot be detected by routine screening.

### Lutheran blood group system (ISBT 005)

Lutheran antigens reside on B-CAM/LU (Figure 11.2), a pair of spliceosomes (protein products arising from the same gene because of alternative splicing of hnRNA) that belong to the immunoglobulin superfamily (IgSF).<sup>15,16</sup> Basal cell adhesion molecule (B-CAM) is involved in the adhesion of the basal surface of epithelial cells to the basement membrane. B-CAM/LU is a receptor for laminin.<sup>17,18</sup> Expression of B-CAM/LU is increased on erythrocytes from patients with sickle cell disease<sup>17</sup> and also on a number of malignant epithelial tumors,<sup>19</sup> which lose the polarity of B-CAM/LU expression found in normal tissues.

The Lutheran blood group system contains multiple antigens; however, clinically significant antibodies are rarely encountered. The most important antigens are Lu<sup>a</sup> and Lu<sup>b</sup>. The prevalence of Lu<sup>a</sup> is less than 10% in most populations, whereas Lu<sup>b</sup> is a high-prevalence antigen with an average occurrence of 99.8% in all populations. Lutheran antigens are poorly developed at birth and, not surprisingly, anti-Lu<sup>a</sup> has been associated only rarely with mild cases of HDFN. It does not cause transfusion reactions. Lu<sup>b</sup> is somewhat more immunogenic, and anti-Lu<sup>b</sup> has caused mild or moderate HTRs and mild HDFN. Of historical note, Lu and Se (Chapter 9) were the first two loci for which an autosomal linkage in humans was demonstrated.

The null phenotype, Lu(a–b–), is rare but quite interesting. It may result from three different patterns of inheritance. A recessive



**Figure 11.2** Members of the immunoglobulin superfamily carrying blood group antigens. B-CAM/LU contains five immunoglobulin domains and carries antigens of the Lutheran blood group system. CD147, or basigin, contains two Ig domains and carries the Ok<sup>a</sup> antigen. CD147 is similar to the LW protein, which is discussed in Chapter 8.

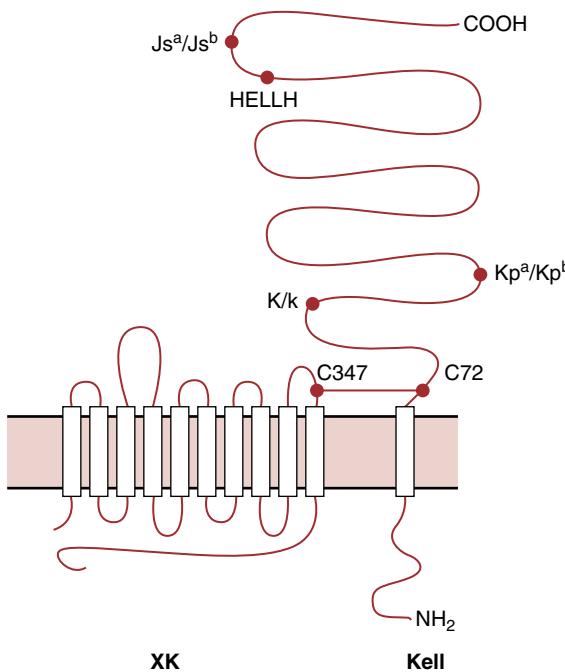
pattern of inheritance is associated with exceedingly rare null alleles of the LU gene. In the most common dominant type (so-called In(Lu)), heterozygous inheritance of mutations in the gene encoding KLF1, a common erythroid transcription factor, results in marked suppression of not only the Lutheran glycoprotein but also P1, i, In<sup>a</sup> and In<sup>b</sup>, and AnWj (see the Indian Blood Group System section).<sup>20,21</sup> The third cause of the Lu(a–b–) phenotype was first hypothesized to be the consequence of an X-linked recessive suppressor gene XS2.<sup>22</sup> Similar to In(Lu), the molecular basis has been identified as heterozygosity for a mutation in the GATA1 gene.<sup>23</sup> GATA1 is an important transcription factor in the differentiation of erythrocytes and megakaryocytes, and mutations in GATA1 account for cases of dyserythropoiesis, thrombocytopenia, and anemia.<sup>24</sup>

### Kell and Kx blood group systems (ISBT 006 and 019)

#### Structure, function, and interaction of the Kell and XK proteins

Antigens of the Kell blood group system are carried by a 93-kD red cell membrane glycoprotein, which consists of a short cytoplasmic N-terminal portion, a single membrane-spanning  $\alpha$ -helical segment, and a large, 665-amino-acid extracellular C-terminal portion held in a globular conformation by multiple disulfide bonds (Figure 11.3).<sup>25</sup> Kell antigens are disrupted by reducing agents such as dithiothreitol, suggesting that disulfide bonds are important in maintaining its antigenic conformation.

The Kell glycoprotein is a member of the neprilysin (M13) family of zinc metalloproteases. This family consists of Kell, neutral endopeptidase 24.11, two different endothelin-converting enzymes, the product of the PEX gene, and XCE.<sup>26</sup> Members of the M13 subfamily of membrane zinc endopeptidases have widely different roles, including processing of opioid peptides, Met- and Leu-enkephalin,



**Figure 11.3** Schematic representation of the Kell/XK complex in the red cell membrane. The XK protein is a multipass membrane protein, while Kell has only one transmembrane domain, most of which is exposed on the extracellular side. Due to multiple disulfide bonds, the extracellular portion of Kell is a globular structure; however, it is represented here schematically so that the positions of the main antigens can be shown. A disulfide bond between Cys72 of Kell and Cys347 of XK connects the two proteins. The position of the pentameric sequence HELLH is shown which is important for zinc binding and catalytic activity of zinc endopeptidases. The K/k polymorphism at amino acid 193 changes the consensus sequence for N-glycosylation at Asn191, which is not glycosylated in K. This difference in glycosylation may be important for the marked antigenicity of K. Positions of two additional sets of antithetical antigens Kp<sup>a</sup>/Kp<sup>b</sup> and Js<sup>a</sup>/Js<sup>b</sup> at amino acids 281 and 597 are indicated.

oxytocin, bradykinin, angiotensin, endothelins, and parathyroid hormone. Kell protein has been shown to preferentially activate endothelin-3,<sup>27</sup> however, the *in vivo* physiologic role of Kell protein is probably complex because K<sub>0</sub> (null) persons are apparently healthy.

Kell glycoprotein interacts in the erythrocyte membrane with the 37-kD protein XK, which plays an important role in the expression of Kell system antigens. In contrast to the Kell protein, XK is a multiple membrane-spanning protein with both of its N- and C termini located intracellularly (Figure 11.3). Absence of XK results in McLeod neuroacanthocytosis syndrome, an X-linked, late-onset neuromuscular disorder.<sup>28,29</sup> Structurally, XK resembles the glutamate transporters, but it has very little amino acid sequence homology with this group of transport proteins and a role in divalent cation homeostasis has been suggested since perturbation of the Kell/XK complex disturbs cell volume regulation. The Kell and XK proteins are covalently associated in the membrane by a disulfide link between cysteine 72 of Kell and cysteine 347 of XK (Figure 11.3).<sup>30,31</sup> The gene encoding the Kx antigen is located on the short arm of the X chromosome near the loci for X-linked chronic granulomatous disease (CGD) and Duchenne muscular dystrophy (DMD).<sup>32</sup>

### Kell in transfusion medicine

The Kell blood group system is the second most important protein blood group system in transfusion medicine after Rh because the antibodies can cause HTRs and HDFN. The most important antigens in this system are K (KEL1) and the antithetical k (KEL2). K and k are codominant autosomal alleles. While approximately 9% of whites and 2% of blacks are K-positive (i.e., K+k- or K+k+), the majority are K-negative (i.e., K-k+). Antigens of the Kell blood group system are highly immunogenic and, excluding ABO, K is second only to RhD in its potential to elicit production of alloantibodies.

Anti-K is a commonly encountered red cell alloantibody. Fortunately, it is easy to obtain blood for transfusion purposes since more than 90% of donor units are K-negative. In contrast, although anti-k is relatively rare, it is also of clinical significance and only 1 in 500 random donor units is antigen-negative. The other two sets of antithetical antigens to which antibodies are often found are Kp<sup>a</sup>/Kp<sup>b</sup> and Js<sup>a</sup>/Js<sup>b</sup> (Figure 11.3), although there are many other rare polymorphisms.

Mothers with anti-K are relatively rare, but since the introduction of Rh prophylaxis, anti-K accounts for nearly 10% of cases of severe HDFN. In contrast to RhD, anti-K titers are poor predictors of fetal anemia. In addition, affected Kell-alloimmunized infants have lower reticulocyte counts and amniotic fluid bilirubin concentrations than RhD-sensitized infants. Because Kell glycoprotein is synthesized early in erythropoiesis, anti-K has been hypothesized to suppress erythropoiesis at the progenitor cell level.<sup>33,34</sup> Administration of recombinant erythropoietin (rHuEPO) to the newborn has been tried with some success in rare cases where the mother has a Kell blood group system antibody causing prolonged anemia in a newborn whose RBCs and their progenitors express the cognate antigen in question.<sup>35,36</sup>

It is important to determine if a fetus is at risk when the mother has anti-K. The putative father should be typed; if he carries the K antigen, genotyping from amniocentesis or cell-free fetal DNA (cffDNA) in the mother's plasma can be performed using molecular techniques.

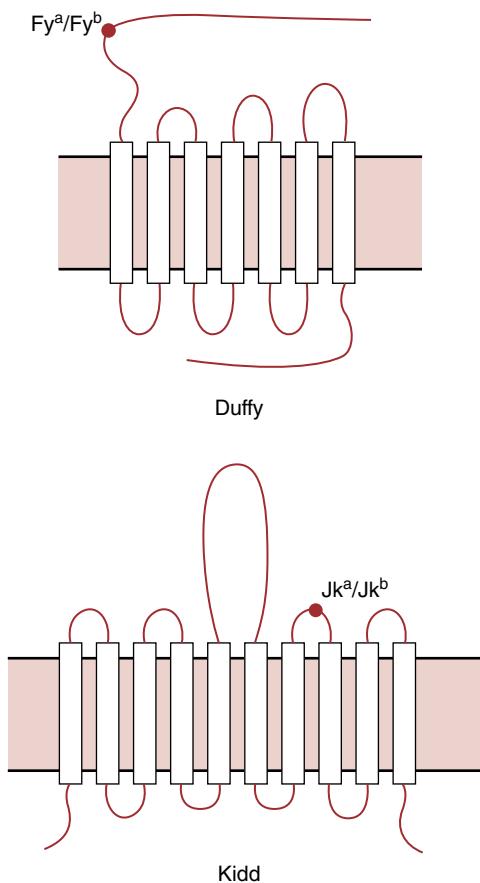
### Null phenotypes

There are two rare but clinically interesting null phenotypes. Rare individuals have erythrocytes that completely lack the Kell glycoprotein. Although these cells exhibit the null phenotype (K<sub>0</sub>), they are morphologically normal and survive normally *in vivo*. Yet, individuals lacking the XK protein, and thus Kx antigen, exhibit depressed levels of the Kell glycoprotein.<sup>25</sup> This phenotype, known as the McLeod neuroacanthocytosis syndrome, is associated with acanthocytic erythrocytes and a mild chronic hemolytic anemia.<sup>28</sup> It is coupled with late onset of neuromuscular symptoms that include muscle weakness or atrophy, cognitive alterations, as well as psychiatric symptoms. The phenotype is caused by large gene deletions encompassing XK.<sup>37</sup>

### Duffy blood group system (ISBT 008)

#### Structure and function of the Duffy protein

The Duffy gene (ACKR1) encodes a glycoprotein of 336 amino acids with a molecular weight of 36 kD. The Duffy glycoprotein has seven transmembrane  $\alpha$ -helical domains and is homologous with proteins of the G-coupled protein receptor family.<sup>38</sup> The Fy<sup>a</sup> and Fy<sup>b</sup> antigens are carried on the extracellular N-terminus; the C-terminus is intracellular (see Figure 11.4). Duffy protein func-



**Figure 11.4** Schematic depiction of proteins carrying the Duffy and Kidd blood group antigens. The Duffy antigen receptor for chemokines (DARC) consists of seven transmembrane segments. The N-terminus is located extracellularly, functions as both the chemokine and *P. vivax* attachment site, and carries the Fy<sup>a</sup>/Fy<sup>b</sup> polymorphism. The Kidd protein has 10 transmembrane segments, both N- and C-termini are intracellular, and the amino acid determining the Jk<sup>a</sup>/Jk<sup>b</sup> polymorphism is located in the fourth extracellular loop.

tions as a chemokine receptor and is also known as the Duffy atypical chemokine receptor 1 (ACKR1).<sup>39</sup> Duffy binds CXC chemokines, such as IL8 (interleukin-8) and MGSA (melanoma growth stimulatory activity), as well as CC chemokines, for instance RANTES (Regulated upon Activation, Normal T Cell Expressed and Presumably Secreted) and MCP1 (macrophage chemoattractant protein-1).<sup>40</sup> It is currently not known why a red cell, with its limited metabolism and response to chemokine binding, would carry a significant number of chemokine receptors at its surface. One possible explanation is that the chemokine receptor acts as a scavenger for locally released chemokines. However, individuals who do not express the Duffy protein either on erythrocytes or in all tissues are phenotypically normal, suggesting that the Duffy protein is dispensable.

Duffy is the primary receptor for the human malarial parasite *Plasmodium vivax*, which infects the erythrocytes of Duffy-positive individuals.<sup>41</sup> Evolutionary pressure exerted by *P. vivax* has led to the selective inactivation of erythroid ACKR1 in West and Central Africa (the Fy(a–b–) phenotype). Recently, Duchene *et al.* identified a role for ACKR1 in normal hematopoiesis and showed that in Fy(a–b–) individuals, phenotypically distinct polymorphonuclear

(PMN) leucocytes were produced that readily left the bloodstream, resulting in an apparent neutropenia (benign ethnic neutropenia, BEN).<sup>42</sup> Genome wide association studies confirmed that rs2814778, the SNV that inactivates the GATA1 binding site responsible for the Fy(a–b–) phenotype in these individuals, was also responsible for the BEN phenomenon and showed for the first time that ACKR1 was normally expressed on neutrophils.<sup>43</sup> In ACKR1-null individuals, erythroid progenitors preferentially differentiated to myeloid cells, and that the absence of ACKR1 on neutrophils resulted in premature egression to the spleen.

### Duffy in transfusion medicine

The two main alleles of the Duffy blood group system are the anti-thetical codominant antigens Fy<sup>a</sup> and Fy<sup>b</sup> whose genetic determinant is a Gly/Asp polymorphism in position 44 (Figure 11.4).<sup>44</sup> These two alleles occur with similar frequencies in persons of European ancestry, with Fy<sup>a</sup> being somewhat more common. The Fy(a–b–) phenotype is extremely rare among whites, but approximately two-thirds of African Americans and more than 90% of native West Africans are Fy(a–b–). This is most likely caused by the genetic adaptation for resistance to *P. vivax* malaria, although this hypothesis is contradicted by some data. For instance, in contrast to the other malarial parasites, *P. vivax* causes a relatively mild form of malaria.<sup>38</sup> Furthermore, despite the high prevalence of Duffy-negative individuals in West Africa, the Duffy protein has recently been shown to be a cofactor in platelet factor 4 (PF4)-dependent killing of *P. falciparum* parasites, suggesting that it has a protective role in this disease.<sup>45</sup>

Fy<sup>a</sup> antibodies are found relatively frequently, constituting 6–10% of the clinically significant antibodies identified by immunohematology laboratories. Both immediate and delayed HTRs caused by Fy<sup>a</sup> incompatibility have been described, ranging from mild to severe hemolysis. HDFN due to anti-Fy<sup>a</sup> is usually mild; only a few cases of severe HDFN have been reported. Fy<sup>b</sup> is a relatively poor immunogen and, consequently, anti-Fy<sup>b</sup> is considerably less common. Although, anti-Fy<sup>b</sup> is usually found in delayed HTRs, it has caused severe acute hemolysis on rare occasions. Only a few cases of mild HDFN have been attributed to the presence of anti-Fy<sup>b</sup>.

The underlying molecular mechanism for the Fy(a–b–) phenotype in individuals of African descent is a single nucleotide substitution c.–67T>C upstream of ACKR1 exon 1. This change interrupts a GATA1 site and abolishes transcription of the gene in the erythroid cells, while leaving the transcription in other tissues unaffected.<sup>46</sup> Consequently transfused Fy(a–b–) people of African ancestry do not develop anti-Fy<sup>b</sup>, and matching is not required for this antigen. In Africans, the change is carried exclusively on the FY\*B allele; however, it has been found on the FY\*A allele in individuals from Papua New Guinea.<sup>47</sup>

### Kidd blood group system (ISBT 009)

Kidd (SLC14A1) is an integral protein with 10 transmembrane domains and both N- and C-termini located intracellularly (Figure 11.4). Of the five extracellular loops, the longest third loop is N-glycosylated, and the relatively short fourth loop carries an Asp280Asn polymorphism corresponding to the Jk<sup>a</sup>/Jk<sup>b</sup> antigens.<sup>48</sup> Kidd protein is expressed not only on the red cell surface but also on neutrophils and in the kidney.

The two main antigens of the Kidd blood group system, Jk<sup>a</sup> and Jk<sup>b</sup>, are found with almost identical frequencies in white populations, while Jk<sup>a</sup> has a higher prevalence in people of African origin.

$\text{Jk}^a$  is a better immunogen, and anti- $\text{Jk}^a$  is found more frequently than anti- $\text{Jk}^b$ . Anti- $\text{Jk}^a$  may cause severe immediate or delayed HTRs and, occasionally, HDFN. It is one of the most dangerous immune antibodies because of its tendency to decrease to undetectable levels in between transfusions, its relatively weak reactivity in standard antibody identification tests, and its ability to bind complement. For these reasons, it accounts for a large proportion of delayed HTRs. Anti- $\text{Jk}^b$  may also cause immediate or delayed HTRs, albeit less severe than those caused by anti- $\text{Jk}^a$  (see <https://www.shotuk.org/shot-reports/> for more information). Several cases of mild HDFN caused by anti- $\text{Jk}^b$  have been reported. Weakly expressed forms of the protein have been described, in which the  $\text{Jk}^a$  and/or  $\text{Jk}^b$  antigens may be barely detectable.<sup>49–51</sup>

The early discovery that cells of the  $\text{Jk}(\text{a}-\text{b}-)$  phenotype are resistant to lysis by 2M urea alluded to the function of the glycoprotein carrying the Kidd antigens. Based on this finding and in vitro expression of the cloned Kidd cDNA,<sup>52–54</sup> it is now known that the protein is the primary erythrocyte urea transporter (SLC14A1). While the importance of urea transport for RBCs is not completely understood, its presence or absence may not be critical for red cell structure and function because carriers of the  $\text{Jk}(\text{a}-\text{b}-)$  phenotype have erythrocytes indistinguishable from those of controls.

## Diego blood group system (ISBT 010)

### Structure and function of the Diego protein

Antigens of the Diego blood group system are carried by band 3 (SLC4A1, anion exchanger 1), the most abundant integral protein of the red cell membrane together with GPA (see above). Band 3 is also one of the most important proteins for the structure and function of the membrane because it maintains red cell integrity by linking the red cell membrane to the underlying spectrin-based membrane skeleton. It also mediates exchange of chloride and bicarbonate anions across the plasma membrane, thereby significantly increasing the carbon dioxide carrying capacity of blood. Several disorders of red cell structure and function have been associated with mutations in the band 3 gene, including Southeast Asian ovalocytosis,<sup>55</sup> autosomal dominant spherocytosis, and distal renal tubular acidosis.<sup>56</sup>

Band 3 consists of an N-terminal cytosolic domain that interacts with the cytoskeleton and a membrane domain, responsible for gas transport. The membrane domain contains 14 transmembrane helices connected by ecto- and endoplasmic loops (Figure 11.1).<sup>57,58</sup> The fourth loop of band 3 is N-glycosylated, and the attached carbohydrate chain carries over half of the red cell ABO blood group epitopes.<sup>59</sup>

### Diego in transfusion medicine

$\text{Di}^a$  was originally described in South American Indians by Layrisse *et al.* in 1955<sup>60</sup> and was reported later to be carried on the Memphis II variant of erythroid band 3.<sup>61</sup> The antithetical antigen  $\text{Di}^b$  was reported in 1967.<sup>62</sup>  $\text{Di}^a$  and  $\text{Di}^b$  represent codominantly expressed gene products.  $\text{Di}^a$  is a low-prevalence blood group antigen in persons of European ancestry who carry the antithetical high-prevalence antigen  $\text{Di}^b$ . However,  $\text{Di}^a$  was used as one of the original markers for studying migration of people from Southeast Asia across the Bering Strait and southward through the American continents.<sup>63</sup> Prevalence of  $\text{Di}^a$  is as high as 8% in certain areas of Southeast Asia and reaches up to 40% in some groups of South American Indians. The  $\text{Di}^a/\text{Di}^b$  antigens are defined by the amino acids p.Pro854Leu in the last extracellular loop of band 3.<sup>64</sup>

Another antithetical pair of antigens,  $\text{Wr}^a/\text{Wr}^b$ , were mapped to the fourth extracellular loop (p.Glu658Lys).<sup>12</sup> The  $\text{Wr}^b$  antigen is only expressed when both GPA and band 3 protein are expressed in the erythrocyte membrane; thus, erythrocytes that lack GPA (so-called En(a–) phenotype) but have normal band 3 are also  $\text{Wr}(\text{b}-)$  (Figure 11.1).

A further 17 low-prevalence antigens are associated with single-point mutations on band 3 and included in the Diego system.<sup>65–67</sup> Positions of the amino acid polymorphisms in the band 3 molecule are shown in Figure 11.1, which also depicts the regions of band 3 and GPA that interact in the membrane and are involved in the formation of the  $\text{Wr}^b$  antigen.

Some antigens of the Diego blood group system have been localized to regions of band 3 protein implicated in the adhesion of abnormal erythrocytes, such as sickle cells or malaria-infected erythrocytes, to vascular endothelium.<sup>68,69</sup> Erythrocytes from carriers of low-prevalence blood group antigens in band 3 may serve as a model for the evaluation of the sequence requirements for adhesion. The so-called *senescent* or *aging red cell antigen* may also be located in the extracellular loops of band 3.<sup>70</sup>

In general, antibodies to Diego system antigens have been implicated in HTRs and HDFN and should be considered clinically significant; however, it has been estimated that 30% of autoimmune sera contain anti- $\text{Wr}^a$  and these are not generally considered to be clinically important.

## Xg blood group system (ISBT 012)

The Xg system contains two antigens carried on glycoproteins,  $\text{Xg}^a$  and CD99.<sup>71</sup> The role of  $\text{Xg}^a$  on erythrocytes has not yet been determined; however, it is 48% homologous to CD99, an adhesion molecule. A single nucleotide variation (SNV) at rs311103 upstream of the *XG* gene, which disrupts binding to the GATA1 box in erythropoietic cells, differentiates between the  $\text{Xg}(\text{a}+)$  and the  $\text{Xg}(\text{a}-)$  phenotype, as well as controlling expression levels of CD99.<sup>72,73</sup>

$\text{Xg}^a$  was the first blood group antigen to be assigned to a chromosome due to its differential distribution between men and women.<sup>74</sup> The *CD99* gene and the rs311103 locus lie within pseudoautosomal region 1 (PAR1) on the X and Y chromosomes, while *XG* on the X chromosome straddles the pseudoautosomal boundary (PAB) and consequently escapes lyonization (X-inactivation).<sup>71</sup> As such, men carry two copies of *CD99* but only a single copy of *XG*. On the Y chromosome, a pseudogene *XGY2* consisting of the first three exons of *XG* is present in PAR1 and does not express a protein product.  $\text{Xg}^a$  antibodies are clinically insignificant.

## Scianna blood group system (ISBT 013)

The seven antigens of the Scianna blood group system are carried by the erythroblast membrane-associated protein (ERMAP), potentially a receptor/signal transduction molecule specific for erythroid cells.<sup>75</sup> Mild delayed HTRs and mild HDFN caused by anti-Sc-2 and anti-Sc-3 antibodies have been reported.

## Colton and GIL blood group systems (ISBT 015 and 029)

Antigens of these two blood group systems are carried by members of the large aquaporin family. Antibodies against the two antigens of the Colton blood group system, the high-prevalence  $\text{Co}^a$  and the less common antithetical  $\text{Co}^b$ , are rare and have only occasionally been

associated with mild HTRs and mild HDFN. These two antigens, together with the high-prevalence Co3 and Co4, are present on all erythrocytes except those of the very rare null phenotype Co (a–b–). They are carried by aquaporin-1 (AQP1),<sup>76</sup> a member of a large family of water channels.<sup>77</sup> The absence of AQP1 in the rare Co(a–b–) phenotype is associated with only slightly abnormal erythrocytes and with normal kidney function,<sup>78</sup> suggesting an alternative water regulating mechanism. The GIL antigen is carried by aquaporin-3 (AQP3), which differs from AQP1 in that it transports not only water, but also glycerol and urea.<sup>79</sup> Anti-Gil is rare, but transfusion reactions have been reported. No HDFN has been described.

### Chido/Rodgers blood group system (ISBT 017)

Antigens of the Chido/Rodgers blood group system are the only protein antigens that are not produced by erythrocytes but instead adhere to the red cell surface (Lewis antigens are glycolipids, see Chapter 9). They are carried by the complement component C4 and adsorbed onto RBCs. Although antibodies against the nine known antigens of the system are generally benign, a severe anaphylactic reaction following a transfusion of platelets to a patient with anti-Ch3 has been described.<sup>80</sup> In the laboratory, anti-Ch/-Rg are readily inhibited by pooled plasma.

### Gerbich blood group system (ISBT 020)

There are 13 antigens in the Gerbich blood group system, and similarly to the MNS system, the antigens are located on glycophorins, i.e., glycophorin C and D (GPC and GPD). However, the glycophorin terminology is the only common feature of these two classes of glycophorins. GPC and GPD are the products of a single gene, GYPC, and are the products of alternative splicing. GPC is produced by a full-length gene transcript, whereas the less abundant GPD is produced from a second initiation methionine that encodes a protein that is 21 amino acids shorter. Although present in much fewer copy numbers than GPA and GPB, GPC plays an important role in the structural integrity of the red cell membrane.<sup>81</sup> In the Leach phenotype, deletions of exons 3 and 4 or a frameshift mutation leads to complete absence of glycophorins C and D from the plasma membrane.<sup>82</sup> The affected individuals have moderate elliptocytosis and decreased red cell deformability and mechanical stability. Antibodies in the Gerbich system, to both the high- and low-prevalence antigens, are rare although anti-Ge2 is more commonly found in individuals from the Middle East. Anti-Ge2 is generally not clinically significant and has not been implicated in HDFN; however, anti-Ge3 may cause a transfusion reaction and has been implicated in severe HDFN. GPC is expressed early on erythroid progenitors, and corresponding antibodies may result in severe anemia in fetuses similar to HDFN in women with anti-K.

### Knops blood group system (ISBT 022)

Antigens are located on the C3b/C4b complement receptor 1 (CR1, CD35). CR1 protects erythrocytes from autohemolysis by inhibiting the classical and alternative complement pathways through cleavage of C4b and C3b. CR1 is a large 190- to 280-kD molecule. It contains 30 complement control protein domains (CCPDs) of about 60 amino acids and seven CCPDs form a long homologous repeat (LHR) of about 450 amino acids. Various forms of CR1 contain up to six LHRs.<sup>83</sup> Erythrocyte CR1 binds immune complexes and carries them to the liver and spleen for removal.

The majority of blood group antigens in the Knops system are carried primarily in LHR-D, including: the antithetical antigen pairs Kn(a/b), McC(a/b), and KCAM/KDAS; the antigen trio Sl1/Sl2/Sl3; and Yk(a). Antibodies to the high-prevalence antigens Kn<sup>a</sup>, McC<sup>a</sup>, and Yk<sup>a</sup> are relatively common but they are readily inhibited by commercially available soluble recombinant LHR-D peptides. Failure of some Knops antibodies to be inhibited by these peptides led to the recent identification of another antithetical antigen pair DACY/YCAD located on LHR-C.<sup>84</sup> Antibodies to Knops system antigens are not clinically significant but can take time to identify. Expression of CR1 on erythrocytes of one individual to another varies widely from 20 to 1500 molecules and is decreased in hemolytic anemias, AIDS, systemic lupus erythematosus, and other autoimmune disorders. *Plasmodium falciparum*-infected erythrocytes deficient in CR1 have greatly reduced rosetting capacity, indicating an essential role for CR1 in rosette formation. This suggests the possibility that CR1 polymorphisms in Africans that affect the interaction between erythrocytes and parasite-encoded protein PfEMP1 may protect against severe malaria.<sup>85</sup> CR1 could therefore be a potential target for future therapeutic interventions to treat severe malaria.

### Indian blood group system (ISBT 023)

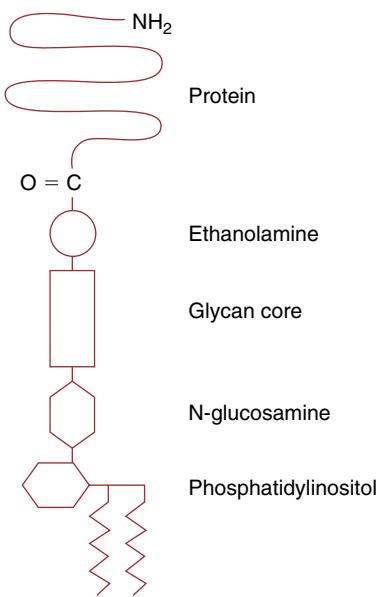
This system consists of the antithetical pair, In<sup>a</sup>/In<sup>b</sup>, and high-prevalence antigens INFI, INJA, INRA, and INSL. These antigens reside on CD44, an adhesion molecule also expressed in leukocytes, fibroblasts, epithelial cells, and other tissues. CD44 is an important lymphocyte marker that functions as a hyaluronan receptor and a lymphocyte homing receptor (Figure 11.2).<sup>86</sup> Transfection of non-adherent cell lines with CD44 cDNA confers an adherent phenotype.<sup>87</sup>

The rare In(a+b–) phenotype is found in individuals from South Asia, stretching east from Iran to India. As with the Lutheran antigens, the expression of Indian blood group system antigens is suppressed in the In(Lu) phenotype (see above). The high-prevalence antigen, AnWj, is also associated with CD44 and is a receptor for *Haemophilus influenzae*.<sup>88</sup> Antibodies to Indian blood group system antigens are rare and of limited clinical significance. Anti-AnWj is extremely rare.

### Blood group antigens on glycosylphosphatidylinositol-linked proteins: Cartwright (ISBT 011), Dombrock (ISBT 014), Cromer (ISBT 021), JMH (ISBT 026), CD59 (ISBT 035), KANNO (ISBT 037), and EMM (ISBT 042)

The common denominator of antigens in these blood group systems is the linkage of the carrier protein to the glycosylphosphatidylinositol (GPI) anchor (Figure 11.5). The Cartwright (Yt) blood group system antigens are located on red cell acetylcholinesterase (AChE) and include the antithetical pair Yt<sup>a</sup>/Yt<sup>b</sup> and three high-prevalence antigens,YTEG, YTLI, and YTOT. In the laboratory, the antibodies are readily inhibited by soluble AChE. Anti-Yt<sup>a</sup> is usually benign; however, continuously transfusing against patient phenotype may result in reduced survival of RBCs.<sup>89</sup> The function of acetylcholinesterase on erythrocytes is not understood, although it appears to play a role in vascular signaling.<sup>90</sup>

The Dombrock blood group system consists of the antigen pair Do<sup>a</sup>/Do<sup>b</sup> and eight other high-prevalence antigens located on



**Figure 11.5** Schematic representation of a GPI-anchored protein. The membrane anchor is provided by phosphatidylinositol. The inositol moiety binds to a glycan core via a molecule of N-glucosamine. The glycan core is attached via ethanolamine to the C-terminus of the protein.

ART-4, a member of the adenosine 5'-diphosphate (ADP)-ribosyltransferase ectoenzyme gene family.<sup>91</sup> Absence of ART4 is defined by the Gy(a–) phenotype. Although rare, antibodies to Dombrock blood group antigens can be of clinical importance. ART4 expression is developmentally regulated during erythroid differentiation and occurs at highest levels in the fetal liver.

The polymorphic Cromer blood group system contains 20 antigens located on decay-accelerating factor (DAF, CD55), a complement regulatory protein. Extraordinarily, many of the polymorphisms appear restricted to one ethnic group or another, making it a very interesting blood group system.<sup>92</sup> Antibodies to Cromer blood group antigens are rare and of limited clinical importance, and due to the expression of DAF on the placenta, antibodies disappear over the course of pregnancy and pose no threat to the fetus.<sup>93</sup> Although DAF was the first complement regulatory protein identified, it plays only a minor role in complement-mediated lysis, the more important being CD59 (MIRL). This was clearly demonstrated in the case of the null phenotype, IFC-negative (Inab), which is associated with lack of DAF expression on all circulating cells but not with increased hemolysis.<sup>94</sup>

The eight high-prevalence antigens of the JMH blood group system reside on a GPI-linked protein, semaphorin 7A (CD108), which is part of a plasma membrane complex associated with intracellular protein kinases.<sup>95</sup> CD108 is expressed in multiple tissues and may play a role in signal transduction. Anti-JMH is generally a weak, clinically benign antibody found in older people, leading to the colloquial terms the “old boys club” or the “over 60 group” for antibody producers.

CD59, also known as MAC-inhibitory protein, was assigned blood group system status following the report of an antibody produced in response to transfusion in a CD59-deficient girl.<sup>96</sup> Although the absence of CD59 in this and other patients has severe clinical consequences, the clinical significance of the antibody is not known.

The high-prevalence antigen KANNO was recently shown to be due to a missense mutation in *PRNP*, the gene encoding the prion protein.<sup>97</sup> Anti-KANNO was first described in 1991 and is most commonly found in Japanese women with a history of pregnancy.<sup>98</sup>

There have been no reports of HTRs or HDFN, and its clinical significance remains unknown.

Another high-prevalence antigen located on a GPI-linked protein belongs to the EMM blood group system. The Emm-negative phenotype is caused by different mutations ranging from single nucleotide changes to partial gene deletions in *PIGG*, a gene in the GPI synthesis pathway.<sup>99,100</sup> Anti-Emm is rare and is most often naturally occurring.<sup>3</sup>

Not surprisingly, the expression of all GPI-linked antigens is decreased in paroxysmal nocturnal hemoglobinuria (PNH), a multi-symptomatic disorder caused by defects in the X-linked phosphatidylinositol glycan class A (*PIG-A*) gene, which participates in an early step of GPI anchor synthesis.<sup>101</sup> The pathophysiology of PNH is due almost exclusively to the absence of CD55 and CD59, which are important regulators of the complement system: CD55 accelerates the rate of destruction of membrane-bound C3 convertase and thus limits C3 activation; and CD59 reduces the amount of the membrane attack complex (MAC) formed by preventing C9 accumulating and thus lytic pore formation. Anemia, due to both hemolysis and bone marrow failure, and thrombosis are also common in PNH patients.<sup>101</sup> See Chapter 29 for a complete discussion of PNH.

### Other minor blood group systems: OK (ISBT 024), RAPH (ISBT 025), JR (ISBT 032), LAN (ISBT 033), VEL (ISBT 034), AUG (ISBT 036), CTL2 (ISBT 039), PEL (ISBT 040), MAM (ISBT 041), and ABCC1 (ISBT 043)

The three high-prevalence antigens of the OK blood group system are carried on basigin (CD147), a widely distributed IgSF molecule.<sup>102</sup> Rare Ok(a–) individuals have so far been reported only in Japan, and the absence of the high-prevalence antigens OK2 and OK3 has been described in single families.<sup>103</sup> As with LW, its extracellular domain contains two immunoglobulin domains. The function of the Ok glycoprotein in erythrocytes is not known, but it has recently been described as a novel receptor of *P. falciparum*.<sup>104</sup>

MER2 is the only antigen of the RAPH system carried on CD151.<sup>105</sup> CD151 is a tetraspannin that is expressed not only on erythrocytes but also on basement membranes, where in the kidney and in skin it is thought to facilitate binding of integrins to the extracellular matrix to maintain integrity.<sup>106</sup>

To date, four proteins in the ABC transporter family have been shown to carry blood group antigens. In 2012, the molecular basis for the Jr(a–) and Lan– phenotypes was identified, and in 2020, the PEL– negative phenotype was resolved.

The Jr<sup>a</sup> antigen was localized to ABCG2, where the Jr(a–) phenotype represented the null phenotype.<sup>107,108</sup> ABCG2 is a well-characterized protein that confers multidrug resistance (e.g., in breast cancer) and is also important in porphyrin homeostasis. It has a high affinity for urate, and Japanese Jr(a–) individuals have a higher incidence of gout. Anti-Jr<sup>a</sup> is not usually considered clinically important; however, it has caused severe HDFN.<sup>109</sup>

The high-prevalence Lan antigen is carried on ABCB6, a mitochondrial porphyrin transporter considered essential for heme synthesis.<sup>110</sup> Like Jr(a–), Lan– individuals represent the null phenotype and lack the protein. ABCB6 is highly expressed during erythropoiesis. Anti-Lan is not generally considered clinically important, and antigen expression is variable.<sup>111</sup>

The PEL antigen is carried by the ABC transporter ABCC4, also called MRP4 for multidrug resistance protein 4. Whole genome sequencing combined with comparative global proteomic

investigations has revealed a large deletion in *ABCC4/MRP4* responsible for the PEL negative phenotype.<sup>112</sup> While this rare phenotype is associated with impaired platelet aggregation, no implications regarding RBC function have been suggested. Even though reduced survival of incompatible RBCs has been observed, anti-PEL is not generally considered clinically important.

The most recent blood group system to be described is named simply ABCC1 after the protein carrier also known as multidrug resistance protein 1. One high-prevalence antigen, WLF, has been described. WLF negative individuals represent the null phenotype, similar to the blood group antigens described above in this section. Even though the ABCC1<sub>null</sub> phenotype seemingly has little effect on the RBCs, possibly due to compensatory mechanisms, it might have an impact on renal function.<sup>113</sup>

Expression of the clinically important Vel antigen has been shown to be dependent on SMIM1, a small transmembrane protein of unknown function.<sup>114–116</sup> The protein is well-conserved across species, suggesting that it is an important protein, and GWA studies suggest that it might play a role in iron metabolism.<sup>117</sup> Anti-Vel is considered clinically significant and has caused severe hemolytic transfusion reactions.

At<sup>a</sup> is a high-prevalence antigen belonging to the Augustine (AUG) blood group system. The At(a–) phenotype has mainly been reported in people of African origin. Lack of the At<sup>a</sup> antigen is due to an SNP in *SLC29A1*, encoding the equilibrative nucleoside transporter 1 (ENT1). Although the SNP does not affect the function of ENT1, anti-At<sup>a</sup> can cause severe HTRs and mild HDTN. While ENT1 is abundant on the membrane of RBCs, the biological function in RBCs remains unclear. Complete loss of ENT1 as in the AUG<sub>null</sub> phenotype, associated with ectopic calcification, does not seem to affect the RBCs.<sup>118</sup>

*SLC44A2* encodes the choline transporter-like 2 protein (CTL2). Two antigens, VER and RIF, make up the CTL2 blood group system. The rare RIF– phenotype is due to a missense mutation in *SLC44A2*, whereas a large deletion underlies the rare SLC44A2<sub>null</sub> (VER–) phenotype, causing complete loss of CTL2 expression.<sup>119</sup> Neither anti-RIF nor anti-VER have been associated with HTRs. However, both antibodies are implicated in TRALI (transfusion related acute lung injury) since they react with CTL2, carrying the human neutrophil antigen 3 (HNA-3), on neutrophils. Despite CTL2 having an evident biological function, as seen by multiple disease associations, the role of CTL2 on RBCs appears to be redundant.

The rare MAM– phenotype was shown recently to be due to different genetic aberrations in *EMP3*, the gene that encodes the tumor suppressor epithelial membrane protein 3 (EMP3), and which had not previously been described on erythroid cells.<sup>120</sup> *EMP3* plays an important role as a regulator of erythropoiesis as shown by increased early progenitor proliferation in cell cultures from MAM– individuals and interacts with CD44 in the RBC membrane. Anti-MAM has been shown to cause severe HDFN and, in some cases, neonatal thrombocytopenia.

## Summary

The century-long history of modern transfusion medicine and immunohematology practice led to the identification of an enormous number of blood group antigens. These have been catalogued into a

framework of blood group systems, collections, and low- and high-prevalence antigen series.<sup>121</sup>

Advances in biochemical and molecular biology techniques over the past decades have led to better understanding regarding gene variation, amino acid polymorphisms, protein structure and function, as well as the immunogenicity of individual antigens. Despite increasingly sophisticated and high throughput applications such as whole genome sequencing and variant filtering strategies, unravelling of a novel blood group antigen still, interestingly, begins with a patient presenting an antibody with an atypical RBC agglutination pattern. Careful sequential serological investigations need to be performed to resolve these antibodies. Although some may have little clinical significance, it is still important to determine their specificity since they may cloud the field, masking clinically relevant antibodies capable of causing HTRs and HDFN. Fortunately, the transfusion medical services have a wide array of techniques at their disposal, and the development of tools such as recombinant soluble proteins is an important contribution to antibody identification (see Chapter 12). The availability of increasingly advanced patient applications coupled with time and cost-efficient methods for phenotyping large volumes of blood donors has undoubtedly contributed to accurate compatibility testing and to safe transfusion of erythrocytes.

## Disclaimer

The authors have no conflicts of interest with regard to the content of this chapter.

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- A full reference list for this chapter is available at: [www.wiley.com/go/simon/Rossi6](http://www.wiley.com/go/simon/Rossi6)
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## CHAPTER 12

# Immunohematology and compatibility testing

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### Introduction

Immunohematology is the study of the immune response to blood group antigens following stimulation with foreign blood cells through transfusion or pregnancy and the resulting impact on donor-recipient compatibility. Although numerous differences (polymorphisms) in protein and carbohydrate molecules are present on blood cells of individuals, by definition, the foreign protein epitope or carbohydrate must have stimulated production of a specific antibody to be considered a “blood group antigen.” This is especially relevant today as extensive polymorphisms in molecules on blood cells will be cataloged in genomic databases, but until found to be the target of an immune response in a patient, these differences are not given blood group antigen status.

This chapter reviews the basic concepts of immunity as they apply to the production of antibodies to foreign red blood cell (RBC) antigens called “atypical” alloantibodies to distinguish them from anti-A and anti-B that are found in the plasma depending on ABO blood type (referred to as “natural” antibodies). Antibodies to platelets and neutrophils, as well as autoantibodies and drug-induced antibodies, are discussed elsewhere.

Identification of the specificity of alloantibodies (antibody identification) is performed by testing plasma or serum against commercial red cells of known phenotypes. Modern immunohematology uses knowledge of the extended blood group profile of the patient to aid and inform the process of antibody identification and the selection of red cell units for transfusion. The chapter also includes a discussion of the steps and requirements for pretransfusion compatibility testing and release of blood components for transfusion, as well as efforts to track adverse transfusion events through biovigilance programs and to reduce alloimmunization by extended antigen matching.

### Red cell immunology

#### The basics of the immune response

Immune responses are divided into two categories: humoral immunity and cell-mediated events. Humoral immunity is B-cell mediated and results in the production of antibody. Cell-mediated immunity involves the activation of T cells, which regulate the

immune response through the production of cytokines and direct interactions with other cells of the immune system.<sup>1</sup>

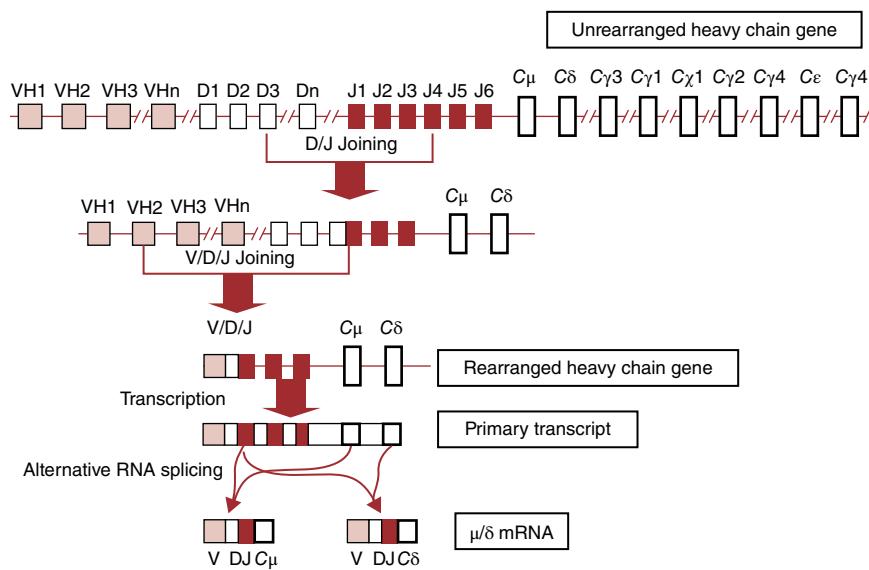
#### Antigen receptors on T cells and B cells

The antigen receptors of both T and B cells consist of two polypeptide chains synthesized under the direction of two different chromosomal loci. In the early stages of lymphocyte development, the genetic material at each of these loci undergoes a process of rearrangement, known as *somatic recombination*, which is unique to each cell.<sup>1</sup> The gene segments, grouped into families based on sequence similarity, and the rearrangement process entail selection and joining of the segments for each portion of the immunoglobulin molecule (Figure 12.1).

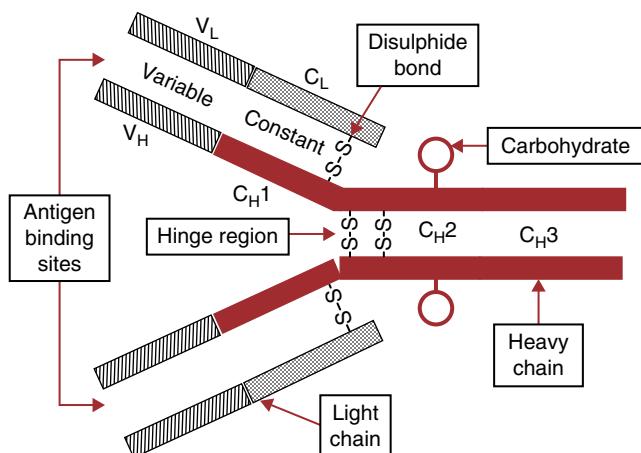
Each B-cell and T-cell receptor chain consists of a variable region, associated with antigen binding, and a constant region. B cell receptors are immunoglobulin molecules; the amino acid sequences of the constant region determine the isotype; and amino acid sequences of the variable region determine the antigenic specificity. The human immunoglobulin heavy chain loci are polymorphic between individuals in the number of genomic V, D, and J segments, but the heavy chain locus has at least 51 V gene segments, 6 J region gene segments, and 27 D region gene segments, and the number of possible rearrangements for different antibody binding regions is over 8000. At the light chain loci, there are at least 71 V gene segments and 9 J gene segments. Further variation is introduced by imprecise splicing of the gene segments and addition of extra nucleotides (called *N nucleotides*), which are not encoded in the genome, to V-D and D-J joints in the heavy chain variable region. Given all these mechanisms and assuming the combination of gene segments is random, the number of different antibody variable regions, and hence antigen binding specificities, is virtually unlimited.<sup>1</sup>

#### Immunoglobulin molecules

Figure 12.2 portrays the basic structure of an antibody molecule, which consists of four polypeptide chains: two identical light chains of 211–217 amino acids and two identical heavy chains of 440 or more amino acids. Antigenic specificity is conferred by the N-terminal amino acids of the heavy and light chains; these are described as *variable regions*. The remaining portions are referred to as *constant regions*.<sup>1</sup>



**Figure 12.1** Diagram of gene rearrangement generating antibody diversity at the immunoglobulin heavy chain locus on chromosome 14. The unrearranged locus (top) shows the V, D, and J gene segments on the left and the constant region segments to the right. Gene rearrangement occurs by selection and joining of one D and one J segment (exon) to form a D/J unit. This is then joined by one V exon. The V/D/J unit, via alternative splicing, is joined to C $\mu$  or C $\delta$  heavy chain gene segments. In later generations, clonal progeny can undergo isotype switching in which the same variable region V/D/J is joined to other sequences from the constant region.



**Figure 12.2** Basic structure of an immunoglobulin molecule.

### Light chains

The constant region of light chains can have one of two different amino acid sequences, designated kappa ( $\kappa$ ) and lambda ( $\lambda$ ). A single B cell will synthesize either  $\kappa$  or  $\lambda$  chains, but not both.

### Heavy chains

The constant region of heavy chains, designated by the Greek letters  $\alpha$  (alpha),  $\delta$  (delta),  $\epsilon$  (epsilon),  $\gamma$  (gamma), and  $\mu$  (mu), gives rise to five types of immunoglobulin classes or isotypes, termed IgA, IgD, IgE, IgG, and IgM, respectively. A single B cell will synthesize only one type of heavy chain.

### Exposure to foreign protein antigens

If foreign protein antigens enter the host, antigen-presenting cells (APCs) process the antigen and display antigen-derived peptides in association with host Class II MHC molecules on the cell surfaces. This peptide–MHC complex is recognized as foreign by the T-cell receptor on T lymphocytes. In contrast, B-cell receptor antibody molecules on B lymphocytes recognize foreign epitopes directly on the protein. Cytokines produced by the activated T cells cause antigen-specific B cells to develop into immunoglobulin-secreting

plasma cells. Plasma cells have a short but active life of antibody production. Some antigen-specific B cells develop into memory cells that persist in the circulation long after their initial activation.<sup>1</sup>

### Primary response

For foreign blood group antigens, as for other immune responses, the first immunoglobulin class to appear is immunoglobulin M (IgM), and the time between antigen exposure and appearance of antibody can vary from days to weeks. Shortly after the appearance of IgM antibody, IgG antibody of the same specificity usually becomes detectable. The IgM component disappears over time, whereas the IgG component persists indefinitely, but in regard to testing for atypical antibodies prior to transfusion the antibody can drop in titer to undetectable levels in laboratory testing. Cytokines from activated T cells are essential for immunoglobulin class switching and for the generation of memory B cells.

### Secondary (anamnestic) response

On re-exposure to a foreign antigen, a memory response is activated within a few hours or days, resulting in a sharp rise in the level of IgG antibody, higher than that was produced in the primary response. In regard to response to foreign blood group antigens and blood transfusion, this can result in a hemolytic transfusion reaction with the destruction of transfused cells.

### T-cell independent response

Antibodies produced in response to foreign carbohydrate antigens are primarily IgM. This is because antigen receptors on B-cells can be activated by direct binding to repeating polysaccharide epitopes, such as the blood group A or B antigens. Structures that have multiple identical repeat carbohydrate chains can initiate B-cell proliferation and antibody secretion without T-cell help. In the absence of T-cell help, isotype switching from IgM to other isotypes does not occur.<sup>1,2</sup>

### Affinity maturation

The affinity of IgG antibody for a specific antigen increases progressively during the immune response. Affinity maturation involves somatic mutation and clonal selection of B cells with

higher affinity antibody receptors for the antigen. This is sometimes seen early in the response to foreign RBC antigens as nonspecific or panagglutinin reactivity in laboratory testing. The following process occurs in the germinal centers of the secondary lymphoid organs:

- 1 **Somatic hypermutation:** Mutations occur in the variable, antigen-binding sequences (complementarity-determining regions [CDRs]) of the immunoglobulin genes. The mutation rate is up to 1,000,000 times higher than normal resulting in 1–2 mutations per CDR per cell generation which alter the binding specificity and affinity of the antibody.<sup>2</sup>
- 2 **Clonal selection:** Only B cells with the highest affinities for antigen will survive. Over several rounds of selection, the resultant secreted antibodies will have increased specificity and affinity for the antigen.<sup>2</sup>

### Clonality

Only antigen-activated B cells proliferate and mature into antibody secreting plasma cells. A polyclonal response occurs when many different B cells are activated simultaneously, best exemplified by anti-D made in D-negative individuals who lack the RhD protein, which carries multiple foreign epitopes. A more restricted oligoclonal response occurs when the antibody-producing cells originate from a few B cells, exemplified by anti-Fy<sup>a</sup> produced by a Fy(b+) individual, which represents a response to a single amino acid difference.

Monoclonal antibodies, which are commonly used as reagents in the laboratory, are specific for a single epitope. Antigen-specific monoclonal antibody responses are rarely found *in vivo* and are usually associated with some leukemias and multiple myeloma. Monoclonal antibodies are isolated *in vitro* from polyclonal responses using hybridoma technology.

### Blood group antibodies

Blood group antibodies bind to antigens on the surface of red cells, platelets, or neutrophils. The focus of this chapter is primarily on antigens expressed on red cells. Most blood group antibodies are either IgG or IgM; only occasionally they are IgA.<sup>3</sup> IgD and IgE immunoglobulins have not been implicated as blood group antibodies.

### Physical properties

The physical properties and serologic characteristics of the three antibody classes with blood group specificity are summarized in Table 12.1. IgM antibodies are pentameric and have 10 antigen binding sites, are direct agglutinating, and often fix complement to the red cells. Antibodies to A and B antigens, which are carbohydrates, are predominantly IgM and are found in persons whose red cells lack the corresponding antigen. They are acquired “naturally,” meaning they are stimulated by plants and bacteria that carry blood group A- and B-like polysaccharides.

Most other blood group antibodies are immune in origin and do not appear in plasma unless the host is exposed to foreign red cell antigens through blood transfusion, pregnancy, or transplantation. The majority are IgG, which have two antigen binding sites (bivalent) (Figure 12.2). Most IgG antibodies do not activate complement, but this depends on both the subclass of IgG and the number and nature of the antigen on the cells.

Antibodies to red cell antigens are considered “unexpected” alloantibodies and are directed to antigens absent on the RBCs of the individual. “Clinically significant” antibodies are those that can

**Table 12.1** Characteristics of Blood Group Antibodies

Immunoglobulin Characteristic	IgM	IgG	IgA
H-chain isotype	μ	γ	α
Subclasses	2	4	1
L-chain types	κλ	κλ	κλ
Sedimentation constant	19 S	7 S	11 S
Molecular weight	900–1000 kD	150 kD	180–500 kD
Electrophoretic mobility	Between β and γ	γ	Γ
Serum concentration (mg/dL)	85–205	1000–1500	200–350
Antigen binding sites	10 (pentameric)	2	4 (dimeric)
Fixes complement	Often	Some	No
Placental transfer	No	Yes	No
Direct agglutinin	Yes	Usually not*	Usually not
Example	Anti-A, anti-B	Anti-D	Anti-Lu

\* Some examples of IgG anti-M are an exception.

cause accelerated destruction or removal of antigen-positive transfused red cells. In pregnancy, IgG antibodies may cross the placenta and cause hemolytic disease of the fetus and newborn (HDFN).<sup>3</sup> There are more than 350 different blood group antigens described.<sup>4</sup> The immunoglobulin class and clinical relevance of some of the antibodies encountered are shown in Table 12.2.

### Red cell antigen–antibody interactions

Red cells normally repel one another due to the zeta potential at the surface which depends on the electronegative surface charge and the ionic cloud that surrounds it (Figure 12.3).<sup>5</sup> In pretransfusion laboratory testing, interaction between red cells and specific blood group antibodies must overcome this repulsion. Interaction between antibody and red cells is observed as agglutination (clumping), or rarely hemolysis, and often requires the use of a secondary antihuman globulin reagent.

### Agglutination reactions

There are two phases of red cell antigen–antibody agglutination interactions that often occur simultaneously. The first is one of association, involving binding of antibody to antigens on the red cell membrane. The second involves the formation of an agglutination lattice of antibody-coated cells. For the latter to occur, antibody molecules must be able to span the distance between adjacent red cells.

IgM antibody molecules can cause direct agglutination of RBCs carrying the corresponding antigen (Figure 12.3C). IgG antibodies often do not cause direct agglutination unless there are many antigens on the RBC (for example, the M antigen located on glycoprotein A). IgG antibodies coating RBCs can be detected with the addition of antihuman IgG (Figure 12.3D). Detection of agglutination routinely requires incubating plasma or serum with red cells at 37°C, followed by centrifugation. For *in vitro* enhancement of the interaction of red cell antigens and antibodies, the surface charge of the red cells can be decreased through a variety of methods: addition of bovine serum albumin; addition of low ionic strength solution (LISS); addition of polyethylene glycol (PEG) solution; or by treating the cells with proteolytic enzymes such as papain or ficin, which cleave the proteins that carry the negatively charged sialic acid residues.

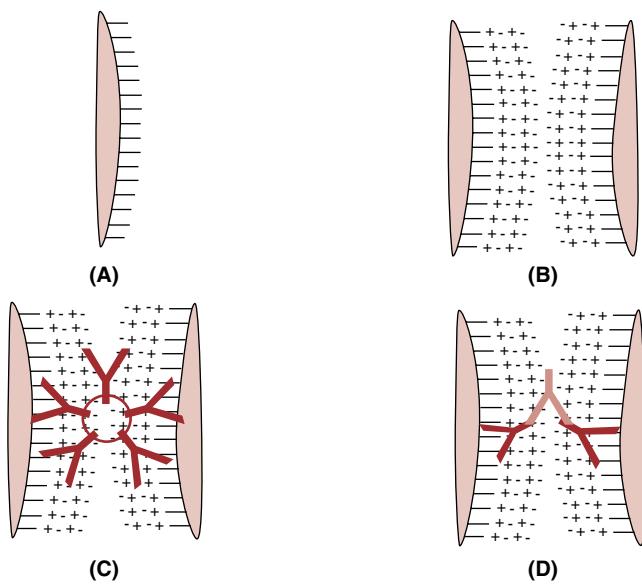
To aid the identification of the antibody specificity, especially when multiple antibodies are present, the strength of the agglutination reactions is graded, and numerical values are assigned based on a scoring system.<sup>6</sup>

**Table 12.2** Blood Group Antibody, Class, Clinical Significance, and % Antigen-Negative (Compatible) Donors<sup>7</sup>

AntiBody	Ig Class <sup>†</sup>	C3-Binding	HDFN <sup>‡</sup>	HTR <sup>§</sup>	%Compatible		Comments
					Caucasian	African Descent	
A	M, G	Yes	Moderate	Yes	53	69	No dosage
A <sub>1</sub>	M	rare	No	Rare	64	78	Present in 1–2% A <sub>2</sub> , 25% A <sub>2</sub> B
A <sub>t<sup>a</sup></sub>	G	No	No	Yes	<1		1 case mild HDFN
B	M, G	Yes	Moderate	Yes	87	76	No dosage
Bg	G	No	No	No	99	99	HLA <sup>  </sup>
C	G, M	No	Mild	Yes	32	73	
c	G, M	No	Yes	Yes	20	2	Often detected with anti-E
Ch	G	No	No	No <sup>#</sup>	4	4	Antibody to C4d <sup>#</sup>
C <sup>w</sup>	G, M	No	Yes	Yes	98	99	
Co <sup>a</sup>	G	Some	Yes	Yes	Rare	Rare	
ACo <sup>b</sup>	G	Rare	Mild	Yes	90	90	
Cr <sup>a</sup>	G	No	No	Mild	None	Rare	
Cs <sup>a</sup>	G	No	No	No	2	5	
D	G, M	No	Yes	Yes	15	8	
Di <sup>a</sup>	G	Some	Yes	Yes	>99	>99	Antigen prevalence up to 54% in some American Indians
Di <sup>b</sup>	G	No	Mild	Yes	Rare	Rare	Antigen prevalence of 99% in Native Americans
Do <sup>a</sup>	G	Yes	No	Yes	33	45	
Do <sup>b</sup>	G	Yes	No	Yes	18	11	
E	G, M	No	Mild	Yes	71	78	Often detected with anti-c
e	G, M	No	Rare	Yes	2	2	
f (ce)	G, M	No	Mild	Mild	35	8	Compatible with c- or e-RBCs
Fy <sup>a</sup>	G	Rare	Yes	Yes	34	90	
Fy <sup>b</sup>	G	Rare	Mild	Yes	17	77	
H	M, G	Yes	No	Yes	Rare	Rare	Antibody found in O <sub>h</sub> individuals
Hy	G	No	No	Mild	Rare	<1	Antibody found in individuals of African descent
I	M, G	Yes	No	Yes	Rare	Rare	Antibody found in i adults
Jk <sup>a</sup>	G	Yes	Yes	Yes	23	8	Antibody levels deteriorate over time
Jk <sup>b</sup>	G	Yes	Mild	Yes	26	51	Antibody levels deteriorate over time
JMH	G	No	No	No	Rare	Rare	Antibody specificity identification within JMH system not clinically relevant
Js <sup>a</sup>	G	No	Yes	Yes	>99	80	
Js <sup>b</sup>	G	No	Yes	Yes	Rare	1	
K	G, M	Rare	Yes	Yes	91	98	
k	G	No	Yes	Yes	Rare	Rare	
Kn <sup>a</sup>	G	No	No	No	5	Rare	Antibody specificity identification within Knops system not generally clinically relevant
Kp <sup>a</sup>	G	No	Yes	Mild	98	>99	
Kp <sup>b</sup>	G	No	Mild	Mild	Rare	Rare	
Lan	G	Some	Mild	Yes	Rare	Rare	
Le <sup>a</sup>	M	Yes	No	Rare	78	77	
Le <sup>b</sup>	M	Yes	No	No	28	45	
Lu <sup>a</sup>	G, A	Rare	Rare	No	92	95	
Lu <sup>b</sup>	G, A	Rare	Mild	Mild	Rare	Rare	
M	M, G	Rare	Rare	Rare	22	26	
McC <sup>a</sup>	G	No	No	No	2	6	Antibody specificity identification within Knops system not generally clinically relevant
N	M, G	No	Rare	Rare	28	25	Clinically significant in N-U- individuals
P1	M	Rare	No	Rare	21	6	
PP1P <sup>k</sup>	M, G	Yes	Yes	Yes	Rare	Rare	Antibody associated with spontaneous abortion
Rg	G	No	No	No <sup>#</sup>	2	2	Antibody to C4d <sup>#</sup>
S	G	Some	Yes	Yes	45	69	
s	G	Rare	Yes	Yes	11	7	
Sc1	G	No	No	No	Rare	Rare	
Sc2	G	No	Mild	No	99	99	
Sl <sup>a</sup>	G	No	No	No	2	40–50%	Antibody specificity identification within Knops system not generally clinically relevant
U	G	No	Yes	Yes	Rare	1	
V	G	No	No	Mild	99	70	
Vel	M, G	Yes	Yes	Yes	Rare	Rare	
Wr <sup>a</sup>	M, G	No	Yes	Yes	>99	>99	Antibody often without known RBC stimulus
Xg <sup>a</sup>	G	Some	No	No	23	23	
Yk <sup>a</sup>	G	No	No	No	8	2	
Yt <sup>a</sup>	G	Some	No	Some	Rare	Rare	

DTT: dithiothreitol.

<sup>†</sup>Predominant immunoglobulin class shown first.<sup>‡</sup>HDFN: reported to cause hemolytic disease of the fetus and newborn.<sup>||</sup>HLA: antibody to antigen present on white blood cells that is variably expressed on red cells.<sup>#</sup>C4d: antibody to epitopes found on the fourth component of human complement (C4). Hives and anaphylaxis reported with plasma containing products. Identification of antibody specificity within CH/RG blood group system not required for clinical purposes.<sup>§</sup>Hemolytic transfusion reaction.



**Figure 12.3** (A) Red cells carry a strong negative electric charge, imparted primarily by the carboxyl ( $\text{COO}^-$ ) group of *N*-acetyl-neuraminic acid. (B) When suspended in saline, red cells are kept apart by virtue of their negative surface charge. The magnitude of this charge is modified by the formation of an ionic cloud of positively charged (+) sodium ions and negatively charged (-) chloride ions at the red cell membrane surface. The force of repulsion that exists between red cells in suspension is referred to as the *zeta potential*. (C) In order for antibody molecules to cause agglutination of adjacent red cells, they must be able to span the intercellular distance. Pentameric IgM antibody molecules can readily bridge the distance between adjacent red cells causing agglutination. (D) IgG molecules cannot readily cause direct agglutination. The bound IgG antibody can be detected by antihuman IgG antibody (e.g., rabbit antihuman IgG), depicted here as lighter color, causing agglutination. Source: Based on Pollack *et al.* (1965).<sup>5</sup>

### Hemolysis

IgM antibodies, in particular anti-A and anti-B, can cause direct lysis of antigen-positive red cells in the presence of complement. Hemolysis results from the action of complement, a series of  $\alpha$  and  $\beta$  globulins that act to form a complex (membrane attack complex) that causes rupture of the red cell membrane. For initiation of the complement cascade, the Fc portions of two immunoglobulin heavy chains must be in proximity on the red cell surface. Initiation of the complement cascade readily occurs when a single pentameric IgM antibody molecule is bound; for it to occur with IgG antibodies, there must be closely adjacent heavy chain regions of two IgG molecules. Not all IgM antibodies bind complement to red cells, and complement binding does not always proceed to complete red cell lysis (intravascular hemolysis). Rather, activated C3b may be cleaved to C3d, which remains bound to red cells and can be detected by the antiglobulin test if anticomplement is included in the reagent.<sup>6</sup> Red cells coated with C3b are also removed by cells of the reticuloendothelial system (extravascular hemolysis).

### Antiglobulin test

IgG antibodies bound to red cells (and complement as well, if the reagent also contains anticomplement) are detected by the antiglobulin test. Once called the Coombs' test after the originator, the test entails the use of (IgM) antibodies raised in animals (rabbit), or prepared from a hybridoma (murine), to detect human IgG and/or

**Table 12.3** Overview of Antiglobulin Tests

Direct Antiglobulin Test (DAT)	Indirect Antiglobulin Test (IAT)
<ol style="list-style-type: none"> <li>Wash red cells to remove plasma/serum.</li> <li>Add antihuman globulin and/or anticomplement reagent.</li> <li>Centrifuge.</li> <li>Examine for agglutination and hemolysis.</li> </ol>	<ol style="list-style-type: none"> <li>Mix serum or plasma and red cells.*</li> <li>Incubate at 37 °C.</li> <li>Centrifuge.</li> <li>Examine for agglutination and hemolysis.</li> <li>Wash, to remove unbound immunoglobulins.</li> <li>Add antihuman globulin reagent.</li> <li>Centrifuge.</li> <li>Examine for agglutination.</li> </ol>

\* An enhancement reagent to promote antibody uptake is often incorporated here.

complement bound to red cells. These antibodies, generally referred to as antihuman globulin (AHG) reagent, will react with human globulins, either bound to red cells or free in plasma or serum. Red cells must be washed free of unbound antibodies before the addition of the AHG to avoid false-negative tests caused by the neutralization of AHG by the unbound globulins.

### Direct antiglobulin test

The direct antiglobulin test (DAT) is used to detect antibodies bound to red cells *in vivo*; such antibodies may be seen in patients with autoimmune hemolytic anemia, infants with hemolytic disease of the fetus and newborn, and patients manifesting an immune response to a recent transfusion.<sup>6</sup>

### Indirect antiglobulin test

An indirect antiglobulin test (IAT) is used to detect and identify unexpected IgG antibodies in the serum or plasma. These antibodies may be seen in individuals who have been previously transfused or pregnant.<sup>6</sup>

For the steps involved in direct and indirect antiglobulin testing refer to Table 12.3.

### Compatibility testing

Pretransfusion compatibility testing involves donor unit testing, patient sample collection and testing, and labeling and clerical checks between the donor unit and intended recipient (Table 12.4). Proper performance and critical attention to each element are needed to ensure that the right unit of blood is transfused to the right patient. A critical part of the process is proper identification of the patient, both at sample collection and at administration of the blood product. An avoidable cause of morbidity and mortality following blood transfusion is ABO incompatibility due to mislabeling of the sample, wrong blood in tube (WBIT), or misidentification and transfusion to the wrong patient.<sup>8</sup>

### Donor testing

#### Collection facility

ABO, RhD, and antibody detection tests on donor blood, tests for infectious diseases, and labeling of donor units are functions performed by a blood donor center; however, some hospital-based transfusion services continue to procure a portion of their blood needs, and thus perform these activities.

The volume of tests performed at donor centers necessitates the use of automated equipment. ABO grouping entails testing both red

**Table 12.4** Pretransfusion Compatibility Testing<sup>a</sup>

	<b>Element</b>	<b>Requirement</b>
Donor unit testing	Collection facility	ABO RBCs tested with anti-A and anti-B Plasma/serum tested with A <sub>1</sub> and B red cells Concordance between red cell and plasma/serum
		RhD Use a method to detect weak D
		Antibody detection Use a method to detect clinically significant antibodies
		Product labeling Process to verify correct information on the label
		Infectious disease testing Per FDA regulations
	Confirmation of ABO	Confirmation of ABO Test integrally attached segment, after label has been affixed
		Red cell testing only
	Confirmation of RhD Patient consent	Confirmation of RhD Confirm only red cells units labeled Rh-negative (weak D testing not required)
		Patient consent Blood bank or transfusion service medical director participates in the development of policies, processes, and procedures
Patient sample collection and testing	Transfusion facility	<b>Patient Sample Collection Identification</b> Positive identification of intended recipient and blood sample at the time of collection Mechanism to identify phlebotomist
		<b>Label</b> Contains two independent identifiers (e.g., first and last names, hospital registration number, and/or date of birth) Mechanism to identify date and time of collection
		<b>Timing</b> Affixed to sample before leaving side of intended recipient Within 3 days of red cell transfusion if patient was transfused or pregnant within previous 3 months or if patient history is uncertain or unavailable
		<b>Patient Sample Testing ABO</b> RBCs tested with anti-A and anti-B Plasma/serum tested against A <sub>1</sub> and B reagent red cells Concordance between red cell result and plasma/serum result
	<i>RhD</i>	RBCs tested with anti-D (weak D testing optional)
		<i>Unexpected antibodies</i> Utilize method that demonstrates clinically significant antibodies using reagent red cells (that are not pooled) Comparison of current ABO, RhD, and antibody screen with historical records. If no previous record, second sample must be tested to confirm ABO and RhD.
	<b>Serologic Crossmatch</b>	Antiglobulin test if clinically significant antibodies detected, currently or in the past
		Tests for ABO incompatibility only if no clinically significant antibodies detected, currently or in the past
	<b>Electronic Crossmatch</b>	Validated computer system used to detect ABO incompatibility

cells and plasma, and the results must be concordant for the unit to be labeled. Red cells are tested with anti-A and anti-B; and the plasma is tested against A<sub>1</sub> and B red cells. Anti-A,B and/or A<sub>2</sub> red cells are often used to detect weak subgroups of A that may be non-reactive in direct tests with anti-A.

Rh typing for the D antigen on blood donor samples must be performed by a method that will detect weak expression of the D antigen, as D is highly immunogenic and weak expression of the antigen can evoke an immune response if transfused to a D negative person. Donor red cells that initially type as Rh-negative are further tested for weak D expression by an indirect antiglobulin test (IAT), or more commonly at donor centers enzyme treatment of the RBCs is used to enhance the reactivity of weak D antigen. All D-positive and weak D-positive donor blood is labeled as Rh-positive.<sup>6,9</sup> Rare RBCs that express very weak D antigen, including those only detected serologically by adsorption/elution of anti-D (termed Del), are not detected as Rh-positive.<sup>6</sup> This is a limitation of serologic methods, and these units are labeled as Rh-negative. Hence, the production of anti-D in a patient receiving blood products labeled as Rh-negative should be reported to the blood center for follow-up testing of the donor.

### Transfusing facility

Confirmation of the ABO/Rh of a red cell donor unit is performed on a segment from the unit after the unit has been labeled and before transfusion by the transfusion facility. Testing of the red cells only is required, and Group O units are confirmed by testing with anti-A,B only. Red cell units labeled as Rh-negative must be confirmed by direct testing with anti-D; weak D testing is not required.<sup>6,9</sup> Repeat testing of donor units for unexpected antibodies, markers of infectious diseases or minor antigens (non-ABO/Rh) for which a unit is labeled, is not required.

### Patient testing

#### Sample collection and storage

A major avoidable cause of fatal, hemolytic transfusion reactions is ABO-incompatible transfusion resulting from patient and/or sample misidentification somewhere in the process from specimen collection to transfusion.

Although considerable resources have been spent to reduce infectious disease transmission, the elimination of process errors in transfusion deserves equal serious attention and resource allocation. These include misidentification of the patient at the time of sample collection or mislabeling of the collection tube; mistransfusion with transfusion of the wrong unit or transfusion to the wrong patient; and inappropriate transfusion, which exposes patients to the hazards of transfusions that they did not need.

Facilities must have validated processes and procedures that define each step in the sample collection process. Some considerations include:

- 1 Requisition.** Forms requesting blood and blood components must contain two independent patient identifiers, usually the first and last names and a unique numerical identifier such as a hospital identification number. The name of the requesting physician, self-identified gender, date of birth, diagnosis, and previous transfusion or pregnancy history are additional helpful information.
- 2 Patient identity.** The collection of a properly labeled sample for testing from the correct patient is critical to safe blood transfusion. The person collecting the sample must positively identify the patient from the wristband containing two unique patient identifiers that remain attached to the patient throughout hospitalization and blood transfusion. The information on the requisition form must be compared with that on the wristband; any discrepancy encountered must be resolved prior to collection of blood samples.

- 3 **Labeling.** Blood samples must be clearly labeled at the bedside with the patient's unique identifiers. There must be a mechanism to identify the date and time of sample collection and the individual who collected the sample.
- 4 **Confirmation of sample identity.** Prior to testing, the transfusion service compares the information on the blood sample label with that on the requisition. A new sample must be obtained whenever there are discrepancies or if there is any doubt about the identity of the sample. It is unacceptable to correct a mislabeled sample.
- 5 **Type of sample.** Either serum or plasma may be used for pretransfusion testing. EDTA anticoagulated plasma samples are more commonly used.
- 6 **Age of specimen.** The specimen used for compatibility testing must represent the patient's current immune status; thus, if a patient has been pregnant or transfused in the previous three months, or if this information is uncertain or unavailable, pretransfusion samples should be drawn within three days prior to transfusion. It is often simpler to stipulate that all pretransfusion samples must be collected within three days before red cell transfusions rather than ascertain if each patient has been recently transfused or pregnant.
- 7 **Storage.** Blood samples used for compatibility testing, including a segment from the transfused unit, must be stored at refrigerated temperatures for at least seven days after transfusion. This ensures that appropriate samples are available for investigational purposes should adverse reactions occur.

### ABO typing

Reagent antisera and red cells are available commercially. Anti-A and anti-B used to test the red cells are monoclonal antibodies prepared by hybridoma technology. Reagent A1 and B red cells used to test the serum or plasma are usually suspended in a preservative medium containing EDTA to prevent lysis of the red cells by complement-binding anti-A and anti-B. Use of anti-A,B and A2 red cells is optional, but is generally considered unnecessary when routinely ABO typing potential transfusion recipients.<sup>6</sup>

The expected findings for each of the four ABO phenotypes are shown in Table 12.5. When interpreting the results of ABO grouping tests, the reciprocal relationship that exists between the absence of A and/or B antigens on red cells and the presence of anti-A and/or anti-B in the plasma/serum is considered. If cell and plasma/serum ABO tests are discrepant, group O blood must be provided for transfusion until the discrepancy is resolved and reliable interpretation of the patient's ABO type can be made.

**Table 12.5** The Expected Reactions of the Four Common ABO Phenotypes: Results of ABO Typing Tests

Blood Type	Tests with Patient Red Blood Cells		Tests with Patient Plasma/Serum	
	Anti-A	Anti-B	A <sub>1</sub> Red Cells	B Red Cells
O	0	0	4+	4+
A	4+	0	0	4+
B	0	4+	4+	0
AB	4+	4+	0	0

0: no agglutination; 4+: strong agglutination.

### Rh typing

Anti-D for testing patient samples is often a blend of monoclonal IgM and monoclonal/polyclonal human IgG. With blended anti-D reagents, the IgM component causes direct agglutination of D-positive red cells, and the IgG component permits the detection of the weak expression of D by application of the antiglobulin test. For patient samples, the antiglobulin test for weak D is not required. To avoid incorrect designation of a D-negative recipient as D-positive because of autoantibodies coating the cells or abnormal serum proteins causing spontaneous agglutination, a control system appropriate to the anti-D reagent is required.<sup>6</sup>

### Tests for unexpected antibodies

Table 12.2 lists the more common blood group alloantibodies encountered and provides the approximate percentage of compatible units that are likely to be found in donors of Caucasian and African descent.

Methods for testing samples for unexpected antibodies must detect clinically significant antibodies, and an antiglobulin test (IAT) after 37 °C incubation of patient serum or plasma with reagent red cells is required. Several methods for antibody detection exist (Table 12.6). Decisions relative to these options are within the purview of the medical director. They should be made based on the type of patients served and the availability of resources, with the realization that no one method will detect all clinically significant antibodies.

### Tube test methods

A variety of red cell suspending media or additives are used either to enhance antibody uptake or to potentiate the agglutination phase of antibody–antigen interactions (Table 12.6). Low-ionic strength saline (LISS) solution, normal saline, or red cell preservatives (modified Alsever's solution) are used as red cell suspending media. Enhancement media added directly to plasma/serum–red cell mixture include LISS additives, polyethylene glycol (PEG), or less commonly bovine serum albumin (22% or 30% w/v).

Antibody uptake is accelerated when red cells are suspended in LISS or PEG. The net effect of additives is that more antibody can be bound in a shorter period of time using LISS or PEG as opposed to saline. Consequently, incubation times can be reduced to 10–15 minutes (exact times vary by reagent manufacturer), compared to 30–60 minutes for saline. The enhancing effect of albumin on red cell antigen interactions can be attributed to its formulation as a low ionic solution.<sup>11</sup>

It is common practice to examine tube tests for agglutination before subjecting them to an IAT. These examinations can be made immediately after mixing cells and serum followed by centrifugation (immediate-spin tests) and again after incubation at 37 °C. For antiglobulin testing, either anti-IgG or polyspecific AHG (containing anti-IgG and anti-C3) may be used. Use of polyspecific AHG may lead to the detection of a number of unwanted positive reactions due to the detection of cold autoantibodies of no clinical significance. PEG tests are not examined for direct agglutination, and AHG containing anti-C3 is not routinely used when PEG is utilized.

Tube testing is considered the gold standard for antibody detection and is the primary method of testing used in reference laboratories when investigating samples from patients who have become alloimmunized. Tube methods are useful because they can easily be adjusted to incorporate treated RBCs or changes in incubation time or temperature. A significant limitation of tube testing is

**Table 12.6** Methods for Pretransfusion Antibody Detection

	Serum/ Plasma	Enhancement Media	Red Cells	Incubation*	AHG
Saline tube	2 drops	NA	1 drop, 2–5%	30–60 min, 37 °C	IgG/PS <sup>†</sup>
Albumin tube	2 drops	2 drops albumin	1 drop, 2–5%	30–60 min, 37 °C	IgG/PS <sup>†</sup>
Low-ionic-strength saline (LISS) tube	2 drops	2 drops LISS	1 drop, 2–5%	10–15 min, 37 °C	IgG/PS <sup>†</sup>
Polyethylene glycol (PEG) tube	2 drops	4 drops 20% PEG	1 drop, 2–5%	15 min, 37 °C	IgG <sup>‡</sup>
Gel <sup>10</sup>	25 µL	LISS in reagent diluent	50 µL, 0.8%	15 min, 37 °C	IgG in gel column
Solid-phase adherence <sup>11</sup>	1 drop	2 drops LISS	†	15–60 min, 37 °C	IgG on indicator cells

AHG: antihuman globulin; PS: polyclonal AHG; IgG: anti-IgG.

\* Times may vary according to reagent manufacturer.

† Predetermined by reagent supplier.

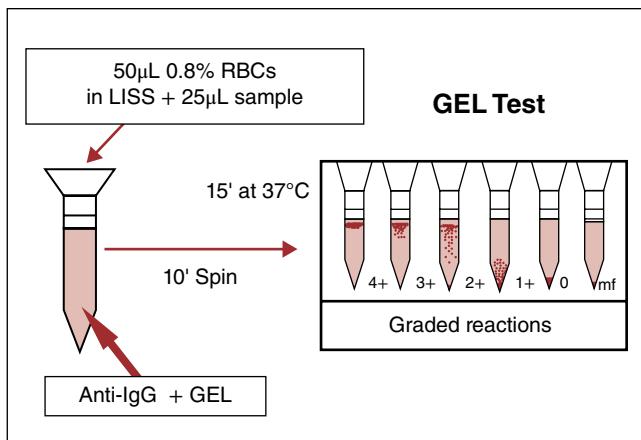
‡ Confirm negative test results with IgG-coated red cells.

technologist time and competence required and the subjective nature of grading agglutination reactions.

#### Gel column testing

A gel matrix material for detecting agglutination due to red cell antigen–antibody interactions was first described in 1990 by Lapierre and colleagues.<sup>12</sup> Cards consisting of six microcolumns, each containing agarose gel suspended in neutral saline or containing anti-IgG, are commercially available. Atop each card is an incubation chamber in which reagent red cells and test plasma are dispensed. The cards are incubated at 37 °C and then centrifuged. As the red cells pass through the gel, if they were coated with antibody during incubation, visible agglutination will be observed as the red cells become trapped in the gel; unagglutinated red cells pellet to the bottom of the microcolumn (Figure 12.4).

When compared to tube tests, the time savings are reflected in the elimination of direct agglutination reading, washing, addition of AHG, and validation of negative tests with IgG-coated red cells. Reproducibility is increased through the use of measured volumes of reactants and less subjective reading of tests. The stability of reactions facilitates validation and review of results. Testing can be automated or semiautomated using liquid sample handling devices. Limitations of this method include increased sensitivity that may detect clinically insignificant reactivity and interference of plasma proteins (rouleaux).

**Figure 12.4** The gel test for detecting unexpected antibodies.

#### Solid-phase adherence methods

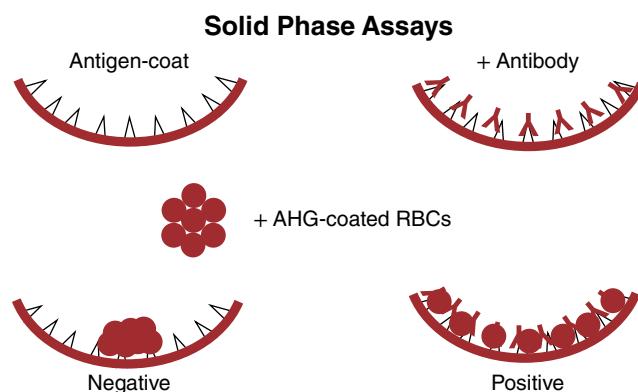
Two forms of solid-phase adherence assays are available for red cell serologic testing. For direct tests, antibody is fixed to wells of a microplate (e.g., anti-A and anti-B for donor/recipient ABO typing), and red cells are added. Following centrifugation, red cells expressing the corresponding antigen will efface across the well; red cells lacking the antigen will pellet to the bottom of the well.

For indirect tests (e.g., for detecting unexpected antibodies), red cells or red cell membranes are affixed to microplate wells; test serum or plasma is added along with LISS and incubated at 37 °C. The plates are washed to remove unbound globulins. Indicator red cells, which are coated with anti-IgG, are added and the plates are centrifuged. The indicator red cells efface across the well in a positive test and pellet to the center of the well in a negative test (Figure 12.5).

One advantage of solid-phase testing is the ability to automate. Limitations include subjective interpretation of results and reports of nonspecific or unidentified reactivity.<sup>13</sup>

#### Automated pretransfusion testing

Automated systems for pretransfusion testing are particularly suited for large hospital-based transfusion services. Automated systems perform sample and reagent pipetting and analysis of agglutination reactions. Standard features include positive sample identification, process control through automated documentation of reagent lot numbers and expiration dates, STAT sample interrupt, and image analysis. The test results can be transmitted via an interface into the laboratory computer system. The benefits of automation include technologist time savings, positive sample identification, standardized testing, and increased workload capacity.

**Figure 12.5** Solid-phase adherence assays.

## Reagent red cells for antibody screening

The FDA mandates that reagent red cell samples licensed for use in pretransfusion antibody detection tests carry the C, c, D, E, e, Fy<sup>a</sup>, Fy<sup>b</sup>, Jk<sup>a</sup>, Jk<sup>b</sup>, K, k, Le<sup>a</sup>, Le<sup>b</sup>, P<sup>1</sup> M, N, S, and s antigens. Reagent red cells for antibody detection are available commercially as sets of either two or three samples. The Rh phenotypes present in two-sample sets are R<sub>1</sub>R<sub>1</sub> (D+C+c-E-e+) and R<sub>2</sub>R<sub>2</sub> (D+C-c+E+e-). In three-sample sets, an rr (D-C-c+E-e-) sample is also provided. Use of three red cell samples facilitates antibody identification and enables the inclusion of red cells homozygous for particular blood group antigens. Such red cells often have a stronger expression of an antigen when compared to red cells from individuals heterozygous for the same gene; this phenomenon is known as *dosage*.

## The principles of antibody identification

When unexpected antibodies are present, as indicated by positive antibody screening tests, they must be identified. This involves testing the patient's serum or plasma against a panel of fully phenotyped reagent red cell samples and the patient's own cells. A typical panel is shown in Table 12.7, which illustrates the results of antibody identification tests with a sample containing both anti-M and anti-K. Tests performed in this example include a reading for agglutination after room temperature incubation (RT) and after incubation at 37 °C. Results of the reactivity seen with LISS additive or with ficin-treated red cells after incubation and washing with addition of antihuman globulin (IAT) are displayed.

A typical approach follows:

- 1 The reactions with the patient's own red cells (AC: autocontrol) are examined. If the AC is positive, autoantibodies may be present and the patient may have a positive direct antiglobulin test (DAT). Therapy with certain drugs can also cause the AC and DAT to be positive.<sup>6</sup> Alternatively, the AC may react because alloantibodies have formed to recently transfused red cells that are still circulating in the recipient. The AC is negative in the case shown, so autoantibodies likely are not present.
  - 2 The graded reaction strengths are examined. Variability in reaction strength may indicate either multiple antibodies present or dosage; dosage is observed when an antibody reacts stronger with red cells from homozygotes (double-dose) than with red cells from heterozygotes (single-dose). Given the varying degrees of reactivity and significant difference in the reactivity at

different phases (room temperature vs IAT, for example) in Table 12.7, more than one antibody appears to be present.

- 3** A process of “ruling out” antibodies is undertaken by evaluating the antigens present on the nonreactive cells. Only reagent red cells 2 and 4 are nonreactive at all phases of testing. The presence of antibodies to antigens present on these two cells (D, C, c, E, e, N, S, s, P<sub>1</sub>, Le<sup>a</sup>, Le<sup>b</sup>, Jk<sup>a</sup>, Jk<sup>b</sup>, Fy<sup>a</sup>, Fy<sup>b</sup>, and Xg<sup>a</sup>) can be eliminated from initial consideration. This leaves the possibility of antibodies to M, K, C<sup>w</sup>, f, Kp<sup>a</sup>, and Js<sup>a</sup>. C<sup>w</sup>, Kp<sup>a</sup>, and Js<sup>a</sup> are low-prevalence antigens not present on reagent red cells used for antibody detection, so these specificities are not responsible for the positive antibody screen. Given the pattern of reactivity, these results suggest the presence of anti-M and anti-K. Importantly, facilities will have policies for how many “rule outs” of each antibody must be performed, and it is often preferred to rule out antibodies using a cell with a double dose rather than a single-dose expression of the corresponding antigen. Testing patient plasma with additional reagent red cells may be necessary.

**4** The test phase and strength of the reactivity are evaluated. Antibodies that are often IgM (Table 12.2), such as anti-M, anti-N, anti-Le<sup>a</sup>, and anti-P1, react as direct agglutinins at room temperature. Antibodies that are usually IgG (e.g., anti-Rh, anti-K, anti-Fy, anti-Jk, and anti-S) react preferentially by the IAT, although during the early stages of the immune response, IgM antibodies of these specificities can be encountered.

With the example here, the anti-M appears to react best at room temperature, and the anti-K reacts best by IAT. This is consistent with the anti-M being IgM, although many examples of anti-M do have an IgG component,<sup>3,7</sup> whereas the anti-K is most likely IgG (Table 12.2). Anti-f does not appear to be present because f-positive cells 1, 6, 8, and 11 are nonreactive by IAT.

In addition, the anti-M and anti-K in this example are both demonstrating dosage. The double dose M+N- cells react stronger at all phases than the single dose M+N+ cells. Only the double dose M+ cells continue to react at IAT. Reagent red cell 7 is the only double dose K+k- cell; this cell reacts by IAT stronger than single dose K+k+ cells (3 and 10).

- 5 The results of tests with enzyme-treated red cells, if performed, are evaluated. Enzyme treatment denatures some antigens, while antibodies to antigens in other blood group systems are enhanced when tested with enzyme-treated cells. In this way, tests with

**Table 12.7** Results of Antibody Identification Panel with a Serum Containing Anti-M and Anti-K

Panel	RH						MNS			P1	LE		KEL			JK		FY	XG	Results								
	D	C	c	C <sup>w</sup>	E	e	f	M	N	S	s	P1	Le <sup>a</sup>	Le <sup>b</sup>	K	k	Kp <sup>a</sup>	Js <sup>a</sup>	Jk <sup>a</sup>	Jk <sup>b</sup>	Fy <sup>a</sup>	Fy <sup>b</sup>	Xg <sup>a</sup>	LISS		FICIN		
1	r'r	0	+	+	0	0	+	+	+	+	0	0	0	+	0	+	0	0	+	+	0	+	+	3+	1+	0	0	0
2	R <sub>1</sub> R <sub>1</sub>	+	+	0	0	0	+	0	0	+	+	0	+	+	0	0	+	0	0	+	0	+	+	0	0	0	0	0
3	R <sub>1</sub> R <sub>1</sub>	+	+	0	+	0	+	0	+	0	+	+	0	+	+	0	0	+	+	+	0	+	4+	3+	3+	0	3+	
4	R <sub>2</sub> R <sub>2</sub>	+	0	+	0	+	0	0	0	+	0	+	+	0	0	+	0	0	0	+	0	+	+	0	0	0	0	0
5	r'r'	0	0	+	0	+	+	+	0	+	0	+	0	+	0	0	0	0	+	+	0	+	+	4+	3+	3+	0	0
6	rr	0	0	+	0	0	+	+	+	0	+	+	0	0	0	0	0	0	0	+	0	+	+	3+	1+	0	0	0
7	rr	0	0	+	0	0	+	0	+	0	+	0	+	+	0	0	0	0	0	+	+	0	0	0	4+	0	0	4+
8	rr	0	0	+	0	0	+	+	+	+	+	+	+	0	0	+	0	0	+	0	+	+	0	3+	1+	0	0	0
9	rr	0	0	+	0	0	+	+	+	0	+	0	+	0	0	0	0	0	+	0	+	+	4+	3+	3+	0	0	
10	rr	0	0	+	0	0	+	+	+	+	+	0	+	0	+	+	0	0	+	+	+	0	+	3+	1+	3+	0	3+
11	R <sub>0</sub> r	+	0	+	0	0	+	+	+	0	0	+	0	+	0	+	0	+	+	0	0	0	3+	1+	0	0	0	
Patient cells																			0	0	0							

RT: room te

0 0 0

RT: room temperature incubation; IAT: indirect antiglobulin test

enzyme-treated cells can be helpful when dealing with sera containing mixtures of alloantibodies. Common antigens such as M, N, S, s, Fy<sup>a</sup>, Fy<sup>b</sup>, and Xg<sup>a</sup> are cleaved by the treatment of red cells with proteolytic enzymes such as papain or ficin; thus, negative or weak reactions are observed when corresponding antibodies are tested with ficin- or papain-treated red cells. In contrast, the treatment of red cells with proteolytic enzymes enhances reactivity with Rh antibodies, as well as antibodies such as anti-Le<sup>a</sup>, anti-P1, and anti-Jk<sup>a</sup>.

In this example, the reactivity attributed to anti-M is not observed when testing ficin-treated cells, as expected. In contrast, K antigen is not affected by ficin treatment, so there is no difference in the IAT reactions of ficin and LISS tests with cell samples 3, 7, and 10. Anti-f can now be completely ruled out because, if present, it would have reacted with all the K-negative ficin-treated cells from donors with r (ce) haplotype.

- 6 There must be sufficient negative test results with red cells that lack the corresponding antigen and sufficient positive test results with red cells that carry that antigen. To obtain a confidence level of >95% ( $p = 0.05$ ), there should be at least three nonreactive antigen-negative reagent red cells and at least three reactive antigen-positive cells. Importantly, when multiple antibody specificities are present, the antigen positive cells should be positive for just one of the antigens (K+,M- and M+,K- in this example). Utilizing the ficin results, and considering that ficin-treated cells are M-, this requirement has been met for both the anti-M and the anti-K.
- 7 The patient would be negative for the corresponding antigen. If the patient has not been recently transfused, the red cells from the patient are tested with anti-M and anti-K and should lack both M and K antigens. (There are exceptions of alloantibody formation when the corresponding antigen appears to be present on the red cells. Most notably, this is seen in the Rh system in individuals with partial antigens who make antibody to the portions of antigen that are absent from their red cells.)

The above illustration represents a basic approach to antibody identification. More complex cases involving autoantibodies, multiple alloantibodies, mixtures of both auto- and alloantibodies, and antibodies to high-prevalence antigens often will require the resources of an immunohematology reference laboratory.

### **Monoclonal antibody therapy interference in pretransfusion testing**

Use of monoclonal antibody immunotherapy is growing in efforts to treat various malignancies. CD38 is highly expressed on myeloma cells, and various anti-CD38 agents, including Daratumumab and Isatuximab, are approved for use in patients with multiple myeloma. Because red blood cells also express CD38, drug therapy with anti-CD38 interferes in pretransfusion antibody detection and compatibility test.<sup>14</sup> While not affecting ABO typing, anti-CD38 causes positive (1+) agglutination in antibody screens, antibody panels, and crossmatching. This reactivity interferes with the ability to detect and identify clinically significant blood group antibodies and precludes the demonstration of compatibility between patient and donor units, delaying issue of blood for transfusion.

While several strategies exist to circumvent anti-CD38 interference, a commonly used approach is to treat the test reagent red cells with dithiothreitol (DTT) to denature CD38 on the red cell membrane and to use these DTT-treated cells for testing. DTT treatment also destroys some clinically significant blood group antigens, including those in the Kell system. Thus, if DTT treatment is used

to mitigate anti-CD38 interference in pretransfusion testing, units transfused should be negative for the K antigen unless the patient's red cells are known to be K positive. Doing an extended red cell phenotype or genotype on the patient prior to initiation of anti-CD38 therapy is recommended.<sup>15</sup> This informs the transfusion service of alloimmunization risk and allows for the transfusion of antigen-matched donor units to avoid alloimmunization in patients that may be facing long-term transfusion support.

Anti-CD47 therapy also causes interference in pretransfusion compatibility testing. CD47 is expressed on all cells, including platelets and red blood cells, and is thought to regulate phagocytosis. Anti-CD47 agents are in clinical trials to treat hematologic malignancies and solid tumors. Interference with some anti-CD47 agents can be observed as robust reactivity (agglutination) in all phases of testing;<sup>16</sup> thus, ABO typing, antibody screens, and serologic crossmatches may be invalid. Treatment of reagent red cells with enzymes or chemicals does not remove CD47 to mitigate interference. Multiple adsorptions of the anti-CD47 drug onto red cells or platelets have been shown to be successful. For those anti-CD47 agents that are IgG4, utilizing an antihuman globulin reagent that does not detect IgG4 can circumvent the interference in the IAT.<sup>16</sup> Importantly, different anti-CD47 agents differ in ability to interfere in pretransfusion testing.<sup>17</sup>

In summary, monoclonal antibody therapies that target molecules also presented on red cells and platelets represent a challenge for pretransfusion laboratory testing that relies on serologic methods to demonstrate compatibility. A key component to reduce delays in selection of units for transfusion is effective communication between the clinical team and the transfusion service when patients are receiving monoclonal antibody therapy.

### **Blood group DNA-based typing in pretransfusion testing**

Blood group antigens other than ABO and RhD are often referred to as *minor blood group antigens*. Testing for antigens beyond ABO and RhD is referred to as performing an *extended phenotype*. Knowing which common antigens are expressed on the patient's red cells and which antigens the patient lacks, and hence could make the corresponding antibody, is very useful for clinical decision making in transfusion, especially if the patient has made a red cell antibody. In the past, this information has not been readily available because performing extended typing for minor antigens by serologic methods is time consuming and costly. In contrast, with DNA-based testing, an extensive antigen profile on the patient (and blood donor) can be obtained in a single assay.<sup>18</sup>

DNA-based typing was introduced more than two decades ago following cloning of several blood group genes. Many blood group antigens result from single nucleotide gene polymorphisms (SNPs) inherited in a Mendelian manner, making assay design and interpretation straightforward. Current methods for testing include the amplification of target gene sequences by polymerase chain reaction (PCR), followed by manual or automated downstream analysis. Semiautomated systems enable large-scale typing of patients and donors for multiple antigens.<sup>18,19</sup> There are FDA-licensed assays that interrogate dozens of minor blood group antigens; however, ABO and RhD genotyping is more complex and requires higher resolution than is required for minor antigen typing. Next generation sequencing (NGS) of whole genomes or exomes, or targeted exome sequencing, or use of massive arrays offers the potential to provide a comprehensive determination of all blood groups. Test design and development of algorithms for interpretation are being developed and validated.<sup>20</sup>

There is nearly complete concordance between DNA (genotype) and serology (phenotype). Discrepancies are most often due to gene mutations that weaken or silence expression of the antigen, or in many cases they are due to human error in the manual process associated with serologic testing and recording of results.<sup>20</sup>

DNA-based typing is useful in several clinical situations (Table 12.8). These include typing of patients who are multiply transfused, who have a positive DAT (with or without serum autoantibody), or who are receiving chronic transfusion therapy for sickle cell disease or thalassemia.<sup>21</sup> DNA-based testing also assists in locating donor units with rare blood types, especially when no serologic reagent is available. Applications in prenatal medicine include the assessment of risk for hemolytic disease when the mother has a blood group antibody and the accurate determination of the RhD status of a mother to guide Rh immune globulin prophylaxis.<sup>22,23</sup>

## Donor-recipient testing

### Recipient prior records check

As part of blood transfusion safety and quality assurance, the results of current pretransfusion tests must be checked against records of any previous tests, if performed. This must be performed before blood is released for transfusion, preferably when a sample is first received for testing. The records must be reviewed for ABO and Rh, difficulties in typing, clinically significant antibodies, severe adverse events related to transfusion, and any special transfusion requirements.<sup>9</sup> Discrepancies between past and present ABO and Rh typing results must be thoroughly investigated. Previously identified clinically significant antibodies must be taken into consideration when selecting blood for present and future transfusions to avoid delayed transfusion reactions, as a majority of clinically significant antibodies drop to serologically undetectable levels.<sup>24</sup>

### Selection of blood for transfusion

#### ABO and Rh

Red blood cells or whole blood selected for transfusion must be compatible with the plasma or serum of the intended recipient. To avoid the hemolytic and potentially fatal consequences of an ABO-mismatched transfusion, red cells carrying A and/or B antigens should not be transfused to a patient unless the patient's red cells also carry those antigens. Group O individuals must receive only group O red cells, whereas AB individuals can receive red cells of any ABO type. Rh-negative individuals, particularly females of childbearing potential, should receive Rh-negative blood.<sup>9</sup>

### Unexpected antibodies

When a patient currently has, or has previously had a clinically significant antibody, defined as one known to cause accelerated destruction of transfused incompatible red cells, blood selected for transfusion must be shown to lack the corresponding antigen by testing with FDA-licensed methods and reagents, if available.<sup>9</sup>

Examples of potentially significant antibodies include those directed toward antigens of the RH, JK, KEL, and FY systems, and the S and s antigens of the MNS system, as well as some other antibodies reactive at 37 °C and/or by the IAT. In the case of antibodies against M, N, P<sub>1</sub>, and Lewis antigens, particularly those that react best at or below room temperature, blood selected for transfusion should be compatible by IAT following 37 °C incubation; demonstrating that compatible units lack the relevant antigen(s) is optional and not required. When autoantibodies are present, testing must be performed to demonstrate the autoantibody is not masking a concomitant, clinically significant alloantibody.<sup>6,9</sup>

### Crossmatch

Before issue of red cells or whole blood, a sample of the recipient's serum or plasma must be tested against the donor cells from an integrally attached segment (exception for emergency release). The method used should be capable of detecting ABO incompatibility and demonstrate clinically significant antibodies, including an IAT. However, if no clinically significant antibodies were detected in the antibody screening and there is no history of clinically significant alloantibodies, only testing to detect ABO incompatibility is required.<sup>9</sup>

### Serologic detection of ABO incompatibility

Detection of ABO incompatibility can be done serologically by performing an immediate-spin crossmatch between the prospective recipient's serum or plasma and donor red cells.

### Computer detection of ABO incompatibility

Computer software may be used to detect ABO incompatibility between the patient sample and the donor unit selected for transfusion.<sup>9</sup> This is termed an *electronic crossmatch* (EXM) and replaces the immediate-spin test for detecting ABO incompatibility.

There are several requirements, including that the computer system be an FDA-cleared medical device that is validated on site. The system must recognize and correlate antibody detection results, comparison of previous records, concordant recipient ABO from at least two determinations, donor component, unit number, and ABO/Rh retype results, as well as logic to alert the user to

**Table 12.8** DNA-Based Typing for Patient and Donor Testing

Transfusion Recipients	Prenatal Practice	Blood Donors
<ul style="list-style-type: none"> <li>• Type patients who have been recently transfused</li> <li>• Type patients whose RBCs are coated with IgG (+DAT)</li> <li>• Distinguish alloantibody from autoantibody</li> <li>• Determine antigen profile in patients who have received an allogeneic stem cell transplant</li> <li>• Determine antigen profile in patients receiving monoclonal antibody therapy</li> <li>• Identify patients with RBCs expressing variant or partial antigens</li> <li>• Type RBCs when no serologic reagents available: Do<sup>a</sup>, Do<sup>b</sup> Js<sup>a</sup>, Js<sup>b</sup>; VVVS, etc.</li> <li>• Resolve serologic typing discrepancies</li> </ul>	<ul style="list-style-type: none"> <li>• Determine gene copy number (zygosity) in paternal sample</li> <li>• Type amniocytes to identify a fetus at risk for hemolytic disease of the fetus/newborn (HDFN)</li> <li>• RHD genotyping of maternal sample for weak D or partial D</li> </ul>	<ul style="list-style-type: none"> <li>• Large-scale typing to locate donors negative for multiple antigens</li> <li>• Identify donors with rare types</li> <li>• Detect weak D antigen expression to confirm D negative status of donors</li> <li>• Detect weak expression of antigens (Fy<sup>b</sup>, e, etc.)</li> <li>• Determine zygosity of reagent panel cells: D, S, and Fy</li> </ul>

discrepancies in ABO/Rh confirmatory testing and ABO/Rh incompatibilities between the recipient and the donor unit. FDA guidance on requirements is available.<sup>25</sup> The advantages of implementing an EXM include technologist time savings, reduced sample handling, avoidance of “false”-positive test results on immediate-spin crossmatch due to cold agglutinins or rouleaux, decreased turnaround time, and potential increased inventory management efficiency.

#### ***Antiglobulin crossmatch***

When clinically significant unexpected antibodies are present, or a patient's records indicate that such antibodies were detected previously, blood selected for transfusion must be negative for the corresponding antigen and tested with the patient's serum or plasma by the IAT. Any of the methods described in this chapter for antibody detection can be used.

An antiglobulin crossmatch can also be performed routinely on recipients with nonreactive screening tests for unexpected antibodies. However, the predictive value of a positive IAT crossmatch following nonreactive screening tests for unexpected antibodies is sufficiently low that many large hospital transfusion services do not perform an IAT crossmatch except as required above. Those that choose to perform an optional IAT crossmatch aim to detect ABO incompatibility, detect unexpected antibodies to low-incidence antigens that are missed in pretransfusion screening tests, detect antibodies manifesting dosage, and consider this testing as a second safety check for antibodies potentially missed in screening tests.

#### ***Extended antigen matching by DNA-based typing***

Increased use of genotyping of both patients and donors also allows for matching the antigen profile of donors to that of patients for the most common blood group antigens. This strategy is especially helpful for chronically transfused patients or in cases where serologically compatible units cannot be provided, such as in cases of warm autoantibodies or in patients receiving monoclonal antibody therapy. In these cases, rather than issuing “least incompatible” red cells for transfusion, units that are “antigen matched for common blood group antigens” can be issued to mitigate delayed transfusion reactions and minimize alloimmunization.

#### **Labeling**

Before blood is released for transfusion, the unit shall have an affixed label or tie tag indicating the recipient's two independent identifiers (usually first and last names, and hospital identification number), the donor unit number, and the interpretation of compatibility tests, if performed.<sup>9</sup>

#### **Issue**

When a blood component is issued, there must be a final check of the following items: transfusion service records to include the recipient's two independent identifiers, ABO group and Rh type; the unit number (or pool number for platelets and cryoprecipitate), ABO group and, if required, Rh of the donor; the product expiration date and, if applicable, time; the interpretation of crossmatch tests, if performed; special transfusion requirements, if applicable; and the date and time of issue.<sup>9</sup> Visual inspection of the unit must be performed and documented, and it should not be issued if any abnormality in appearance is noted.

#### **Emergency release**

When a patient's ABO group is not known, group O red cells are issued.<sup>9</sup> For female patients of future childbearing potential, these should also be Rh-negative. If the ABO group and Rh type have been determined on a current sample, type-specific or ABO-compatible red cells may be issued. The container label or tie tag should conspicuously indicate that compatibility testing was not completed at the time the unit was released. Testing should be completed expeditiously with a patient sample collected as early as possible, and the records should contain a signed statement from the requesting physician indicating the need for transfusion before completion of compatibility testing (or any other testing, including for infectious diseases) testing.

#### **Administration of blood and blood components**

The transfusion service director should participate in the development of policies regarding recipient consent for transfusion, as well as protocols for administration of blood and components, including the use of infusion devices and the identification, evaluation, and reporting of adverse events related to transfusion. At a minimum, recipient consent should include an explanation of the risks and benefits of transfusion, along with treatment alternatives, the opportunity to ask questions, and the right to accept or refuse transfusion.<sup>9</sup>

The physician's written order must be reviewed, and after issue from the laboratory and immediately before transfusion all information must be verified bedside, including the recipient's two independent identifiers and ABO/Rh, donation number and donor ABO/Rh, interpretation of compatibility test, special transfusion requirements, and that the unit is satisfactory in appearance and has not expired.

The patient's medical records must include the transfusion order, documentation of patient consent, name of component, donation number, date and time of transfusion, pre- and post-transfusion vital signs, amount transfused, identification of the transfusionist, and documentation and follow up of any adverse events.

#### **Biovigilance and transfusion safety**

Blood transfusion is not without risks, ranging in severity from febrile nonhemolytic reactions to red cell alloimmunization, disease transmission, and death from bacterial contamination or transfusion of incompatible blood.

Biovigilance systems and networks have been established to track adverse reactions and incidents associated with blood collection and transfusion. In many countries, these programs gather and analyze data to identify trends and risks and to recommend best practices and interventions with the goal of providing improved patient care and safety, while reducing overall costs. Biovigilance programs provide benchmarking internally and against national averages, consistent definitions for adverse reactions, and analysis by experts for the purpose of recommending nationwide health and safety enhancements, for risk mitigation, and to facilitate better understanding of reactions.<sup>26,27</sup>

Adverse event reporting of patient outcomes in the United States occurs through AABB working with patient safety organizations (PSOs) dedicated to confidentially analyzing data on adverse reactions and incidents associated with blood transfusion to communicate best practices and to design interventions to improve patient safety. Some hospitals have created a position of transfusion safety officers (TSOs) to identify, resolve, and monitor organizational weakness leading to unsafe transfusion practice. New technology is becoming available to improve sample labeling and the bedside

clerical checks including wireless handheld portable devices, bar coding, radiofrequency identification, and chip technology.<sup>28</sup>

## Conclusion

Compatibility testing is performed to detect serologic incompatibility between the donor unit and the intended recipient, and to prevent both clerical and technical errors that may have serious, if not fatal, consequences. Most importantly, there can be no substitute for proper patient identification, proper sample labeling, and proper performance of serologic tests.

## Disclaimer

The authors have disclosed no conflicts of interest.

## Key references

- A full reference list for this chapter is available at: [www.wiley.com/go/simon/Rossi6](http://www.wiley.com/go/simon/Rossi6)
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## **SECTION IV**

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# **Blood components**

CHAPTER 13

## Red blood cell production and kinetics

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### Introduction

The main function of red blood cells, the erythrocytes, is to transport oxygen from the lungs to the other tissues of the body. Oxygen delivery is finely controlled by the number of erythrocytes circulating in the blood, which is a function of the rate of senescent erythrocyte removal and the rate of new erythrocyte (reticulocyte) entry. Circulating erythrocytes are maintained in an extremely narrow range because the normal bone marrow produces almost the same number of new erythrocytes each day as is lost through senescence. This daily turnover of approximately 1% of circulating erythrocytes represents 200–250 billion erythrocytes in a healthy adult. When increased numbers of erythrocytes are lost, such as with bleeding or hemolysis, the production of new erythrocytes increases rapidly, replacing the lost erythrocytes and reestablishing the steady-state number of erythrocytes. The rapid expansion of erythrocyte production in response to bleeding or hemolysis is so well regulated that rebound polycythemia does not occur. This exquisitely controlled production of erythrocytes is mediated through a negative feedback mechanism that involves renal oxygen supply and utilization, the hormone erythropoietin (EPO) that is produced in the kidneys, and the erythroid progenitor cells in the bone marrow that depend upon EPO to survive. Normal red blood cell production also depends upon adequate supplies of specific nutrients, among which iron, folate, and vitamin B<sub>12</sub> are the most important. Disorders of the hematopoietic system or other diseases such as those associated with chronic inflammation inhibit the erythropoietic process.

mainly from studies of mice and humans. These studies have included direct morphologic and immunologic analyses of cells in hematopoietic tissues, in vitro culture of hematopoietic cells, transplantation studies with hematopoietic cells, and genetic studies of mice with natural mutations, transgene expressions, or targeted gene knockouts.

Labeled endothelial cells in the ventral part of the aorta in developing mice transform into HSCs<sup>1</sup> by a mechanism that does not require mitosis.<sup>2</sup> Among the various functions of blood cells, tissue oxygenation by the erythrocytes is the first required during embryonic development and the most tightly regulated in postnatal life. Erythropoiesis has two sequential but overlapping phases during development. In the first or primitive phase, erythrocytes are produced in “blood islands” of the yolk sac during weeks 3–6 of human gestation, with primitive erythrocytes comprising the large majority of circulating erythrocytes at 8 weeks but declining to undetectable levels by 12 weeks of gestation.<sup>3</sup> In the subsequent definitive erythropoiesis phase, erythrocytes are produced mainly in the human fetal liver from 6 to 22 weeks of gestation, and mostly in the bone marrow at later times.<sup>3</sup> Definitive erythroid cells arise from HSCs that are first detected in the aortogonadomesonephros (AGM) region of the mesoderm,<sup>4</sup> circulate, seed the fetal liver, and then migrate from the fetal liver to the developing bone, where they initiate marrow hematopoiesis.<sup>5–7</sup> The hemoglobin of the primitive erythrocytes contains embryonic ε- and ζ-globins, whereas the hemoglobin of the definitive erythrocytes contains adult α-globin and either fetal γ-globin from mid-gestation through the first few postnatal months or mainly adult β-globin after the first few postnatal months.<sup>8</sup>

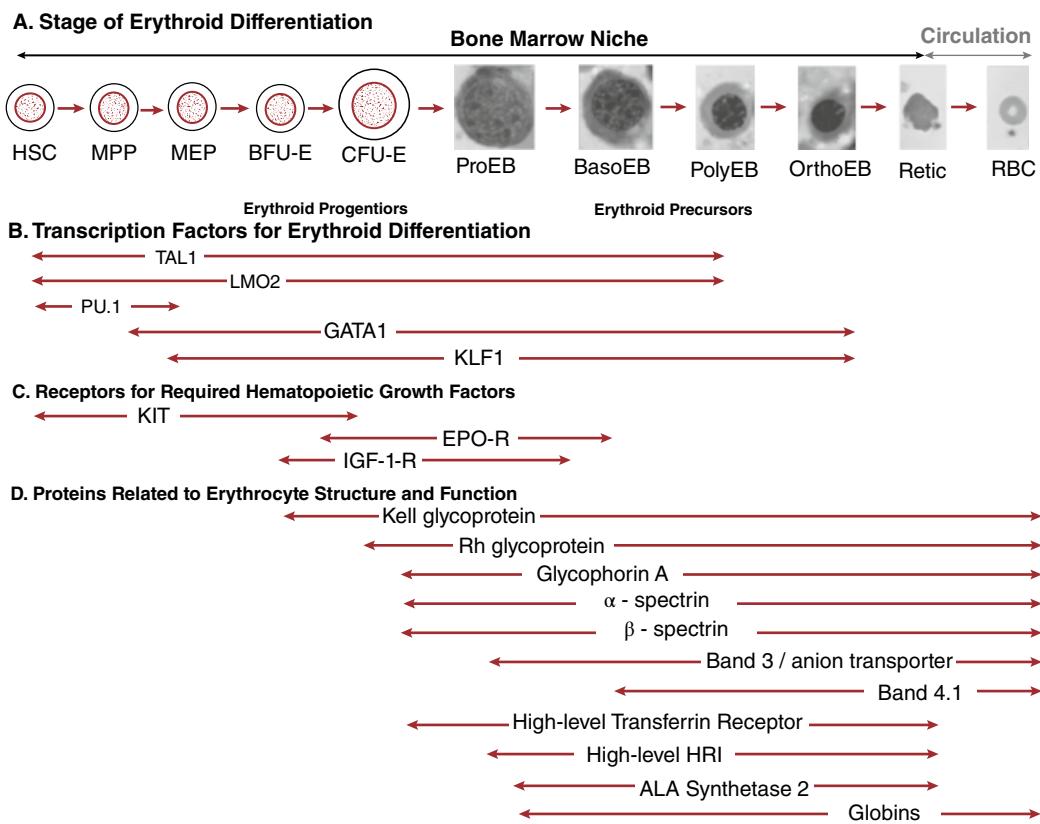
### Erythropoiesis

#### Erythropoiesis: a component of hematopoiesis

Erythropoiesis, the process of erythrocyte production, is part of the larger process by which a pluripotent hematopoietic stem cell (HSC) proliferates and differentiates into all of the cell types of the blood and immune systems, including platelets, granulocytes, monocytes and macrophages, T lymphocytes and B lymphocytes, as well as erythrocytes. Thus, normally regulated hematopoiesis is required for effective hemostasis, inflammation, immune responses, and tissue oxygenation. Current concepts of hematopoiesis are derived

#### Stages of erythropoiesis

Erythroid progenitor cells arise from HSCs that commit to differentiation and are termed *multipotent progenitors* (MPPs). MPPs proliferate and undergo a series of decisions based on specific transcription factor activities that determine their progeny's fate in terms of blood cell lineage (see Figure 13.1). The myeloid transcription factors PU.1 and GATA1 direct differentiation toward the nonlymphoid lineages, and, if the activity of the GATA1 transcription factor is increased, differentiation toward the bipotent megakaryocytic-erythroid progenitor (MEP) is promoted.<sup>9,10</sup> MEP fate, in turn, is determined by the activities of two other competing



**Figure 13.1** Cellular events in erythroid differentiation. (A) Stages of differentiation: hematopoietic stem cells (HSCs), burst-forming units-erythroid (BFU-Es), colony-forming units-erythroid (CFU-Es), proerythroblasts (ProEBs), basophilic erythroblasts (BesoEBs), polychromatophilic erythroblasts (PolyEBs), orthochromatic erythroblasts (OrthoEBs), reticulocytes (Retics), and erythrocytes (RBCs). (B) Transcription factors: basic helix-loop-helix factor (TAL1), Lim domain partner of TAL1 (LMO2), factor binding purine-rich GAGGAA sequence (PU.1), zinc finger factor binding GATA-containing sequences (GATA1), and Krüppel-like factor (KLF1). (C) Growth factor receptors for stem cell factor (KIT), erythropoietin (EPO-R), and insulin-like growth factor-1 (IGF-1-R). (D) Proteins related to erythrocyte structure and function and heme-regulated inhibitor (HRI; eIF2 $\alpha$  kinase). Periods of expression for erythroid-specific forms of proteins are shown. Transferrin receptors are present in all stages, but the period of high-level expression that characterizes hemoglobin-producing erythroblast is shown. Band 4.1 shown for the spliced form found in circulating erythrocytes. Content of each protein may vary during the period shown. Source: Based on Koury.<sup>76</sup>

transcription factors: erythroid Krüppel-like factor-1 (KLF1), which promotes erythroid differentiation, and FLI1, which promotes megakaryocytic differentiation.<sup>9,11</sup>

In Figure 13.1, the hematopoietic stages committed solely to erythroid differentiation begin with the burst-forming units-erythroid (BFU-Es),<sup>12</sup> which produce large colonies or multiple colonies of human erythroblasts after 2–3 weeks in tissue culture. BFU-Es can circulate in the blood, but after they differentiate in marrow to the next defined stage, the colony-forming units-erythroid (CFU-Es),<sup>12</sup> they associate with a macrophage, forming an erythroblastic island (EBI), the basic unit of terminal mammalian erythropoiesis.<sup>13</sup> Coordinated KLF1 activity in both the central macrophage and the erythroid cells<sup>14,15</sup> of an EBI directs the development of as many as 30 or more adherent erythroblasts at various stages of differentiation from CFU-Es through enucleating orthochromatic erythroblasts. BFU-Es and CFU-Es were originally defined by in vitro colony-forming activity and erythroblasts by their morphological appearances in Giemsa-stained films of aspirated marrows. More recent studies using flow cytometry have enabled the identification and isolation of all stages of human erythroid progenitors BFU-Es, CFU-Es and their erythroblast progeny, the

proerythroblasts (ProEBs), basophilic erythroblasts (BesoEBs), polychromatophilic erythroblasts (PolyEBs), and orthochromatic erythroblasts (OrthoEBs) based on the surface expressions of CD34, CD36, endoglin (CD105), and the transferrin receptor (CD71) for the progenitors; or glycophorin A, anion transporter (Band 3), and  $\alpha 4$  integrin for the precursors.<sup>16</sup>

OrthoEBs enucleate within the bone marrow forming reticulocytes, very irregularly shaped cells containing hemoglobin and residual organelles (the “reticulum”) that allow them to be distinguished from the mature erythrocytes. The extruded nucleus with its thin shell of hemoglobin-containing cytoplasm, termed a *pyrénocyte*, is rapidly phagocytosed by the central macrophage, which degrades the nucleus and hemoglobin and recycles the nucleosides and iron.<sup>17</sup> The final stage of differentiation, the erythrocyte, is achieved after the reticulocytes have entered the circulation, lost their residual internal organelles via autophagy,<sup>18</sup> and remodeled their irregular shapes by shedding of exosomes<sup>19,20</sup> and forming uniform biconcave disks. Reticulocyte maturation to an erythrocyte occurs within 1–2 days after entering the circulation, but mature erythrocytes continue to shed microvesicles until they are removed 110–120 days later as senescent cells.<sup>21</sup>

## Intracellular requirements for normal erythroid differentiation

A series of intracellular and extracellular events are needed for successful completion of the erythroid differentiation scheme as shown in Figure 13.1. The intracellular events include the expression of (1) hematopoietic and erythroid-specific transcription factors; (2) specific microRNAs and long, noncoding RNAs involved in the differentiation process; (3) proteins involved in the proliferation and differentiation of the erythroid cells; and (4) proteins such as hemoglobin, intrinsic membrane, and membrane skeleton proteins that comprise the mature erythrocyte.

GATA1, KLF1, and the transcription factor complex of TAL1/SCL, LMO2, and LDB1 are essential for erythropoiesis from the pre-EPO-dependent stages through late erythroblast stages.<sup>11,22,23</sup> In addition to regulating expression of erythroid-specific genes such as those encoding the EPO receptor, globins, and glycophorins, these transcription factors also regulate long, noncoding RNAs that can influence other erythroid gene expressions in the later stages of differentiation, such as the gene encoding Band 3 and the anion transporter.<sup>24</sup> The expression of transcription factors and other crucial erythroid proteins, in turn, are partially controlled by specific microRNAs, which regulate mRNA stability and translation at all stages of erythroid differentiation.<sup>23,25</sup> In fact, post-transcriptional regulation of protein synthesis by microRNAs allows the control of the reticulocyte maturation process that occurs days after the erythroid cell has lost its nucleus.<sup>26</sup>

During the terminal stages of erythropoiesis in the EBI, the erythroblasts undergo progressive decreases in size, nuclear condensation, and subsequent enucleation. Decreased cell size between the ProEB and OrthoEB stages is achieved by a shortened duration of the G1 phase of the cell cycle, resulting in less protein accumulation between cytokineses.<sup>27,28</sup> These terminal erythroblast divisions are regulated by cyclin D3, a G1-phase cyclin,<sup>29</sup> and direct contact with the central macrophage shortens the G1 phase of the erythroblasts.<sup>30</sup> During these more rapid cell divisions, the ratio of heterochromatin to euchromatin increases with a progressive condensation and reduction in nuclear size<sup>31</sup> that are associated with histone deacetylation<sup>32</sup> and DNA demethylation in the mouse.<sup>33</sup> In humans, nuclear condensation occurs earlier (around the BasoEB stage) and involves the nuclear lamins.<sup>34,35</sup> In the formation of the reticulocyte and pyrenocyte, the condensed erythroblast nucleus is extruded by an active process similar to cytokinesis that requires filamentous actin,<sup>36</sup> nonmuscle myosin IIB,<sup>37</sup> and tropomodulin 1 (Tmod1).<sup>34</sup> KLF1 expression in the central macrophage regulates the phagocytosis of the pyrenocyte and subsequent degradation of its DNA and hemoglobin.<sup>14,17</sup>

Hemoglobin, the predominant protein of erythrocytes, is synthesized in a highly regulated process that begins in the BasoEBs and continues through the reticulocyte stage. Extremely large quantities of heme are produced without intracellular accumulations of iron or protoporphyrin. As iron accumulates in the basophilic erythroblast, it upregulates heme synthesis through a 5'-iron-responsive element (IRE) in erythroid-specific 5-aminolevulinic acid synthase (ALAS2, the first step of heme synthesis) mRNA that controls translation;<sup>38</sup> GATA1 activity induces heme synthetic enzymes and protein kinase A phosphorylates and activates ferrochelatase, which catalyzes the last step of iron incorporation into protoporphyrin;<sup>39</sup> and the amino acids glycine and glutamine (as source of succinyl CoA) provide the substrates for porphyrin synthesis.<sup>40</sup> Similarly, heme is incorporated into globin chains without accumulating

intracellular excesses of globin chains or heme, and 2 $\alpha$ -hemoglobin and 2 $\beta$ -hemoglobin chains are assembled into hemoglobin A tetramers without accumulating unpaired hemoglobin chains.<sup>41</sup>

Multiple layers of regulation that are specific to erythroid cells control heme and hemoglobin synthesis including (1) heme regulation of erythroblast GATA1 activity<sup>42</sup> and iron acquisition from endocytosed transferrin receptors,<sup>38</sup> (2) heme upregulation of erythroblast protein synthesis through inactivation of heme-regulated eIF2 $\alpha$  kinase (heme-regulated inhibitor [HRI]), which in the absence of heme phosphorylates the translation initiation factor eIF2 $\alpha$ , thereby inhibiting its ability to initiate general mRNA translation,<sup>43</sup> and (3) heme induction of rRNA and ribosomal proteins.<sup>42</sup> In the regulation of hemoglobin production, heme derepresses  $\beta$ -globin transcription by binding and enhancing the degradation of BACH1, a transcription repressor at the locus control region (LCR). With the loss of BACH1, the NFE2-Mafk transcription factor complex binds and activates  $\beta$ -globin transcription,<sup>44,45</sup> which combined with GATA1 and KLF1 activities leads to coordinated  $\alpha$ -globin and  $\beta$ -globin transcriptions.<sup>46</sup> While  $\beta$ -globin transcription is upregulated, heme indirectly downregulates  $\gamma$ -globin transcription through increased translation of its major target gene, translation of activating transcription factor-4 (ATF4), which in turn induces BCL11A that inhibits  $\gamma$ -globin transcription.<sup>47</sup> Finally,  $\alpha$ -hemoglobin-stabilizing protein (AHSP) regulates intracellular free  $\alpha$ -globin chain content by coordination of heme insertion, appropriate folding, and assembly of  $\alpha$ -globin chains into hemoglobin.<sup>48</sup>

In the terminal stages of erythroid differentiation, the plasma membrane and associated membrane skeleton undergo large changes in their composition. From the CFU-E through reticulocyte stages, several patterns of intrinsic membrane protein expression are found: (1) from a baseline of little or no expression, large increases occur in proteins that are major components of erythrocyte membranes, such as glycophorin A, glucose transporter 1 (GLUT1), and Band 3; (2) more gradual increases from low baseline levels occur in glycophorin C, and RhAG, RhD, and Lutheran antigens; (3) from a stable baseline, late declines of moderate degree occur in Kell antigen and transferrin receptor 1 (CD71); and (4) prominent declines occur in adhesion proteins such as CD36 and CD44, and integrin components  $\alpha$ 4,  $\alpha$ 5, and  $\beta$ 1.<sup>16</sup> Most of the membrane skeletal proteins, including  $\alpha$ - and  $\beta$ -spectrins, ankyrin, adducin, Band 4.1, Band 4.9, and tropomodulin, increase, whereas actin declines slowly during terminal erythroid differentiation.<sup>16</sup> This pattern of accumulation of membrane skeletal proteins during terminal erythropoiesis is mainly related to the accumulation pattern of Band 3, to which the membrane skeleton is bound,<sup>49</sup> and mRNA splicing of the skeletal proteins, such as Band 4.1.<sup>50</sup> In addition to regulating cellular structure, alternative splicing of transcripts plays a role in the regulating cell cycle and chromatin function during terminal erythropoiesis.<sup>51</sup> Once hemoglobin synthesis has been completed during normal steady-state erythropoiesis, the transferrin receptor 1 (CD71) is completely lost during reticulocyte maturation through the exosomal pathway.<sup>52</sup>

## Extracellular requirements for erythroid differentiation

The extracellular requirements for erythroid differentiation include (1) stromal cell and matrix support within the marrow, (2) required hematopoietic growth factors, and (3) nutrients required for progenitor cell proliferation and differentiation. HSCs and BFU-Es can circulate in the blood, but to complete differentiation they must

adhere to and be retained in specific areas in marrow termed *niches*. HSCs home to and are retained in the marrow by cytokines and chemokines that are produced by mesenchymal stem cells. The most prominent marrow cytokine is secreted and membrane-bound KIT ligand (SCF), which binds its receptor, KIT, on HSCs. The most prevalent marrow chemokine is stromal-cell-derived factor 1 (CXCL12), which binds its receptor, CXCR4, on HSCs.<sup>53</sup> In the marrow, HSCs differentiate through the MPP and MEP stages to reach the BFU-E stage. The marrow matrix protein laminin binds the p67 nonintegrin receptor on circulating BFU-Es, thereby promoting their retention and proliferation in the marrow.<sup>54</sup> When BFU-Es differentiate to CFU-Es, they associate with stromal macrophages forming the EBIs. At least five interacting surface membrane protein pairs mediate macrophage–erythroid interactions in EBIs: (1) macrophage vascular cell adhesion molecule 1 (VCAM1) binds erythroblast  $\alpha 4\beta 1$  integrin, (2) macrophage  $\alpha_v$  integrin binds erythroblast interstitial cell adhesion molecule-4 (ICAM4/LW), (3) erythroblast–macrophage protein (EMP) on both macrophages and erythroblasts binds with itself on the other cell type, (4) macrophage CD169–Siglec1 binds erythroblast sialylated glycoproteins, and (5) macrophage hemoglobin–haptoglobin receptor (CD163) binds an unknown erythroblast ligand.<sup>13</sup>

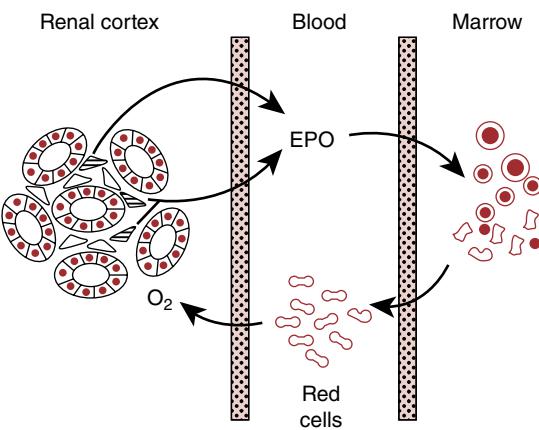
In Figure 13.1, receptors for the hematopoietic growth factors necessary for normal erythropoiesis are shown for the period when they are required. The principal growth factor regulating erythropoiesis is EPO, which is discussed in detail in the “Erythropoietin” section. Prior to EPO dependence, specific growth factors maintain progenitor cell survival and proliferation, with the most prominent being SCF and insulin-like growth factor-1 (IGF1) supplied by the marrow environment.<sup>55</sup> CFU-Es and ProEBs lose SCF and IGF-1 responsiveness, respectively, while they are dependent on EPO for survival. However, in contrast to normal erythropoiesis, during periods of hypoxic stress, CFU-Es and ProEBs can expand their numbers greatly without any further differentiation. The two main extracellular mediators of this expansion are (1) glucocorticoids,<sup>56,57</sup> which are produced in the adrenals and appear to induce a protein in erythroid progenitors that binds the mRNAs that direct terminal erythroid differentiation;<sup>58</sup> and (2) bone morphogenetic protein 4 (BMP4),<sup>59</sup> a member of the transforming growth factor- $\beta$  family of cytokines that can be produced by the central macrophage of the erythroblastic islands.<sup>60</sup> A major intracellular mediator of the erythroblast expansion in response to hypoxia is the upregulation of *Bmi-1* and *BMI-1* expressions in mice and humans, respectively.<sup>61,62</sup>

Included among the vitamins and minerals that cause anemia during deficiency states are copper; cobalt; vitamins A, C, and E; pyridoxine; riboflavin; and nicotinic acid.<sup>63</sup> However, the most common nutritional deficiencies that cause anemia are those of folate, vitamin B<sub>12</sub>, and iron. The roles of these three nutrients are described in the “Nutritional Requirements for Erythropoiesis” section.

## Erythropoietin

### Regulation of EPO production by tissue hypoxia

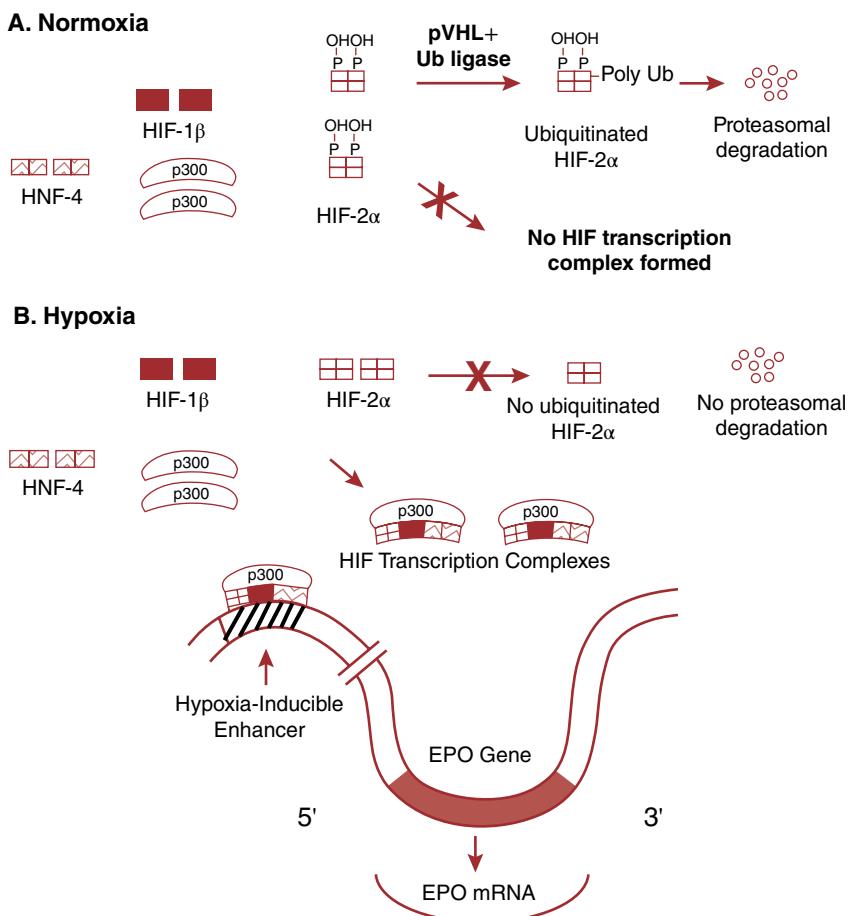
EPO, a glycoprotein hormone, is a major component of the oxygenation–EPO negative feedback mechanism shown in Figure 13.2. The major determinant of oxygen delivery from the lungs to the peripheral tissues is the number of circulating erythrocytes. With anemia, when erythrocyte numbers are decreased, oxygen delivery decreases and the peripheral tissues become hypoxic. All tissues



**Figure 13.2** Oxygenation–erythropoietin (EPO) negative feedback mechanism. Circulating erythrocyte numbers determine the amount of oxygen ( $O_2$ ) delivered from the lungs to other tissues. A specific subset of renal cortical interstitial cells (hatched) produces EPO when hypoxic. EPO is promptly secreted into the blood and prevents programmed death (apoptosis) of marrow erythroid progenitor cells. Erythroid progenitors that survive the EPO-dependent period of differentiation develop into reticulocytes (irregularly shaped, anucleate cells in marrow and blood) and subsequently mature into biconcave erythrocytes. Increased erythrocyte numbers resulting from increased plasma EPO deliver more oxygen to the kidneys, relieve renal hypoxia, and thus decrease EPO production.

experience hypoxia during anemia, but those organs that respond with EPO production are the kidneys and, to a much lesser extent, the liver.<sup>64</sup> The kidney cells that produce EPO are a subset of interstitial fibroblasts located adjacent to proximal tubules, with EPO-producing cells in small foci of the inner cortex in slight anemia, larger areas within the inner half of the cortex in moderate anemia, and distributed throughout the cortex in severe anemia.<sup>65,66</sup> These progressive increases in the areas of EPO production in the kidney correspond to increasing areas of cortical hypoxia, which are a function of oxygen supply from the blood and local oxygen tissue utilization, which is mainly determined by the metabolic demands of the tubular epithelium. Rapid increases in EPO production after blood loss or hemolysis are not due to increased production by each EPO-producing cell but rather to recruitment to active EPO production of increased numbers of cells with the potential to produce EPO.<sup>65</sup> With a linear decrease in hematocrit, the number of cells actively producing EPO and the resultant plasma EPO levels increase exponentially,<sup>65,66</sup> as was originally reported for plasma EPO levels in most clinical anemias, except for those involving patients with renal disease, chronic inflammation, or malignancies.<sup>67–69</sup>

Hypoxia sensing by EPO-producing cells involves hypoxia-inducible transcription factors (HIFs), a multicomponent complex that binds hypoxia-inducible transcription enhancer elements of various genes, including *EPO*, *VEGF*, and genes encoding several glycolytic enzymes.<sup>70,71</sup> Under normoxic conditions, the steady-state HIF- $\alpha$  component of the complex does not accumulate intracellularly because it is rapidly degraded by the ubiquitin–proteasome pathway (Figure 13.3).<sup>72</sup> However, when a cell with EPO-producing capacity experiences hypoxia, the degradation of HIF- $\alpha$  decreases and intracellular levels promptly increase. Polyubiquitination of HIF- $\alpha$  depends upon the von Hippel-Lindau protein (pVHL) interacting with those HIF- $\alpha$  molecules that have hydroxylation of two specific proline residues (Figure 13.3).<sup>73–75</sup> These prolyl hydroxylations are directly linked to the oxygenation because they are catalyzed by prolyl



**Figure 13.3** Induction of erythropoietin (EPO) gene transcription by hypoxia in renal cortical fibroblasts. (A) In cells capable of producing EPO, two components of hypoxia-inducible factor (HIF-2 $\alpha$  and HIF-1 $\beta$ ) are constitutively produced under normoxic conditions. However, the molecular oxygen present in the EPO-producing cells under normoxic conditions is used in the hydroxylation of two prolines in HIF-2 $\alpha$  in a reaction mediated by specific HIF-prolylhydroxylases (HIF-PHDs). The prolyl hydroxylations (P-OH) lead to recognition by von Hippel–Lindau protein (pVHL), which targets HIF-2 $\alpha$  for polyubiquitination (Poly Ub) by ubiquitin ligase. The polyubiquitinated HIF-2 $\alpha$  is rapidly degraded by proteasomes. (B) When renal EPO-producing cells are hypoxic, HIF-2 $\alpha$  is not hydroxylated and accumulates because it is not degraded by the ubiquitin–proteasomal pathway. HIF-2 $\alpha$  forms heterodimers with HIF-1 $\beta$  and associates with two other components of the HIF transcription complex, hepatocyte nuclear factor-4 (HNF4) and p300. The HIF transcription complex binds to a hypoxia-inducible enhancer located 6–14 kilobase pairs upstream of the EPO coding sequences, and thereby increases EPO transcription and accumulation of EPO messenger RNAs. EPO mRNA is promptly translated, and EPO is secreted into the blood such that increases in circulating EPO can be detected within two hours of experiencing hypoxia.

hydroxylases (HIF-PHDs) that contain nonheme iron at their active sites and have molecular oxygen as a substrate. The transcription complex containing HIF-2 $\alpha$  regulates renal EPO transcription through an enhancer that is located 6–14 kbp upstream of the EPO coding region.<sup>71</sup> Once hypoxia reaches the threshold that triggers EPO transcription, the resultant EPO messenger RNA is translated into the EPO glycoprotein, which is immediately secreted.<sup>65</sup> When an individual cell is triggered to produce EPO, it does so in an all-or-none manner.<sup>65,76</sup> Thus, EPO concentrations in the blood increase sharply within two hours after loss of blood, hemolysis, or a sudden decrease in atmospheric oxygen.

### Effects of erythropoietin on erythroid progenitor cells

In the marrow, EPO binds to transmembrane glycoprotein erythropoietin receptors (EPO-Rs), which are first displayed on the surface of erythroid progenitor cells before the CFU-E stage and persist until the late basophilic erythroblast stage (Figure 13.1).<sup>76</sup> The bind-

ing of EPO to EPO-Rs leads to three major events: (1) homodimerization and conformational alterations of EPO-Rs, (2) initiation of intracellular signaling by the EPO-Rs, and (3) endocytosis of the EPO–EPO-R complexes, which are degraded via lysosomal activity.<sup>77,78</sup> Dimerization and structural changes of EPO-Rs after EPO binding induce both signaling and endocytosis. The endocytosis and intracellular degradation of the EPO–EPO-R complexes appear to be the normal mechanism for the clearance of EPO from the blood.<sup>79</sup> EPO-Rs have no intrinsic enzyme activity, but they interact with several signal transduction pathways through Janus tyrosine kinase-2 (JAK2). JAK2 physically associates with the cytoplasmic portion of EPO-Rs, chaperones EPO-Rs to the surface of the erythroid cell, and is activated by the conformational changes in the EPO-Rs produced by the binding of EPO.<sup>80,81</sup> Activated JAK2 phosphorylates itself and EPO-Rs as well as initiates signal transduction pathways that include signal transduction and activator of transcription-5 (STAT5), RAS–RAF–MAP kinases, and phosphoinositol-3 kinase/AKT kinase (protein kinase B).<sup>82,83</sup>

Although the mechanisms linking EPO-R signaling to the biological effects of EPO have not been determined, EPO prevents the apoptotic death of erythroid progenitor cells in CFU-E through early BasoEB stages.<sup>84–87</sup> During EPO dependence, individual erythroid cells at the same stage of differentiation can display wide variation in their degree of dependence on EPO for survival.<sup>88</sup> Such variable susceptibility to apoptosis among EPO-dependent progenitors appears to be due to expression levels of FAS, a membrane protein of the tumor necrosis factor (TNF) family, which triggers apoptosis when it binds FAS ligand.<sup>89</sup> EPO, in turn, acts to decrease FAS expression in erythroid progenitors. FAS ligand, which binds and activates FAS, is produced in a constitutive manner in the marrow, mainly by mature erythroblasts in humans.<sup>90</sup> Thus, within the EBI, a negative feedback loop from the terminally differentiating erythroblasts can modulate the rate of CFU-E–ProEB apoptosis and indirectly control rates of erythrocyte production.<sup>89</sup> By a more indirect mechanism, EPO signaling also appears to protect late-stage erythroblasts from apoptosis, including in the post-EPO-dependent period, by inducing large amounts of the antiapoptotic protein BCL-X<sub>L</sub>.<sup>91,92</sup>

### Erythrocyte production kinetics based on EPO levels

A model that incorporates varying plasma EPO levels and heterogeneity in EPO dependence among the EPO-dependent progenitors has been proposed to explain various physiologic and pathologic rates of erythrocyte production.<sup>93</sup> In an expanded version of this model, erythroid progenitors enter the EPO-dependent period of differentiation, left of the dotted line in Figure 13.4, extending from the CFU-E through the early BasoEB stage and encompassing three generations of cells. The proportion of total cells that survive in a generation is shown under the population. The surviving cells are represented by circles, each of which contains a large black dot representing an intact nucleus. The cells lost to apoptosis are shown by circles containing an X. The number of surviving cells in a generation results in twice that number for the total cells in the subsequent generation. Most cells reaching the CFU-E stage need more EPO than the low levels found in normal plasma to sustain them and their progeny through the EPO-dependent period of differentiation. As a result, approximately 200–250 billion erythrocytes produced daily by a normal, healthy adult are the descendants of a minority of all the potential erythroid progenitor cells that could be generated during the EPO-dependent period (Figure 13.4A). When blood loss, hemolysis, or decreased atmospheric oxygen is encountered, plasma EPO increases, allowing the survival of many EPO-dependent progenitors that would die by apoptosis under normal conditions (Figure 13.4B). This enhanced survival increases reticulocyte production within a few days after encountering blood loss, hemolysis, or decreased atmospheric oxygen. The increased reticulocytosis leads to increasing erythrocyte numbers until oxygen delivery recovers to normal, accompanied by declining plasma EPO levels until normal levels are achieved. In pathologic states of chronically decreased oxygen delivery, such as lung disease or cardiac diseases with right-to-left shunts, the persistently increased EPO levels (and increased glucocorticoids and BMP4) allow greater-than-normal survival of EPO-dependent cells such that the total number of erythrocytes is maintained in the polycythemic range. Likewise, the acquired somatic mutation of JAK2 (V617F) that is associated with hyperactivity of EPO-R signaling most commonly results in polycythemia vera.<sup>94</sup>

When plasma EPO levels fall below normal, many erythroid progenitor cells that would survive the EPO-dependent period of

differentiation under normal conditions die by apoptosis resulting in anemia from decreased reticulocyte production (Figure 13.4C). Renal disease is the major cause of decreased EPO. In mouse models of renal disease, EPO-producing renal cortical fibroblasts are transformed by TNF $\alpha$  signaling through NF $\kappa$ B into proliferating myofibroblasts that do not produce EPO.<sup>95</sup> Other clinical diseases noted to have decreased EPO levels are inflammatory disorders<sup>68</sup> and malignancies,<sup>69</sup> which are associated with increased inflammatory cytokines including TNF $\alpha$ , indicating that decreases in plasma EPO contribute to the anemia of chronic inflammation.

### Nutritional requirements for erythropoiesis

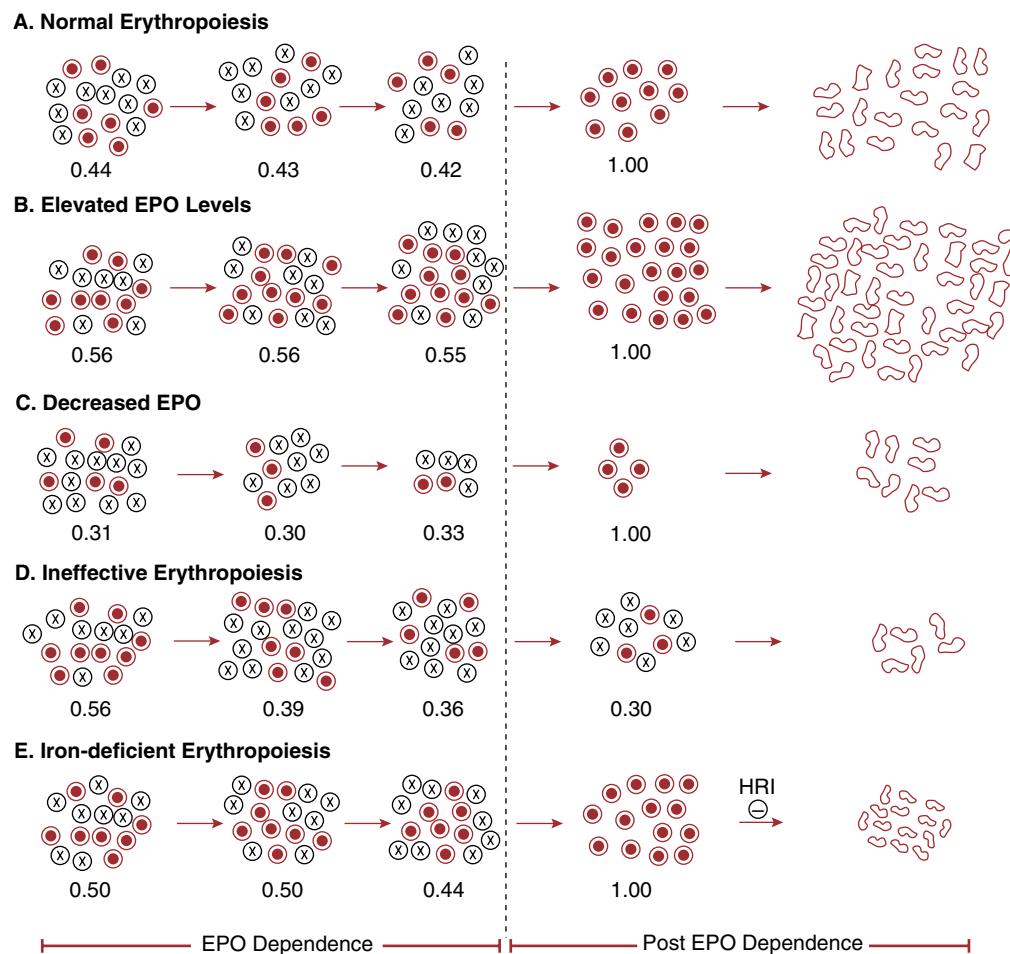
Although erythropoiesis is finely regulated by the oxygenation-EPO feedback mechanism shown in Figure 13.2, the erythropoietic process is frequently limited by an insufficient supply of folate, vitamin B<sub>12</sub>, or iron. Folate and vitamin B<sub>12</sub> (cobalamin) are required for the synthesis of DNA, and the daily production of very large numbers of erythrocytes results in a large DNA synthesis requirement. Although iron is also needed by all proliferating cell populations, the erythroblasts need much more iron than any other cell type because they produce hemoglobin. Through the hypoxia-feedback mechanism, these nutrition-related anemias are associated with increased EPO levels,<sup>67</sup> but the increase in EPO is more limited in iron deficiency, as described in the “Iron Deficiency and Development of Microcytic Anemia” section, and increased EPO can only partially compensate for the decreased erythropoiesis caused by a specific nutrient deficiency. Administration of the deficient nutrient, however, results in the resolution of anemia in each of the deficiency states.

### Deficiencies of folate or cobalamin and development of macrocytic anemia

After reduction to tetrahydrofolate (THF), folate functions as a carrier of one-carbon molecules and becomes a cofactor in the synthesis of three deoxyribonucleosides (dGTP, dATP, and TTP) that are required for DNA synthesis.<sup>96</sup> In two separate reactions, formyltetrahydrofolate (CHO-THF) provides two of the carbons in the synthesis of the purine precursor of adenosyl and guanosyl deoxyribonucleosides; in both of these reactions, 10-CHO-THF is converted to THF. In a third reaction, methylenetetrahydrofolate (CH<sub>2</sub>-THF) provides a methylene group and reducing equivalents in the methylation of deoxyuridylate to form thymidylate; in the process, CH<sub>2</sub>-THF is converted to dihydrofolate (DHF). To regenerate THF, the active one-carbon acceptor-donor form, DHF must be reduced by dihydrofolate reductase (DHFR).

Thus, drugs that inhibit DHFR such as methotrexate or trimethoprim-sulfamethoxazole cause a deficiency of THF, the functional form of folate. Cobalamin is a cofactor in the conversion of methyl-tetrahydrofolate (CH<sub>3</sub>-THF) to THF. CH<sub>3</sub>-THF, the most prevalent form of folate in plasma, is imported into cells and retained there by addition of polyglutamates. Cobalamin deficiency results in the trapping of folate in the CH<sub>3</sub>-THF form, from which it cannot be converted to THF and subsequently to the CHO-THF and CH<sub>2</sub>-THF forms required for deoxyribonucleoside synthesis.<sup>97,98</sup> Furthermore, CH<sub>3</sub>-THF is the poorest THF form for polyglutamation, resulting in generalized loss of intracellular folate.<sup>96</sup>

Folate deficiency, cobalamin deficiency, or drugs that inhibit DHFR will decrease intracellular levels of folate coenzymes needed for de novo synthesis of all the deoxynucleosides used in DNA synthesis, except for deoxycytidine. An inadequate supply of deoxynucleosides



**Figure 13.4** Model of erythropoiesis based on suppression of apoptosis by EPO and heterogeneity in EPO dependence among erythroid cells. From CFU-E through early basophilic erythroblast stages, erythroid progenitor/precursor cells depend on EPO for survival. The EPO-dependent period is left of the dotted line and encompasses three generations and two cell divisions. Each division is represented by an arrow. In the post-EPO-dependent period, to the right of the dotted line, two cell divisions occur. Surviving cells in each generation are shown as circles containing large black dots representing intact nuclei. Cells succumbing to apoptosis are shown as circles containing Xs. The proportion of the total cells that survive is shown below each generation. The number of surviving cells in a generation results in twice that number of total cells in the subsequent generation. The final populations of cells shown on the right represent the anucleate, irregular reticulocytes. (A) Normal erythropoiesis, with average survival rates of 43% in the EPO-dependent generations, produces 200–250 billion reticulocytes daily. (B) Elevated EPO levels as found after acute blood loss or hemolysis increase survival rates to 56% in the EPO-dependent generation, and reticulocyte production rate more than doubles. (C) Decreased EPO levels, as found in renal failure, decrease survival rates to 32% in the EPO-dependent generation, and reticulocyte production is less than one-half of normal. (D) Ineffective erythropoiesis increases rates of apoptosis due to a pathologic process such as folate or vitamin B<sub>12</sub> deficiency or β-thalassemia. High EPO levels in response to decreased erythrocyte production expand surviving cells in the early EPO-dependent generations, but the increased rates of apoptosis in the late EPO-dependent and post-EPO-dependent stages decrease daily reticulocyte production to less than one-third of normal. (E) Iron-deficient erythropoiesis with moderately elevated EPO activity for the degree of anemia slightly increases the survival rate during the EPO-dependent period, but in the post-EPO-dependent period, when hemoglobin is synthesized, heme-regulated inhibitor (HRI) prevents apoptosis by inhibiting general protein synthesis while enhancing the production of the mediator of stress erythropoiesis, the transcription factor ATF4. The inhibited protein synthesis decreases reticulocyte production rate, size, and hemoglobin content.

causes the accumulation of erythroid progenitors in the S phase of the cell cycle, which is followed by the induction of apoptosis.<sup>99</sup> Erythroid cells at the end of the EPO-dependent stage and the beginning of the period of hemoglobin synthesis appear to be most susceptible to this apoptosis. EPO-induced expansion of the EPO-dependent population at the CFU-E and proerythroblast stages leads to the presence of even greater numbers of these progenitor cells that subsequently undergo apoptosis just as they are beginning to produce hemoglobin.<sup>100</sup> The resultant clinical disease is megaloblastic anemia, which is characterized by ineffective erythropoiesis and macrocytic erythrocytes (Figure 13.4D). In ineffective erythropoiesis, progenitor cells in the

EPO-dependent period expand due to increased EPO levels. The number of reticulocytes formed, however, is less than normal because of the increased rates of pathologic apoptosis in the EPO-dependent and post-EPO-dependent periods of differentiation.

Although the degree of ineffective erythropoiesis is prominent in megaloblastic anemia, the same process of inhibited DNA synthesis but with less apparent apoptosis is common in many macrocytic anemias. Cell size reductions during normal terminal erythroid differentiation result from shortening of the G1 phase of cell cycle while the lengths of S and G2/M phases remain unaffected.<sup>27</sup> Terminally differentiating erythroblasts with delayed or prolonged

cell cycle durations produce larger-than-normal erythrocytes because they accumulate larger amounts of protein during the protracted periods between cell divisions.<sup>41</sup> With folic acid fortification of grain products, which began in the United States in 1998, folate deficiency related to dietary intake has been largely eliminated,<sup>101</sup> except for those with malabsorption disorders.<sup>102</sup> However, cobalamin deficiency and drugs that interfere with folate metabolism such as methotrexate, trimethoprim-sulfamethoxazole, and anticonvulsants remain clinically relevant causes of macrocytic anemias.<sup>103</sup> Drugs that directly inhibit DNA synthesis such as antivirals (azidothymidine or zidovudine), immunosuppressives (azathioprine), and ribonucleotide reductase inhibitors (hydroxyurea) are major causes of macrocytic anemia.<sup>103</sup> In addition, several inherited and acquired marrow failure syndromes that cause macrocytic anemia have either directly or indirectly inhibited DNA synthesis and increased apoptotic loss of erythroid progenitors.<sup>41</sup> Those with direct DNA synthesis inhibition include Fanconi anemia, in which increased DNA crosslinking requires more DNA repair before cell division is completed,<sup>104</sup> and dyskeratosis congenita anemia, in which chromosomal telomeres cannot be maintained.<sup>105</sup> In addition to direct DNA damage, the induction of p53 in these erythroblasts contributes to both delayed cell cycle and apoptosis.<sup>106</sup> Other marrow failure diseases show indirect inhibition of DNA synthesis, such as Diamond-Blackfan anemia<sup>107,108</sup> and 5q-myelodysplastic syndrome anemia,<sup>109</sup> in which impaired ribosomal biogenesis and/or function leads to secondary inhibition of DNA synthesis and accompanying apoptosis by p53 induction.<sup>108</sup>

### **Iron deficiency and development of microcytic anemia**

In addition to its oxygen transport function in hemoglobin, iron has essential roles in heme as part of myoglobin, mitochondrial cytochromes, and peroxidases. Among many nonheme enzymes, iron is required by four enzymatic processes described in other sections of this chapter: prolyl hydroxylation in HIF- $\alpha$  stability, mitochondrial ferrochelatase insertion of iron into protoporphyrin, ribonucleotide reductase in deoxynucleoside synthesis, and aconitase in glucose metabolism. Two-thirds of the body's iron is in the hemoglobin of circulating erythrocytes, and iron deficiency most commonly arises from blood loss. Two milliliters of blood contains about 1 mg of iron, which is approximately the amount absorbed daily by the duodenum, balancing the 1 mg normally lost through shedding of gastrointestinal tract and skin cells. Erythroid progenitor cells are the greatest consumers of iron in the body, using about 25 mg daily under normal conditions, with most of the iron that is supplied to erythroid cells being recycled from macrophages that phagocytose senescent erythrocytes and degrade their hemoglobin. When erythropoietic demands are increased after bleeding or hemolysis, duodenal iron absorption is increased indirectly by erythroferrone, a hormone produced by erythroblasts that decreases hepatic production of hepcidin.<sup>110</sup> Hepcidin, a 25-amino-acid hormone produced in the liver, is induced by increased plasma iron and by inflammatory cytokines.<sup>111</sup> Hepcidin binds and downregulates surface expression of ferroportin, the cellular iron exporter of iron for all cells, including duodenal enterocytes, which are responsible for iron absorption.<sup>112</sup> The increased absorption mediated by erythroferrone is limited, however, and chronic blood loss of as little as 5 mL per day may cause iron deficiency.<sup>113</sup>

Specific regulators of iron and heme metabolism protect cells from the toxic effects of iron while assuring that crucial cellular

processes that rely on iron are sustained in nonerythroid cells during iron deficiency. Therefore, as iron deficiency develops, erythropoietic utilization of iron becomes restricted and anemia develops. In iron-deficient cells, iron regulatory proteins (IRP1 and IRP2) bind to iron responsive elements (IREs) in 5'- and 3'-untranslated regions (UTRs) of mRNAs controlling production of proteins involved in cellular iron import, export, and storage.<sup>114,115</sup> Under iron-replete conditions, IRP1 functions as the enzyme aconitase with an iron-sulfur cluster in its active site; under iron-deficient conditions, IRP1 lacks the iron-sulfur cluster and binds IREs. IRP2 is rapidly degraded under iron-replete conditions but is stable and binds IREs during iron deficiency. IRP binding of IREs in the 5'-UTR of mRNAs inhibits their translation, decreasing their production. Two important examples are mRNAs for ferroportin and ferritin, the intracellular storage protein, both of which decrease during iron deficiency, allowing the maintenance of normal intracellular iron levels. In contrast, IRP binding of IREs in the 3'-UTR of mRNAs stabilizes them and enhances their translation. An example is transferrin receptor mRNAs, where IRP binding increases transferrin receptor production, thereby increasing cellular iron importation.

Although translation of ferroportin mRNA is controlled by the 5'-IRE in most cells, alternative splicing in duodenal enterocytes and erythroid precursor cells produces ferroportin mRNAs without 5'-IREs.<sup>116,117</sup> Thus, during iron deficiency, ferroportin expression is sustained in these two cell types, allowing uncompromised iron exportation into the plasma from duodenum and diminished accumulation within erythroid precursors and erythrocytes.<sup>118,119</sup> During iron deficiency, IRPs increase binding to 5'-IREs of two key mRNAs involved in erythropoiesis. IRP1 binds a 5'-IRE in HIF-2 $\alpha$  mRNAs, leading to decreased translation of HIF-2 $\alpha$  messages in the renal cortical fibroblasts that are capable of producing EPO.<sup>120-122</sup> The decreased intracellular HIF-2 $\alpha$  protein results in less EPO production despite the hypoxia of the renal cortex from the decreased numbers of circulating erythrocytes. As a result, renal EPO production may be relatively diminished in the anemia of iron deficiency when compared to other anemias of similar severity. In erythroid progenitors, HRI-mediated increases in ATF4 and its target *GRB10* can dampen EPO signaling.<sup>43</sup> The decreased EPO activity in iron deficiency, relative to anemia from blood loss or hemolysis, leads to relatively increased apoptosis of erythroid cells in the EPO-dependent stages that immediately precede the stages that synthesize hemoglobin (compare Figures 13.4B and 13.4E).

In the later hemoglobin-producing stages of iron-deficient erythropoiesis, IRP1 binds a 5'-IRE in mRNAs encoding ALAS2, which is the rate-controlling enzyme in porphyrin synthesis.<sup>38</sup> The resultant decreases in ALAS2 lead to less accumulations of protoporphyrin and heme in the erythroblasts. The decreased heme in erythroblasts increases HRI activity, which inhibits protein synthesis in general and globin syntheses in particular.<sup>43</sup> The combined effects of relatively decreased EPO activity and HRI-mediated restriction of protein synthesis in iron deficiency result in a slower rate of completion of the terminal stages of erythroblasts, with decreased rates of red blood cell production resulting in hypochromic, microcytic anemia as shown in Figure 13.4E.

In addition to iron deficiency anemia, HRI plays a role in other microcytic anemias in which heme production is limited. Inherited disorders of ALAS2 cause sideroblastic anemia as iron accumulates in mitochondria when porphyrin synthesis does not provide sufficient protoporphyrin IX for heme formation. Likewise, mutations in ferrochelatase decrease intracellular heme.

Mice deficient in HRI that become iron-deficient or have impaired porphyrin synthesis die from anemia when excess globin chains that cannot form hemoglobin without heme precipitate, denature, and cause oxidative damage resulting in apoptosis of erythroblasts.<sup>123,124</sup> Thus, HRI rescues iron-deficient erythroblasts from the thalassemia-like phenotype of oxidative damage from excess globin chains by restricting globin chain synthesis when heme synthesis is insufficient.

### **Thalassemia and the development of ineffective erythropoiesis and microcytosis**

Thalassemia is the other major type of microcytic anemia. When thalassemia is severe, it is treated with chronic red cell transfusions. Thalassemias are caused by mutations that decrease the synthesis of either  $\alpha$ - or  $\beta$ -globin with intracellular accumulations of the excess unpaired  $\alpha$ - or  $\beta$ -globin chains.<sup>125</sup> Compared to the excess  $\beta$ -globin chains in  $\alpha$ -thalassemia, which form tetramers of hemoglobin H, the excess  $\alpha$ -globin chains in  $\beta$ -thalassemia are relatively insoluble. Excess free  $\alpha$ -globin chains in  $\beta$ -thalassemia are partially decreased by accumulation of  $\gamma$ -globin chains producing fetal hemoglobin, binding to AHSP, ubiquitination–proteasomal degradation, and autophagy of aggregated  $\alpha$ -globins.<sup>126</sup> If unpaired globin chains are not removed by these intracellular adaptations, they can precipitate and denature, leading to the formation of methemoglobin and hemichromes that bind, oxidize, and disrupt the function of erythroid membrane and membrane skeletal proteins.<sup>127</sup> When the cytoplasmic domain of Band 3 is affected by this oxidative damage, it leads to aggregation, deposition of anti-Band 3 IgG, complement fixation, and phosphatidylserine externalization that in turn targets the cells for erytrophagocytosis.<sup>128,129</sup> The decreased solubility of unpaired free  $\alpha$ -globins in  $\beta$ -thalassemias results in ineffective erythropoiesis due to intramedullary apoptosis of erythroblasts, whereas  $\alpha$ -thalassemias have relatively less erythroblast apoptosis but more erythrocyte hemolysis.<sup>130</sup> Apoptosis in the  $\beta$ -thalassemias affects the late stages of erythroblast differentiation, and, because the mitigation of the EPO response due to IRP activity in iron deficiency does not occur in thalassemia, erythroid progenitors and early-stage erythroblasts expand in response to increased EPO. These early-stage erythroid populations expand extensively, with the degree of expansion directly related to the rate of apoptosis in the late-stage erythroblast populations.<sup>131</sup> The large expansion of erythroblast populations in the more severe cases of  $\beta$ -thalassemia increases erythroferrone, which increases iron absorption and results in iron overload that complicates and limits transfusion therapy in these patients.<sup>110</sup>

Depending upon the severity of thalassemia, the oxidative stress due to denatured globin chains, heme, and nonheme iron can overwhelm the erythroblast's normal antioxidant enzymes, such as superoxide dismutase, catalase, and glutathione peroxidase, and toxic oxygen species scavengers, such as reduced glutathione and peroxiredoxin.<sup>132</sup> In these oxidation-stressed thalassemic erythroblasts, HRI has also been found to have an antioxidant effect via increased Atf4, which induces expression of antioxidant genes, including heme oxygenase-1 (HO1), the first step in the degradation of heme.<sup>133</sup> In mice, HRI deficiency converts the moderate anemia of  $\beta$ -thalassemia intermedia into an embryonic lethal anemia with extensive accumulations of precipitated and denatured  $\alpha$ -globin chains.<sup>124</sup> The antioxidant activity of HRI is accompanied by its general restriction of protein synthesis so that the phenotype in thalassemias is a microcytic, hypochromic anemia.

### **Anemia of chronic inflammation**

Diseases that can secondarily decrease erythropoiesis include those that directly displace the EBIs in the bone marrow, such as metastatic neoplasms, lymphoid neoplasms, and myelofibrosis.<sup>134,135</sup> However, the most common cause of secondary inhibition of erythropoiesis is anemia of chronic inflammation (ACI), which occurs in patients with chronic infections, neoplasms, and inflammatory diseases. ACI has multiple components in common with the various mechanisms shown in Figure 13.4. These components include direct and indirect inhibitory effects of specific inflammatory cytokines on erythropoietic cells and their hematopoietic progenitors. Inflammatory cytokines with recognized inhibitory mechanisms include interleukin-1 (IL1), IL6, TNF $\alpha$ , and interferon- $\gamma$  (IFN $\gamma$ ).<sup>136</sup> Direct inhibition of cell survival and growth by IFN $\gamma$  involves the induction of PU.1 in MEPs which suppresses erythroid differentiation and promotes megakaryocytic differentiation. In the subsequent stages of EPO dependence, IFN $\gamma$  enhances the expression of members of the apoptosis-inducing TNF receptor family, including receptors for TNF $\alpha$ , FAS, TNF-related apoptosis-inducing ligand (TRAIL), TNF-like weak inducer of apoptosis (TWEAK), and receptor-binding cancer antigen expressed on SiSo cells (RCAS1).<sup>137–139</sup> The EPO-dependent stages are also affected indirectly by decreased EPO production that is induced by TNF $\alpha$ . Although the concentrations of TNF $\alpha$  to which the EPO-producing fibroblasts in the renal cortices are exposed are lower than when the inflammation is within the renal tissue, plasma EPO levels are lower in ACI than in other anemic states without inflammation.<sup>68,69</sup>

The later stages of erythropoiesis when hemoglobin is produced have relatively restricted iron supplies due to IL6 and members of the bone morphogenetic protein (BMP) family that induce transcription of hepcidin in the liver.<sup>111</sup> Experimental models show that hepcidin induction can be mediated by IL6 signaling through the JAK2–STAT3 pathway<sup>140–142</sup> or by bacterial endotoxin signaling through the BMP–Smad1/5/8 signaling pathway.<sup>143</sup> Hepcidin downregulates ferroportin on all cells, but its effects on three specific types of cells are most important for the inhibition of erythropoiesis in ACI. In macrophages, decreased ferroportin greatly diminishes the recycling of iron recovered from phagocytosed senescent erythrocytes. This sequestration of iron in macrophages is partially offset in ACI erythroblasts because hepcidin-induced decreases in erythroid cell ferroportin limit their iron export compared to erythroid cells in iron deficiency.<sup>118</sup> In severe cases of ACI, the downregulation of ferroportin on duodenal enterocytes restricts iron absorption, and eventually iron deficiency can develop. When iron deficiency complicates ACI, HRI activity causes the usually normocytic anemia to become microcytic. Most of the studies have focused on the acute phase of inflammation. The long-term consequences and the development of an EPO-refractory anemia remain unclear.

### **Erythropoietic therapies**

Enhancement of erythropoiesis to reduce the frequency of RBC transfusion may be achieved by increasing erythropoietic progenitors, supplying exogenous stimulators of erythropoiesis, increasing endogenous production of erythropoietic stimulators, and reducing effects of negative regulators of erythropoiesis. In aplastic anemia, decreased numbers of HSCs and MPPs result in decreased erythroid progenitors that descend from them. Immune suppression with cyclosporine plus antithymocyte globulin and stem cell stimulation with the thrombopoietin mimetic, eltrombopag, separately or in combination are effective in these patients.<sup>144</sup> In Diamond–Blackfan anemia (DBA), decreased progenitor cells are confined to the erythroid lineage and

are the result of mainly mutant genes encoding ribosomal proteins. The majority of DBA patients respond, at least initially, to glucocorticoids hormones, prednisone or prednisolone, with expansion of erythroid progenitors.<sup>145</sup> In chronic, low-risk variants of myelodysplastic syndrome, the abnormal erythroid lineage involvement extends through the late-stage erythroblasts. About half of transfusion-dependent patients with the del(5q) karyotype can become RBC transfusion free with the immunomodulator, lenalidomide.<sup>146</sup> For those patients without del(5q) karyotype and low endogenous EPO levels, erythropoietic stimulating agents, recombinant human erythropoietin and darbepoetin, are effective alone and in combination with granulocyte-stimulating factor.<sup>147</sup> In patients with low-risk MDS<sup>148</sup> or beta-thalassemia,<sup>149</sup> luspatercept, which binds transforming growth factor-beta superfamily ligands including activin and growth-and-differentiation factor 11, increases erythropoiesis and reduces RBC transfusion requirements, but long-term effects are unknown. Patients with renal disease have EPO deficiency that is treated effectively with recombinant human erythropoietin rhEPO<sup>150,151</sup> or its modifications including hyperglycosylated rhEPO (darbepoetin)<sup>152</sup> and polyethylene glycol-conjugated rhEPO (CERA).<sup>153</sup> However, correction to or near normal ranges of hemoglobin or hematocrit is associated with increased thrombotic vascular events. HIF prolyl hydroxylase inhibitors stabilize HIF-2α and, thereby, increase endogenous EPO transcription, plasma EPO levels, and increase erythropoiesis in renal disease patients with anemia.<sup>154–157</sup> However, possible adverse long-term events of increased HIF such as VEGF-induced vessel growth in diabetic retinopathy or malignancies remain unknown.

## Summary and outlook

Erythropoiesis, a component of hematopoiesis, is required for normal maintenance of red blood cell numbers and responds with increased production rates following blood loss or hemolysis. In the erythroid differentiation process, the rate of erythrocyte production is regulated largely by EPO, which is produced in the renal cortex in response to the tissue hypoxia that results from decreased oxygen delivery in anemic states. The oxygen-EPO feedback mechanism finely controls rates of erythrocyte production that never overshoot and result in polycythemia. This feedback mechanism, however, responds promptly to physiologic changes such as blood

loss, hemolysis, or changes in atmospheric oxygen. Chronic underproduction of erythrocytes results in anemia, and effective treatments may prevent or reduce red blood cell transfusions for those with underproduction anemias. Use of recombinant EPO is routine for patients with the anemia of renal disease, an EPO deficiency state. Limited responses to EPO in patients with anemias due to malignancy or myelodysplasia, combined with an increased potential for thrombotic and cardiovascular complications of EPO therapy in general, have resulted in more restricted use of recombinant EPO or its modified forms in clinical practice. New therapeutic agents that increase endogenous EPO production or decrease the activity of negative erythropoietic regulators may help treat underproduction anemias. Deficiencies of folate and vitamin B12, which are required for the extensive cellular proliferation needed to produce erythrocytes, and iron, which is required to produce the major protein of erythrocytes, hemoglobin, are remediable causes of anemia. Chronic inflammation, another very common cause of anemia, is most improved by treating the primary disease, but new agents may enhance erythropoiesis in those cases in which treatment of the primary disease may be limited.

## Key references

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## CHAPTER 14

# Red blood cell metabolism and preservation

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## Introduction

Human red blood cells (RBCs), mature and released from the bone marrow, lack nuclei, ribosomes, and mitochondria. They have a life span of 120 days, after which they are removed from the circulation in the natural course of aging.<sup>1</sup> RBCs pick up oxygen during a one-third second transit through the alveolar capillary (a billion O<sub>2</sub> molecules per cell) and deliver oxygen through capillaries with a diameter smaller than their own. RBCs can neither use oxygen for the extraction of energy nor synthesize proteins or polynucleotides. Their primary functions, transporting oxygen from the lungs to the tissues and carbon dioxide back to the lungs, do not require the expenditure of energy, though oxygen release from hemoglobin is tightly regulated by high-energy phosphate compounds—such as adenosine 5'-triphosphate (ATP) and 2,3-diphosphoglycerate (DPG). As such, maintaining hemoglobin in an optimal state for delivering of oxygen and keeping normal cell flexibility and morphology do require active metabolism and are prerequisites for appropriate RBC function and successful transfusion therapies.

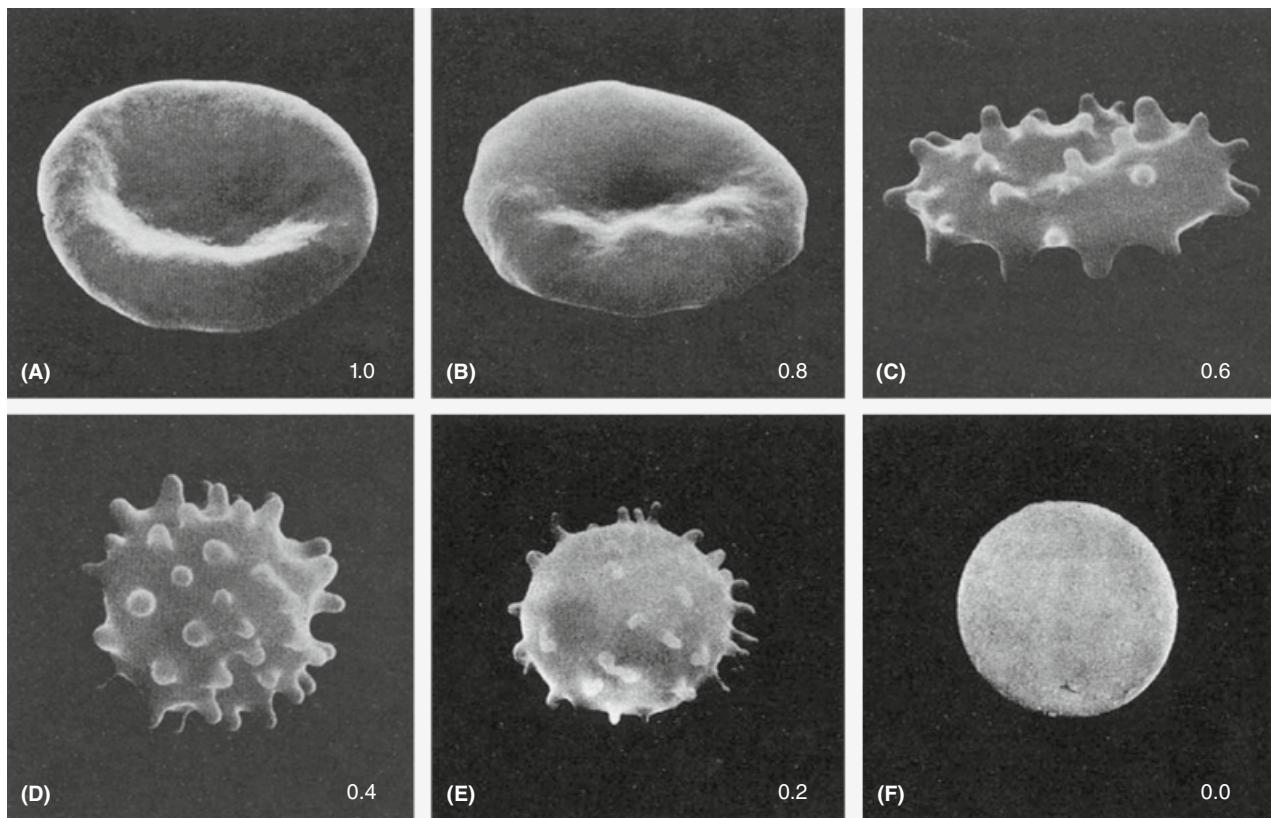
During standard storage at 4 °C for up to 42 days, significant and in part reversible changes in red cell morphology and metabolism occur, called storage lesions.<sup>2</sup> One striking example is the change in red cell shape (Figure 14.1) which can be reversed to a large extent by washing stored RBCs with albumin or by metabolic rejuvenation.<sup>3,4</sup> Recent advances such as imaging flow cytometry and cell sorting allow the precise measurement and isolation of cells with these changes.<sup>5</sup> In order to understand and further improve storage, it is essential to understand basic principles of red cell metabolism.

Critically, the binding and release of oxygen by hemoglobin is not completely reversible. Occasionally, the departing oxygen takes an extra electron, forming methemoglobin (Fe<sup>++</sup> → Fe<sup>+++</sup>) and superoxide (O<sub>2</sub><sup>-</sup>). The RBC is full of methemoglobin reductase and superoxide dismutase but needs energy to drive their activities. As the waste products of energy metabolism, lactate and protons, accumulate in closed storage systems and drive down pH, the reducing potential of the RBC's major antioxidant, glutathione, falls from 0.7 to 0.2 mEv and oxidant damage to proteins, lipids, and carbohydrates accumulates, despite methemoglobin reductase, superoxide dismutase, and other protective mechanisms.<sup>6</sup>

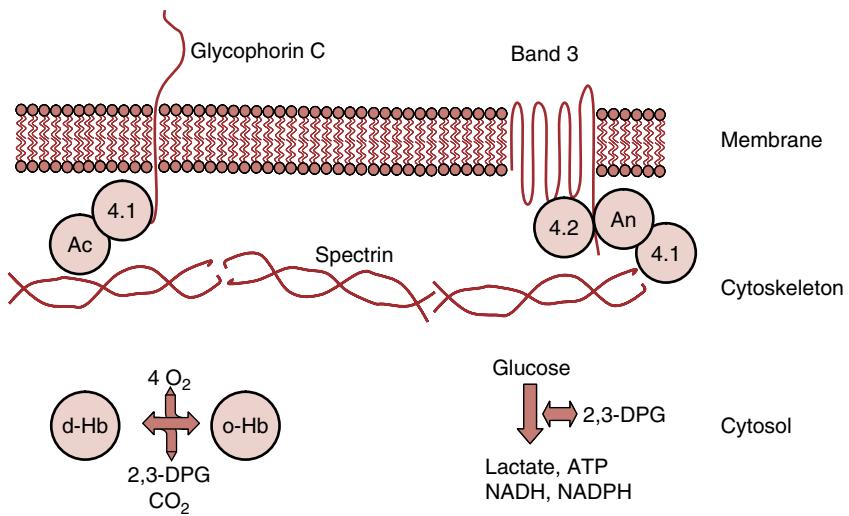
Understanding of RBC metabolism has increased greatly with the application of “-omics” technologies. Proteomics has identified 2200 separate proteins in RBCs that are the products of 5% of all

human genes.<sup>7</sup> This number includes 340 proteins specifically associated with the red cell membrane. Structural proteins, membrane receptors, metabolic enzymes, heat shock proteins, chaperonins, and others have been cataloged, sorted into pathways and functional groups, and provide the most complete overview of the molecule by molecule functioning of a human cell type, suggesting several roles for red blood cells in systems homeostasis beyond oxygen transport in like fashion to a circulating organ. Metabolomics can simultaneously measure the concentrations of thousands of small molecules and follow their activities in isotopic pulse-chase experiments.<sup>8</sup> Lipidomics can enumerate the many classes of lipids present, categorized on the basis of their hydrophobicity and fatty acyl composition (chain length, degree of unsaturation, and oxidation).<sup>9</sup> Transcriptomics reveals the presence of 400 different small RNA molecules serving a variety of roles.<sup>10</sup> Analysis to date confirms the view that red cells are complex, metabolically active cells using glucose to make ATP and reducing equivalents to ensure flexibility and oxygen delivery. The red cell may be 98% hemoglobin by protein weight, but it is much more than “a hapless sac of hemoglobin.”<sup>11</sup>

Integration of biochemical metabolism and biophysical discoveries in the last two decades by several research groups show that many molecular interactions are missed when the red cell is considered by itself without taking into account the physiologic vascular milieu in which it performs its main biological function.<sup>12,13</sup> For example, the interaction of the two most abundant proteins in the red cell cytosol and membrane, hemoglobin and the band 3 protein, respectively, is influenced by conformational changes that accompany oxygen binding to hemoglobin; this interaction in turn modulates the activity of glycolytic enzymes that compete with de-oxyhemoglobin for the same binding domain on band 3, which in turn regulates metabolic fluxes through glycolysis, as well as the structural architecture of the red cell membrane-cytoskeletal linkage (Figure 14.2). During storage, concentrations of red cell ATP, DPG, and glutathione decline leading to cell wall dysfunction and damage. Dysfunction can manifest as stiffer red cells, while damage can include membrane loss. Both of these effects can in turn affect vascular flow of transfused RBCs. On the arterial side, stiffer cells have trouble absorbing the energy of pulsatile flow and are projected more frequently into the normally cell-free layer of plasma at the vascular surface where they absorb endothelium-derived nitric oxide and lead to vasoconstriction.<sup>14</sup>



**Figure 14.1** Scanning electron micrographs showing representative red blood cells in various stages of shape change typical of prolonged storage. The cells progress from discocytes (A) through several stages of echinocytes (B–D), to spherocytoids (E), and finally to spherocytes (F). Scores based on the visual appearance as those shown in the lower right of the individual images can be assigned to several hundred individual stored cells and averaged to produce a morphology score for the unit. Such scores decrease in a linear manner during storage but can be substantially reversed by rejuvenation.



**Figure 14.2** Schemata of red cell components. In the past, it was believed that oxygen transport by hemoglobin and metabolism were cytosolic functions, and that the membrane and cytoskeleton merely enclosed them. We now recognize that the attachments of the membrane to the cytoskeleton from band 3 through ankyrin (An) and proteins 4.2 and 4.1 to spectrin, and from Glycophorin C through protein 4.1 and actin (Ac) to spectrin are destabilized by 2,3-diphosphoglycerate (2,3-DPG). 2,3-DPG released from deoxy-hemoglobin (d-Hb) binds to band 3 and partially detaches the membrane from the cytoskeleton allowing lateral movement of membrane structures. This could have implications for red cell flexibility when slipping into peripheral capillaries. Other abbreviations: oxy-hemoglobin O-Hb.

Band 3—whose main function is to exchange chloride for bicarbonate anions—closely interacts not just with hemoglobin but also with carbonic anhydrase in the cell membrane. In capillaries in the peripheral vasculature, where oxygen tensions are lower and carbon dioxide accumulates as a function of tissue metabolism, this complex in the red cells promotes the production of bicarbonate with the release of protons that, via the so-called Bohr effect, cause the release of oxygen from hemoglobin to the tissues.<sup>8</sup> On the venous side, damaged membranes can lead to procoagulant and proinflammatory events.<sup>15</sup> In the following sections, red cell metabolism will be discussed taking into account intermolecular interactions of importance for the primary function of hemoglobin, which is oxygen and carbon dioxide transport. This section is followed by a review of the development of red cell storage systems.

## Metabolism

### Metabolism of glucose

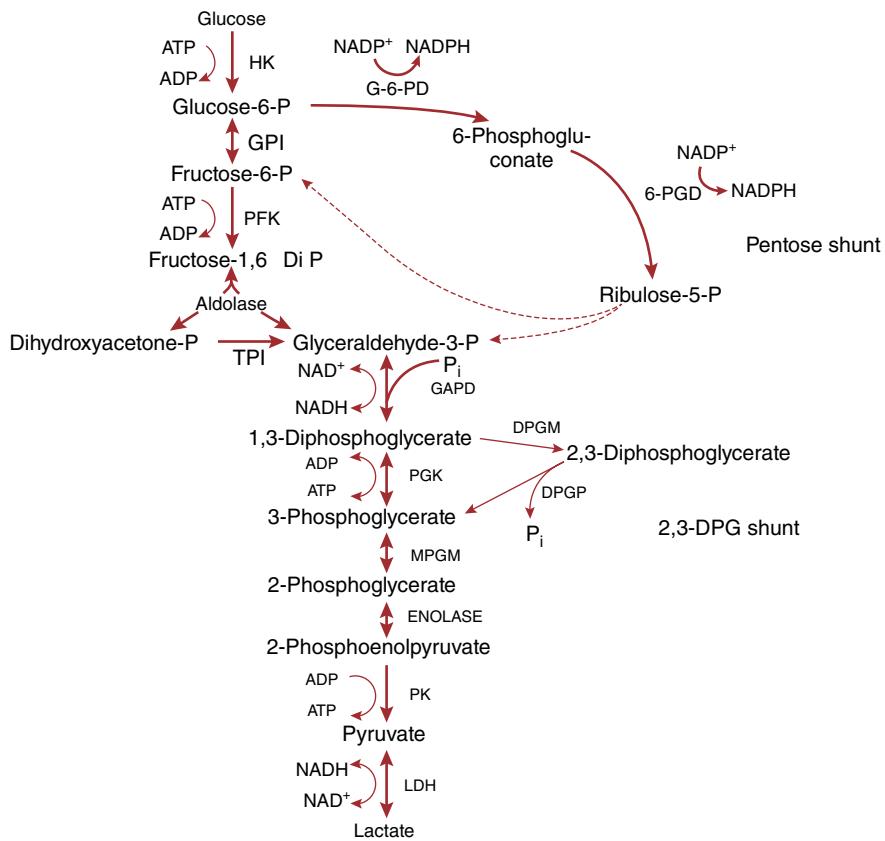
Under physiologic circumstances, the energy that the red cell requires is derived through the breakdown of glucose to lactate or pyruvate. The sequence of reactions is generally known as glycolysis or the Embden–Meyerhof–Parnas (EMP) pathway.<sup>16</sup> This pathway

is phylogenetically very old, and the sequence of reactions is the same in all mammalian tissues as well as in yeast and bacteria. Glycolysis is, however, inefficient, producing only 2 ATP molecules for each glucose broken down in contrast to the 34 ATP made from glucose in mitochondria.

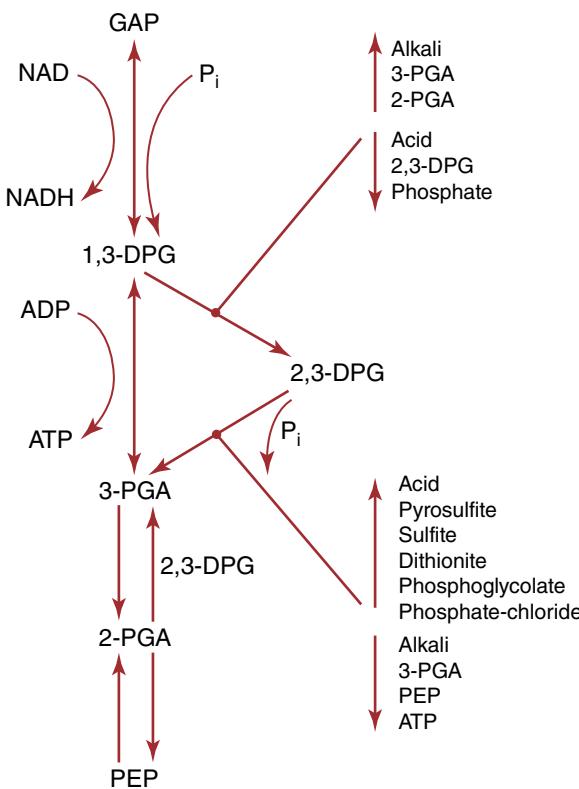
The reactions of the glycolytic pathway are shown in Figure 14.3. In this sequence of reactions, the six-carbon sugar glucose is phosphorylated, isomerized to fructose phosphate, phosphorylated again, and cleaved into three-carbon phospho-sugars. The three-carbon sugars are again phosphorylated. Finally, these carbohydrate-bound high-energy phosphates that have been synthesized are transferred to adenosine diphosphate (ADP), producing the high-energy compound ATP. The ATP synthesized is used by ATPases for the pumping of ions against concentration gradients, secreted to cause vasodilation, and very importantly used for the phosphorylation of glucose to make more ATP in the glycolytic pathway.<sup>17</sup>

### The 2,3-DPG shunt (Rapoport–Luebering shunt)

RBCs express a shunt that branches from the main glycolytic pathway after the formation of 1,3-diphosphoglycerate (1,3-DPG) and returns to it with the formation of 3-phosphoglycerate (3-PGA) (Figure 14.3). The Rapoport–Luebering shunt produces 2,3-DPG from 1,3-DPG, followed by the dephosphorylation of 2,3-DPG to 3-PGA



**Figure 14.3** The glycolytic pathway with the pentose and 2,3-DPG shunts of red cell metabolism. ATP, adenosine triphosphate; ADP, adenosine diphosphate; HK, hexokinase; NADP<sup>+</sup>, nicotinamide adenine dinucleotide phosphate, oxidized form; NADPH, nicotinamide adenine dinucleotide phosphate, reduced form; 6-PGD, 6-phosphogluconate dehydrogenase; P, phosphate; G-6-PD, glucose-6-phosphate dehydrogenase; PFK, phosphofructokinase; TPI, triosephosphate isomerase; NAD<sup>+</sup>, nicotinamide adenine dinucleotide, oxidized form; NADH, nicotinamide adenine dinucleotide, reduced form; GAPD, glyceraldehyde-3-phosphate dehydrogenase; PGK, phosphoglycerate kinase; DPGM, diphosphoglycerate mutase; DPGP, diphosphoglycerate phosphatase; MPGM, monophosphoglycerate mutase; PK, pyruvate kinase; LDH, lactate dehydrogenase. Source: Adapted from the 3rd edition of Rossi's Principles of Transfusion Medicine.



**Figure 14.4** Regulation of the formation and breakdown of 2,3-DPG. The steady-state concentration of 2,3-DPG is governed by the rate of its formation from 1,3-DPG and by its breakdown to 3-phosphoglyceric acid. Both reactions are catalyzed by the same multifunctional enzyme modulated by a variety of substances. Depending on the reaction, the enzyme is abbreviated DPGM or DPGP in the figure. Abbreviations are as in Figure 14.3. Source: Adapted from the 3rd edition of Rossi's Principles of Transfusion Medicine.

(Figure 14.4). Both reactions are catalyzed by the same enzyme and are balanced at physiologic pH.<sup>18</sup> At higher pH, the enzyme acts only as a mutase moving phosphate in 1,3-DPG from position 1 to position 2 in the molecule; at lower pH it acts only as a phosphatase, transforming 2,3-DPG to 3-PG. As the 2,3-DPG shunt bypasses one of the two ATP synthesis steps in glycolysis, 2,3-DPG is made at the expense of ATP. In storage systems, a high pH can shut down ATP production, while a lower than physiologic pH leads to a burst of ATP production driven by the breakdown of 2,3-DPG.

The production of large quantities of 2,3-DPG is a unique feature of glycolysis in the red cell. Red cells contain approximately equimolar amounts of hemoglobin and 2,3-DPG to allow 1:1 binding. In its most well-known function, the binding of 2,3-DPG to the  $\beta$  subunits of deoxyhemoglobin serves to stabilize the T (tense, low oxygen affinity) state of hemoglobin which shifts the oxygen equilibrium curve to the right (favoring dissociation of oxygen). In the R (relaxed, high oxygen affinity) state, approximately 80% of 2,3-DPG is “free,” while in the T state over 80% of 2,3-DPG is bound to hemoglobin.<sup>19</sup> In the “free” state, 2,3-DPG at physiological concentrations performs the second function of modulating properties of the red cell membrane.<sup>8</sup> It binds directly to band 3 and thereby interferes negatively in the interactions between proteins 4.1 and 4.2, ankyrin, and band 3.<sup>20</sup> 2,3-DPG also releases spectrin from the membrane skeleton and interferes negatively in the interactions between spectrin, actin, protein

4.1 and the glycophorin C complex.<sup>8,21,22</sup> These effects decrease the number of connecting links between the cell membrane and the cytoskeleton and increase lateral mobility of integral membrane proteins.<sup>10,23</sup> The rise and fall of “free” 2,3-DGP concentrations with each pass through the circulatory system results in repetitive destabilization and restabilization of the membrane–cytoskeleton architecture (Figure 14.2). “Free” 2,3-DPG increases cell flexibility by weakening the links between the membrane and the cytoskeleton and facilitates gas exchange by allowing the red cell to slip through narrow capillaries and splenic sinusoids. However, further experiments are needed to clarify the full physiologic implications of the interactions between 2,3-DPG, cell membrane proteins, and the cytoskeleton.

### The pentose shunt (hexose monophosphate shunt)

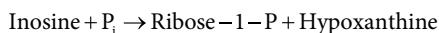
Under normal, steady-state conditions, ~92% of glucose is metabolized in red cells by way of the glycolytic pathway. The residual 8% is oxidized through another important metabolic pathway called the pentose phosphate pathway (PPP) or hexose monophosphate shunt (Figure 14.3). Some of the glucose-6-phosphate (G-6-P) formed when glucose is phosphorylated in the hexokinase reaction enters this pathway. Glucose-6-phosphate dehydrogenase (G-6-PD) catalyzes the rate-limiting oxidation of G-6-P to 6-phosphogluconolactone, reducing nicotinamide adenine dinucleotide phosphate (NADP) to NADPH. After hydrolysis of the lactone to 6-phosphogluconic acid, another oxidative step reduces additional NADP to NADPH, and releases carbon dioxide from the six-carbon compound, forming the pentose sugar ribose-1-phosphate. After a series of rearrangements (in the nonoxidative steps of the PPP), two normal intermediates of the main glycolytic pathway, fructose-6-phosphate and glyceraldehyde-3-phosphate, are formed and rejoin the main metabolic stream.

The pentose shunt is important to the red cell as a source of NADPH, a key reducing equivalent that is involved in the recycling of oxidized glutathione by glutathione reductase,<sup>24</sup> as well as a cofactor for several NADPH-dependent enzymes such as glutathione peroxidase, catalase, peroxiredoxins, glutaredoxins, thioredoxins, biliverdin reductase B, and the ascorbate-tocopherol axis.<sup>25</sup> Reduced glutathione is important for the elimination of peroxide, protection of protein SH-groups, limitation of lipid peroxidation, and detoxification processes. The pentose shunt also plays an important role in the red cell by providing ribose-5-phosphate to make phosphoribosyl pyrophosphate (PRPP), an essential substrate for the synthesis of adenine nucleotides required for continuing synthesis of ATP (see below) and GTP, as required for cyclic purine signaling (e.g., cyclic-AMP and cyclic-GMP) and purine salvage reactions (e.g., hypoxanthine-guanosine-phosphoribosyltransferase).

### Alternative substrates for red cell metabolism

Glucose is the natural substrate for human red cell energy metabolism, but red cells are also capable of metabolizing other sugars (i.e., fructose, mannose, galactose, and the three-carbon sugar dihydroxyacetone). However, none of these other sugars have proven to be useful in the design of blood preservatives.<sup>26</sup>

The presence in red cells of the enzyme nucleoside phosphorylase also makes it possible for RBCs to use nucleosides such as inosine to support ATP synthesis:



In this reaction, ribose-1-phosphate is formed without the expenditure of ATP. Ribose-1-phosphate is then readily converted to

fructose-6-phosphate by the pentose shunt which feeds into the glycolytic pathway leading to the generation of ATP. In the rejuvenation of red cells (see later) inosine allows ATP-depleted red cells to prime their metabolic pump (the glycolytic pathway). It has not been possible to include inosine in blood preservatives since the product of its metabolism, hypoxanthine, is rapidly converted to uric acid with the concomitant generation of the pro-oxidant hydrogen peroxide in the body. Because many patients who receive blood transfusions have impaired liver function or may already have hyperuricemia because of hereditary or acquired factors, a blood product that increases plasma uric acid level is not considered safe.<sup>27</sup>

## Regulation of energy metabolism

### Rate of glucose metabolism

In nucleated cells, metabolic regulation is dependent on protein synthesis which in turn is regulated by increasing or decreasing the rate of DNA transcription or the translation of messenger RNA. Red cells do not have these options. Instead, the rate of glucose metabolism is regulated by feedback mechanisms acting on the glycolytic enzymes.<sup>28</sup> In spite of extensive studies, our understanding of the control of glucose metabolism by red cells is still incomplete.

The N-terminal cytoplasmic domain of the band 3 protein binds hemoglobin, cytoskeletal proteins, and glycolytic enzymes, including the rate-limiting enzymes phosphofructokinase, aldolase, and glyceraldehyde 3-phosphate dehydrogenase.<sup>29</sup> Based on current evidence, de Rosa *et al.*<sup>8</sup> point out that binding between hemoglobin in the T state and band 3 causes a release by displacement of glycolytic enzymes, which results in increased activity of the main glycolytic pathway. In contrast, hemoglobin in the R (highly oxygenated) state is associated with decreased glycolysis, as glycolytic enzymes bind to band 3, but increased activation of the pentose shunt (see later).<sup>30</sup>

The rate of glucose metabolism by red cells is influenced by many factors other than basal enzyme activity. Negative feed-back mechanisms are involved in the control of the glycolytic pathway. Both hexokinase and phosphofructokinase are inhibited by hydrogen ions (low pH)<sup>31</sup> or other metabolites (e.g., citrate inhibits phosphofructokinase in other cell types through the Randle effect).<sup>32</sup> As storage progresses, studies have shown the progressive accumulation of reversible and irreversible oxidation of hemoglobin and glycolytic enzymes at functional cysteines (e.g., Cys152 and 156 of GAPDH), as well as deamidation of asparagines facing the substrate accessible pocket of the active site of several glycolytic enzymes.<sup>33,34,35</sup>

However, the principal reason that the rate of glycolysis slows markedly during red cell storage is the accumulation of lactic acid.

### 2,3-DPG concentration

The concentration of 2,3-DPG depends on the rate of its formation and degradation. Many effectors determine whether the mutase or the phosphatase activity of the diphosphoglycerate mutase/phosphatase predominates (Figure 14.4). The hydrogen ion concentration is the most important physiologic modulator.<sup>36</sup> At low pH, phosphatase activity is stimulated and mutase activity is inhibited. Thus, high pH favors 2,3-DPG maintenance and production during storage at the expense of ATP formation, whereas low pH leads to the rapid loss of 2,3-DPG with an increase in ATP production as long as DPG lasts. Modulation of red cell metabolism by elevating pH has been the principal means used to retard the decline of 2,3-DPG levels that occurs during liquid storage of red cells (see later). After the depletion of ATP during storage, 2,3-DPG levels in transfused red cells return to 50% of normal in 7 hours and almost to 95% at 72 hours.<sup>37</sup>

### The pentose shunt activity

Pentose shunt activity is influenced by the availability of NADP and the concentration of NADPH. Under oxidative stress, NADPH is oxidized to NADP and the activity of the pentose shunt increases, which is consistent with the observation of increased pentose shunt activity when hemoglobin is in the R state as mentioned previously. Oxygenated hemoglobin has decreased affinity for the cytoplasmic N-terminus of band 3, which then becomes accessible for the inhibitory binding of glycolytic enzymes. As such, glycolysis slows and up to 90% of glucose is diverted toward the pentose shunt for NADPH synthesis to counteract oxidant stress that arises through Fenton chemistry in the presence of iron and high oxygen concentrations. Since 2,3-DPG is consumed as storage progresses, increases in hemoglobin oxygen saturation as a function of storage duration (especially after storage week 3) have been associated with an increased activation of the pentose phosphate shunt.<sup>38</sup> Oxidation of GAPDH is an alternative mechanism through which glycolysis slows and the pentose shunt activity increases in red cells as a function of oxidant stress *in vivo* or during storage in the blood bag.<sup>39</sup> However, fluxes through this pathway are constrained by the activity of the enzyme glucose 6-phosphate dehydrogenase (G6PD), a protein that is coded by a gene on the X chromosome and, as such, is expressed at higher levels in red cells from female than male donors. Also, aging of RBCs, or the host in general, has been associated with declines in G6PD expression/activity, in part explaining why older red cells or red cells from older blood donors are characterized by increases in oxidant stress.<sup>40,41</sup>

G6PD activity is also negatively impacted by donor differences.<sup>42,43</sup> Notably, G6PD is highly polymorphic and ~400 million people around the world suffer from impairments to G6PD activity (<10 or even <1% residual activity) as a function of single nucleotide polymorphisms. Recent studies suggest that red blood cells from donors with G6PD deficiency are characterized by improved energy metabolism (as fluxes through glycolysis cannot be diverted through the pentose phosphate shunt) but poorer antioxidant metabolism. Other studies have shown that end-of-storage red cells from G6PD deficient donors (especially the African American variant) show decreases in post-transfusion recoveries—i.e., the percentage of transfused red cells that still circulate at 24 hours from transfusion—in autologous volunteers.<sup>44</sup>

### Adenosine triphosphate

In the red cells, ATP is synthesized by the glycolytic pathway, but its regulation is complex. ATP is used in a number of different metabolic pathways, particularly by the kinases that phosphorylate sugars (i.e., hexokinase and phosphofructokinase) and proteins, and by ATPase-driven ion pumps (i.e., Na<sup>+</sup>-K<sup>+</sup>-ATPase, Mg<sup>2+</sup>-ATPase, and Ca<sup>2+</sup>-ATPase). Moreover, red cell membrane shape and rigidity are controlled by the ATP-dependent cytoskeleton.



ATP is in equilibrium with ADP and adenosine monophosphate (AMP) as shown above. Production of ATP from ADP in glycolysis normally keeps AMP concentrations low. As the level of ADP increases when glycolysis slows, some is converted to AMP. AMP, in turn, is deaminated in the AMP-deaminase (AMPD) reaction and the total red cell pool of adenine decreases. This demonstrates why, during storage, ATP levels are depleted if adenine is not added to the anticoagulant and/or additive solution. Since red cell-specific AMPD3 activity is inhibited by 2,3-DPG, but promoted by

intracellular calcium and oxidant stress, ATP breakdown and purine deamination products such as hypoxanthine have been identified as metabolic markers of the storage lesion and post-transfusion recovery in mice and humans.<sup>45</sup> ATP is also used extensively in red cells to fuel phosphorylation reactions, which is relevant in red cells as erythrocytes have a higher number of kinases than any other cell. Though the topic would require a whole thematic chapter, the interested reader is referred to comprehensive reviews/research articles that document the impact of red cell protein phosphorylation in the maintenance of the homeostasis of membrane cytoskeleton and band 3–hemoglobin interactions through the phosphorylation of tyrosine in the N-terminus of band 3.<sup>46</sup>

ATP, released by RBCs during hypoxia, contributes to vascular tone by signaling to endothelial cells directly or in the form of ADP acting on P2Y receptors. Additionally, further breakdown of ADP into AMP and enzymatic conversion of AMP to adenosine via extracellular ectonucleotidases (e.g., CD73) participate in responses to hypoxia via autocrine signaling to circulating RBCs through adenosine interactions with the receptor A2B (ADORA2b). ADORA2b receptors are G-protein coupled receptors on red cells that produce increased intracellular cAMP and activation of cAMP-dependent protein kinase A (PKA).<sup>47</sup> Following exposure to hypoxia, adenosine uptake via the transporter ENT1 can be decreased via PKA-dependent phosphorylation and subsequent proteasomal degradation.<sup>48</sup> A second exposure to hypoxia thus results in a faster acclimatization because reduced uptake of adenosine by RBCs leads to a faster rate of extracellular accumulation.<sup>49</sup> Since circulating adenosine levels >14 μM are potentially toxic (they can cause arrhythmias), breakdown by adenosine deaminase in the bloodstream is critical in many circumstances, for example in the context of sickle cell disease where the activity of this enzyme limits adenosine signaling through ADORA2b and sickling of deoxy HbS.<sup>50</sup>

### Guanine nucleotides

Red cells contain and turn over guanine nucleotides that perform at least three functions. First, G proteins play a role in the signal transduction of membrane shear into secretion of the local vasodilators cAMP and ATP, as described above.<sup>51</sup> Second, guanosine nucleosides contribute to salvage reactions through the hypoxanthine guanosine phosphoribosyltransferase route. The resulting inosine can be phosphorylated to inosine monophosphate (IMP). Tracing experiments suggest that residual activity of the salvage pathway can convert a minimal fraction (<5%) of IMP back to AMP.<sup>52</sup> Third, high concentrations of GTP inhibit red cell transglutaminase, a primitive coagulation system that also interacts with the cytoplasmic domain of band 3 and with protein 4.1.<sup>53</sup> When GTP concentration is reduced during RBC aging, the resulting disinhibition of transglutaminase, which has a Factor XIII like activity, can facilitate the removal of senescent red cells by binding them to fibrin clots. Finally, like inosine for rejuvenation solutions, guanosine has been used in some novel additives (e.g., PAGGSM or PAGGGSM) to fuel the phosphoribolysis reaction that results in the release of guanine and ribose phosphate, which can in turn fuel the synthesis of ATP without initial energy expenditure.<sup>54</sup>

### Synthetic processes

Red cells are only able to assemble a limited repertoire of important molecules from simpler precursors. They retain the capacity to synthesize nucleotides through a “salvage pathway” and to synthesize glutathione from its precursor amino acids. Adenine is able to enter

the erythrocyte by membrane transporters, and then purine nucleotides can be synthesized through the adenine phosphoribosyl transferase reaction:



This reaction is critical in blood storage. The beneficial effect of addition of adenine to stored blood depends on it. Phosphoribosyl pyrophosphate (PRPP), a substrate for the formation of AMP in this reaction, is synthesized from ribose-phosphate formed in the pentose shunt. Guanine nucleotides form in an analogous reaction that is catalyzed by a different enzyme, hypoxanthine-guanine phosphoribosyl transferase. Red cells also actively synthesize a number of other small molecules, including reduced glutathione, nicotinamide adenine dinucleotide (NAD), and S-adenosyl-L-methionine.<sup>13</sup> The latter is a critical methyl-group donor for methylation reactions, which are required for repairing isoaspartyl damaged by oxidant stress.<sup>55</sup>

### Carboxylic acids and transamination

Recently, omics approaches have identified the presence of cytosolic isoforms of Krebs cycle enzymes in the mature erythrocyte, including malate dehydrogenase 1 (MDH1) and isocitrate dehydrogenase 1 (IDH1). Tracing experiments with stable isotope-labeled glutamine and citrate have helped confirm that these enzymes are active and that they participate in reducing equivalent homeostasis (NADH and NADPH) as a function of oxidant stress and hypoxia.<sup>56</sup> Other than participating in the homeostasis of reducing equivalents, aspartate to fumarate conversion can result from the salvage reactions to restore deaminated purines (e.g., IMP to AMP) described above.<sup>57</sup> In parallel, carboxylate metabolism crosstalks with transamination reactions, to the extent that reactions that use glutamate as an amine group donor (e.g., pyruvate conversion to alanine and vice versa) concomitantly generate alpha-ketoglutarate, a substrate for isocitrate dehydrogenase.<sup>58</sup> This pathway is active in red blood cells and it responds to oxidant stress and hypoxia in the mature erythrocyte in vitro (e.g., blood storage) and in vivo (e.g., in response to hemorrhagic shock).<sup>59,60</sup>

### Glutaminolysis, glutathione synthesis, and cysteine metabolism

Glutamine uptake is increased at lower pH owing to the dependency of the glutamine transporters on proton gradients. Glutamine uptake contributes to intracellular glutamate levels, a precursor for the ATP-dependent synthesis of glutathione, the main soluble antioxidant in red blood cells. The synthesis of glutathione is mostly constrained by the availability of the sulfur-containing amino acid cysteine. As such, cysteine efflux is a marker of red blood cell aging in vivo and in vitro.<sup>61</sup> Cysteine metabolism is associated with iron-induced nonapoptotic cell death (ferroptosis) in other cell types, a phenomenon that may contribute to explaining nonapoptotic cell death in the mature, iron-rich (66% of bodily iron) red blood cell.<sup>62</sup>

### Sulfur metabolism and polyamines

Cysteine metabolism in red blood cells is intertwined with the metabolism of other sulfur-containing amino acids, methionine and taurine. While the transulfuration pathway—which interconverts cysteine into methionine—is not active in red blood cells, scavenging of reactive oxygen species by methionine and taurine has been extensively reported, to the extent that both metabolites have been suggested as supplements to current storage additives to counteract storage-induced oxidant stress.<sup>63,64</sup> Methionine also participates in one carbon metabolism: while only limited residual

activity of folate metabolism is detectable in mature erythrocytes (e.g., the methenyl-tetrahydrofolate metabolism step as it is impacted by NADPH homeostasis), most of methionine metabolism is observed in the context of (i) methylation of isoaspartyl groups resulting from oxidant stress-induced deamidation of asparagine or dehydration of aspartate residues in proteins; and (ii) purine catabolism and polyamine synthesis downstream to the breakdown and recycling of S-adenosylmethionine via methylthioadenosine intermediates. These pathways are relevant not just for protein recycling, but also for the role of newly synthesized polyamines in the scavenging of reactive oxygen species and alkalinization of the intracellular pH, to the extent that sub-millimolar concentrations of these metabolites contribute to scavenging activity.

#### **Arginine metabolism and nitric oxide synthesis**

Owing to the lack of mitochondria, red blood cells are characterized by an incomplete urea cycle. On the other hand, mature red blood cells can metabolize arginine as a substrate for a functional nitric oxide synthase and arginase 1.<sup>65</sup> While the former enzyme consumes arginine to generate citrulline and nitric oxide, a potent vasodilator, the latter competes for the same substrate to generate ornithine with no nitric oxide production. Because of these reactions, autologous transfusion studies have shown increased arginase activity and decreased vasodilatory capacity of red blood cells stored longer than 35 days.<sup>66</sup> Scavenging of circulating nitric oxide—including that released by endothelial cells—has been reported by the Gladwin group as a function of free hemoglobin levels, following red cell intravascular hemolysis, a mechanism that they posited to contribute to some of the rare adverse effects associated with transfusion.<sup>67</sup> On the other hand, intracellular metabolism of nitric oxide by hemoglobin has been proposed to play a role in the mechanisms of metabolic adaptation to high altitude hypoxia.<sup>68</sup>

#### **Membrane metabolism**

The red cell membrane is composed of a phospholipid bilayer containing cholesterol molecules and membrane proteins.<sup>69</sup> The phospholipids are arranged with a predominance of phosphatidyl choline (PC) and sphingomyelin in the outer leaflet and phosphatidylinositol, phosphatidylethanolamine (PE), and phosphatidylserine (PS) in the inner leaflet. Since PCs contain three methyl groups while PEs have two, the ratios of PC/PE contribute to methyl-group homeostasis (in like fashion to what is described for methionine above) and, thus, to isoaspartyl damage repairing following oxidant stress. Loss of phospholipid asymmetry results in exposure of PS that is an important apoptotic marker on the red cell surface. Exposure of PS on the outer cell surface promotes red cell removal from the circulation, while the surface glycoprotein CD47, which decreases during storage, inhibits phagocytosis.<sup>70</sup> The phospholipid asymmetry is maintained by the ATP-dependent flippase (aminophospholipid translocase) activity. This activity counteracts phospholipid scrambling which moves PS from the inner to the outer cell surface. Flippase activity decreases during storage, but can be corrected by rejuvenation of the red cells.<sup>71</sup> Phospholipid scrambling is normally low during storage, but is increased by photodynamic treatment for pathogen inactivation and by cell aging in association with rising intracellular  $[Ca^{++}]$ .<sup>72</sup>

Membrane proteins such as glycophorin and band 3 extend through the lipid membrane. Branches of carbohydrates anchored to membrane proteins protrude from the outer surface to produce the glycocalyx. The inner surface of the membrane is lined by the

cytoskeleton, which is anchored to the membrane through the N-terminal cytoplasmic domains of band 3 and glycophorin C. The tetrameric form of band 3 binds ankyrin that constitutes with proteins 4.1 and 4.2 a major attachment site for spectrin. A second attachment site for spectrin involves actin and protein 4.1 in the glycophorin C complex. Both attachments (Figure 14.2) are weakened when the concentration of “free” (nonhemoglobin-bound) 2,3-DPG is increased. As described above, these weakened interactions appear to be of importance for cell flexibility and gas delivery.<sup>8</sup> Membrane shape and deformability are controlled by the ATP-regulated cytoskeletal attachments. Because some capillaries in the microcirculation have a diameter of only half that of a red cell, loss of flexibility and deformability is a serious consequence of the storage lesion, and is responsible for the removal of rigid cells (Figure 14.1).

Sphingolipid signaling also contributes to membrane stabilization in response to hypoxia in vivo and in vitro. Activation of PKA following exposure to hypoxia (refer to the paragraphs above) also promotes the phosphorylation and activation of the red cell-specific enzyme sphingosine kinase 1.<sup>73</sup> This enzyme generates sphingosine 1-phosphate (S1P), a sphingolipid that binds to deoxyhemoglobin upon 2,3-DPG binding, resulting in the further stabilization of the tense state of hemoglobin.<sup>74</sup> S1P levels decrease during storage in certain additives (e.g., additive solution 3), while it increases with others (e.g., additive solution 1) as a function of the osmolarity of the solution.<sup>75</sup>

The red cell membrane also contains transport proteins such as the glucose transporter,  $Ca^{2+}$ -ATPase, the  $Na^{+}$ - $K^{+}$ -ATPase, the GSSG (oxidized glutathione) transport ATPases, and amino acid transporters, in addition to various other transport channels. A prime metabolic activity of the red cell is maintaining osmotic stability through the activity of its membrane pumps that are ATP driven. The  $Na^{+}$ - $K^{+}$ -ATPase is highly sensitive to changes in temperature and scarcely functions at 4°C. As a consequence, the normal diffusion of sodium into RBCs and the outward leakage of potassium are not effectively mitigated by the  $Na^{+}$ - $K^{+}$ -ATPase during refrigerated storage, and slow potassium leakage is ongoing. The leakage of potassium is further increased by irradiation. Increased potassium content in the plasma or suspending solution of stored red cells units (RBCs) presents a potential hazard to neonates and others receiving high-volume central infusions of RBC products.<sup>14</sup> Citrate in plasma increases potassium toxicity, while reducing RBC supernatant volume results in less potassium leakage before the new equilibrium is reached. Studies performed over 30 years ago indicate that the restoration of potassium after transfusion is slow and can take more than six days.<sup>14</sup> More recent studies in cells with better ATP concentrations show much faster restoration.

Although the macromolecules of the RBC membrane are produced during early erythroid stages prior to enucleation, some of the components of the membrane are metabolically quite active in mature erythrocytes. For example, cholesterol in the membrane exchanges readily with cholesterol in the plasma, phosphatidylinositol undergoes active phosphorylation, and some proteins are phosphorylated by protein kinases and dephosphorylated by phosphatases. These reactions may influence the functional status of membrane components, but their importance is not yet fully understood. Similarly, the fatty acyl composition of the membrane of freshly drawn red blood cells closely mirrors that of plasma and it varies as a function of diet. Diets high in fish oil will for example result in red blood cell membrane lipid enrichment in omega-3 fatty acids, which in turn affects membrane fluidity, mechanical properties, and post-transfusion recoveries upon storage.<sup>76</sup> While red cells are incapable of

synthesizing long chain fatty acids through the elongation of palmitate (the most abundant fatty acid in eukaryotic cells), the degree of fatty acid unsaturation can be affected by the activity of fatty acid desaturases, which have been identified and are active in red blood cells as a function of NADH/NAD<sup>+</sup> ratios and oxidant stress.<sup>77</sup> The degree of unsaturation of fatty acids in the red cell membranes also promotes membrane lipid oxidation since double bonds are more easily attacked by reactive oxygen species. Indeed, as refrigerated storage time increases, lipid peroxidation products accumulate as markers of storage lesions and a predictor of post-transfusion recoveries in humans and rodent models of blood transfusion.<sup>78</sup> Elegant genetic studies in mice have revealed a role for iron metabolism by the membrane ferroreductase STEAP3 (also known as tumor suppressor-activated pathway 6—as this protein is transcriptionally regulated by tumor suppressor protein p53) in the regulation of lipid peroxidation processes.<sup>79</sup> Of note, in nucleated cells p53 plays a central role in ferroptosis, i.e., iron-induced nonapoptotic cell death.<sup>80</sup> The red blood cell enzyme glutathione peroxidase 4 (GPX4) antagonizes ferroptosis by consuming glutathione to repair oxidized lipids. The role of GPX4 in blood storage, ferroptosis, and post-transfusion recoveries of stored red blood cells has not yet been investigated.

To recycle oxidized lipids, red blood cells also leverage a dedicated pathway that is known as the Lands cycle.<sup>81</sup> This pathway is regulated by hypoxia (e.g., pathologically, in sickle cell disease), and it relies on the activity of phospholipase A2 enzymes (to release the oxidized fatty acyl-moiety from the complex lipid) and lysophosphatidylcholine acyltransferase 1 (LPCAT1), which transfers a new fatty acid to the lysophospholipid to replace the missing fatty acyl group. Of note, in response to oxidant stress enzymes such as peroxiredoxin 6 migrate to the membrane and catalyze phospholipase A2-like activities.<sup>82</sup> The intermediate steps of this pathway also involve the transferring and conjugation of the damaged fatty acyl-moiety to carnitines, making carnitine a limiting factor in membrane lipid remodeling and a potential adjuvant to preserve membrane lipid homeostasis in stored units.<sup>83</sup> Alterations in carnitine levels and acyl-carnitines have been observed in stored red cells as a function of genetic defects and osmolarity of the storage additive, as well as the sex of the donor (higher in females).<sup>84</sup>

### **Eryptosis-programmed cell death in red cells**

While RBCs lack the nucleus and mitochondria that are required for apoptosis in most cell populations, they undergo a unique form of programmed cell death called eryptosis associated with low pH, increased cell calcium, or energy loss with low ATP concentrations.<sup>86</sup> The process is marked by irreversible shape change, loss of membrane by microvesiculation, and exposure of negatively charged phospholipids on their cell surface. Oxidative damage appears to play an important role. Alterations in membrane lipid homeostasis (including fatty acid unsaturation and conjugation to carnitines) have been observed in red cells in response to several proeryptotic stimuli.<sup>87</sup> Presumably eryptosis marks RBCs at the end of their normal lifespan for removal from the vascular system by splenic or other macrophages.<sup>88</sup>

Eryptosis is critically limiting in RBC storage because falling pH and ATP concentrations are a concomitant of closed system storage with the accumulation of acid breakdown products of glycolysis leading to acidic inhibition of glycolysis.<sup>89</sup> The accumulation of hemoglobin-containing microvesicles in the supernatant appears to be good marker of the process, as is increasing RBC calcium content with activation of calpain enzymes, but shape change and the appearance of microerythrocytes are now easily measured.<sup>5</sup>

### **Summary**

In the circulation, red cells metabolize glucose through the glycolytic pathway with its pentose and 2,3-DPG shunts. The energy gained provides ATP to maintain ion and glucose concentration gradients between the plasma and erythrocyte, and to support red cell deformability. It also secures NADH to keep hemoglobin in the reduced state and NADPH to protect SH-groups on hemoglobin and membrane proteins. Finally, the production of 2,3-DPG is important for the optimal dissociation of oxygen from hemoglobin while the rise and fall of nonhemoglobin-bound 2,3-DPG with each pass through the circulatory system induces repetitive changes in the membrane–cytoskeleton architecture, which has implications for red cell flexibility and gas transport.

### **Red cell preservation in transfusion medicine**

#### **General considerations and principles**

RBCs are the most commonly transfused blood components, and their use in a variety of physical circumstances and clinical conditions has shaped their development as products. As an example, the US military's need to reduce the weight and breakage of blood bottles during shipment led to the development and adoption of plastic blood bags.<sup>90</sup> This led to closed-system sterile component production and, fortuitously, exposing RBCs to the plasticizer diethylhexyl phthalate (DEHP), which reduced hemolysis four-fold during storage.<sup>91</sup> Arguments over the removal of plasma between blood bankers, who wanted to use plasma to make albumin and coagulation factors, and surgeons, who wanted to increase blood flow during rapid transfusions in trauma patients, led to the development of additive solutions.<sup>92</sup> Again, there were beneficial unforeseen consequences, as the removal of plasma led to better RBC storage and reduced transfusion reactions.

These examples of improved RBC storage methods notwithstanding, the development and adoption of new RBC storage technologies has been slow and limited.<sup>93</sup> There are two main reasons for this. First, the US Food and Drug Administration (FDA) recognizes that four million patients a year receive RBCs and that the product must achieve very high levels of safety.<sup>94</sup> Further modifications require a favorable risk:benefit ratio to pass regulatory review. For example, the idea of adding antibiotics to RBC units has been abandoned since the potential gain, preventing bacterial contamination that kills approximately two patients a year in the United States, would come at the risk of exposing millions to allergenic and toxic drugs with the probability of greater harm than good. Second, within the industry, there is limited perception of the need for change, limited investment in change, lack of understanding of the red cell storage lesions, poor developmental strategies, and conservative regulatory stances.<sup>95</sup> For these reasons, only five major improvements have occurred in the last 60 years: (1) the addition of phosphate, (2) the use of plastic bags, (3) the addition of adenine, (4) the development of additive solutions, and (5) the use of leukoreduction, which reduces hemolysis. The most significant ongoing efforts to further improve RBC products for transfusion, including the development of advanced additive solutions and pathogen reduction methods, have likewise made slow progress for similar reasons.

For 60 years, it has been a societal goal that RBCs for transfusion should be available, safe, effective, and cheap.<sup>37</sup> Making red cells readily available requires both the ability to take liquid units out of the refrigerator and administer them immediately to critically ill or injured patients and the ability to find rare units in frozen national or international inventories. Storage systems contribute to blood

safety by isolating individual units in closed systems and reducing product breakdown and bacterial growth through cold storage. A major goal of storage systems is to maintain effectiveness by preserving the lifespan and function of fresh red cells to the greatest extent possible. Keeping blood cheap requires controlling per-unit costs and not increasing health risks that will make new demands on other parts of the system. At the present time, the unit costs of RBCs remain low, and controlling those costs has been an additional factor in limiting progress on storage systems.

### A short history of RBC storage systems

Rous and Turner developed the first red cell storage solution in 1916, a simple mixture of citrate and glucose.<sup>96</sup> It was initially used to store rabbit red cells for heterophil agglutination testing for syphilis, but when the cells appeared to be intact four weeks later, they were reinfused back into the donor rabbits raising the hematocrit without increasing the reticulocyte count or bile in the urine suggesting that the reinfused cells were circulating.<sup>97</sup> A year later, Rous's postdoctoral fellow, Oswald H. Robertson, used this Rous-Turner solution to build the first successful blood bank in the Harvard Medical Unit attached to the British Expeditionary Force in France.<sup>98</sup>

The major problem with the Rous-Turner solution was that it could not be heat sterilized, as the sugar caramelized, so there was a risk of bacterial contamination from the open mixing of the ingredients and adding the solution to the bottles. In 1943, Loutit and Mollison solved this problem by lowering the pH of the solution to 5.0 to make acid citrate dextrose (ACD) solution.<sup>99</sup> ACD solution could be autoclaved in sterile vacuum bottles and was used as the standard blood collecting solution in the United States and Britain for many years. It was first intended to be used in a 1:4 ratio with collected whole blood; later the formulation was concentrated to be used at a 1:7 ratio, to reduce the dilution of the blood, and this remains the standard today.

In exploring why red cells seemed to survive better in the lower volume ratio of the anticoagulant-nutrient solution, it became clear that the cells passively lose phosphate. This loss could be prevented by adding phosphate to the solution.<sup>100</sup> Citrate phosphate dextrose (CPD) in a 1:7 volume ratio, 63 mL for 450 mL of whole blood, improved red cell survival during storage slightly better than did the older ACD.<sup>101</sup> CPD became the standard anticoagulant in the United States, while ACD persisted in Europe, and both were used for 21-day storage with recovery of 70–80% of the RBC 24 hours after reinfusion of the stored cells back into their original donor.

At about the same time, Gabrio and her colleagues recognized that nucleotides were important for red cell metabolism, but almost a decade passed before researchers determined that adenine was the critical intermediate.<sup>102,103,104</sup> In 1968, Shields formulated a mixture of CPD and adenine, CPDA-1, and showed that it markedly improved whole blood storage.<sup>105</sup> During the subsequent 11 years in which the FDA debated the safety of adenine, plastic bags revolutionized blood banking, allowing blood components to readily be separated from one another. Unfortunately, the excellent CPDA-1 whole blood storage solution turned out to work less well added to "packed" RBCs that were separated from whole blood.<sup>106</sup> Beutler and West showed that the higher the hematocrit of RBCs in concentrates, the lower the red cell ATP concentrations and the *in vivo* recovery.<sup>107</sup> The obvious answer was to add back more volume and nutrients in the form of an "additive solution," but the initial attempt to do this with a solution of bicarbonate, adenine, glucose, and phosphate (BAGP) did not work.<sup>108</sup> CPDA-1 was finally licensed as a five-week storage solution in 1979, but only

after Hogman had developed a simple additive solution of saline, adenine, and glucose (SAG) that also worked for five-week storage but without the adverse effects associated with the high pH.<sup>109</sup> SAG went on to immediate use in Sweden.

The use of SAG was associated with 1% hemolysis by the end of five-week storage, leading to the subsequent inclusion of mannitol (SAG-M) as a "membrane stabilizer."<sup>110</sup> This change reduced hemolysis by more than 50%. Drawing whole blood into CPD, making component products, and then storing the RBC concentrate with an additional 100 mL of SAG-M (CPD/SAG-M) remains the standard RBC storage system in Europe and Canada. Minimal variants of this basic solution, additive solution-1 (AS-1) and additive solution-5 (AS-5), are widely used in the United States. A somewhat different additive solution variant using citrate and phosphate in the place of mannitol (AS-3 or SAG-CP) is also in use. Despite differences in formulation, all these "first-generation" additive solutions appear to be equivalent and provide about 82% recovery with about 0.4% hemolysis after six weeks of storage.<sup>111</sup>

The most important limits of the first generation of additive solutions are the loss of membrane with resulting loss of deformability and viability that occur with prolonged storage. The progressive loss of 2,3-DPG may also be important in some situations, although its clinical impact is not well understood. More advanced additive solutions have been developed and licensed but are not yet routinely available.<sup>99</sup> They work by providing phosphate and buffering capacity, and can effectively maintain the viability of red cells over 7–8 weeks of storage. They are considered to be attractive more for their ability to improve recovery, reduce membrane loss, and improve 2,3-DGP and/or ATP concentrations for all stored RBCs than for their ability to extend the storage period.

### Collection and separation procedures

The volume of whole blood removed for storage, processing, and transfusion was historically 450 mL, a pint, in Western countries. For some newer collection systems, this amount has been increased to 500 mL, a half-liter, to increase the collection with each donation and to offset the losses associated with filtration leukoreduction. Products derived from both collection volumes, whether leukoreduced or not, are considered "one unit." The interdonor differences in hematocrit and platelet count are sufficiently great so that the yields of red cells, platelets, and plasma in components prepared from units of either whole blood collection volume show considerable overlap. Some have argued for a more standard definition of a unit, perhaps based on grams of hemoglobin, but it would make blood collection more difficult and wasteful.<sup>112</sup>

As noted, the volume of anticoagulant-nutrient solution is normally 1/7th the volume of the collected blood, 63 mL for a 450 mL whole blood collection and 71 mL for 500 mL. This volume ratio has been a standard for more than 50 years and has been used with the anticoagulant-nutrient solutions ACD-A, CPD, and CPDA-1. There was long a question of whether the first few drops of blood to enter the collection system are injured by their sudden immersion in the acid anticoagulant, but the red cells seem to tolerate this process with minimal hemolysis or loss of viability.<sup>113,114</sup> At the end of collection, venous blood with a pH of about 7.35 has been mixed with anticoagulant-nutrient solution with a pH of 5.0–5.6 with a resulting pH about 7.05 in the mixture. The low pH of anticoagulants is tolerated because of the high buffering capacity of the hemoglobin molecule which limits the effects of the acidic primary collection solution.

Whole blood in the anticoagulant-nutrient solutions CPD and CPDA-1 are licensed for storage for three or five weeks, respectively,

and there is renewed interest in their use for trauma patients. Increased storage as whole blood competes with making components which serve more patients and improve aspects of the storage of the individual blood elements. For example, red cells are best stored cold, platelets for prophylactic use in oncology patients at room temperature, and plasma as a frozen product. Removing the white blood cells (WBCs) also improves RBC storage by removing a cell population with high energy requirements and potential for damaging red cells by released enzymes.<sup>115</sup>

Schemes for separating whole blood into components are based on centrifugation. The standard “platelet-rich plasma” method used in the United States involves performing a low-speed “soft” spin to sediment the red cells against the pole of the bag opposite the connections to the satellite bags and then squeezing off the supernatant platelet-rich plasma from the top of the bag into the first satellite bag. Thus, typically, 500 mL of whole blood with a hematocrit (Hct) of 42%, consisting of 210 mL of red cells and 290 mL of plasma, is collected into 70 mL of anticoagulant–nutrient solution increasing the volume in the bag to 570 mL and reducing the Hct to 36%. Removing most of the supernatant plasma will increase the Hct in the remaining packed RBC component to 80–90%, consisting of the original 210 mL of red cells and the remaining 22–45 mL of plasma/anticoagulant–nutrient solution mixture. Of the original 290 mL of plasma and 70 mL of anticoagulant–nutrient solution, about 90% is removed in this initial separation process.

In Europe, an initial hard spin is generally preferred in order to harvest buffy coat for the production of platelets and increase the volume of the separated plasma. As noted above, the red cells do better if most of the plasma volume and anticoagulant–nutrient solution are replaced.

Separation of whole blood into plasma and RBCs by using a hollow-fiber filtration system has been described.<sup>116</sup> Red cell parameters were similar to those obtained when routine centrifugation methods were used, and the filters do not cause hemolysis. Levels of plasma Factor VIII and Factor XI were slightly reduced with this prototype; however, there was no evidence of activation of the coagulation or complement systems, and filters that do not interact with coagulation factors can be made.

### Anticoagulant–nutrient solutions

Anticoagulant–nutrient solutions were developed sequentially from ACD to CPD to CPDA-1. This path is the result of the discovery of the critical nutrients required by RBCs during prolonged storage: dextrose, phosphate, and adenine. The recognition that RBC concentrates occasionally ran out of glucose led to the development of CP2D, with twice the amount of glucose in CPD, and a never-licensed CPDA-2 with a third more glucose than CPDA-1.<sup>117,118</sup> The molar contents of the four licensed additive–nutrient solutions are shown in Table 14.1. Note that all of the anticoagulant–nutrient solutions are acidic, reducing the pH of the stored blood below 7.2 and leading to a burst of ATP production at the expense of the rapid depletion of 2,3-DPG as large amounts of 3-phosphoglycerate enter the glycolytic pathway.

The amounts of the nutrients in the additive solutions are critical depending on the intended storage times. There is enough glucose in whole blood to keep the red cells healthy for four days. Whole blood, stored in citrate alone for up to four days, was considered the safest form of storage before autoclavable solutions containing sugar were developed. The high glucose content of CP2D was necessary because it was first used with an additive solution that did not contain sufficient glucose, adenine–saline. Phosphate exists at

**Table 14.1** Compositions and Properties of Some of the Common Acid Citrate Preservative Solutions

	ACD-A	ACD-B	CPD	CPDA-1	CP2D
Citric Acid	35	21	14	14	14
Sodium Citrate	97	58	116	117	117
Dextrose	136	81	141	142	284
Monosodium phosphate			15.8	16	16
Adenine				2	
pH	5.0		5.6	5.6	5.6
Volume ratio used (mL anticoagulant:mL blood)	1:7	1:4	1:7	1:7	1:7

ACD is acid–citrate–dextrose and has two formulations (A and B); CPD is citrate–phosphate–dextrose; CPDA-1 is citrate–phosphate–dextrose, and adenine; a CPDA-2 was developed but never licensed. CP2D is CPD with double-dose dextrose. All concentrations are in mmoles/L.

1–1.3 mmol/L in plasma, but needs to be present at higher concentrations in the nutrient solutions to prevent diffusive loss of RBC phosphate, resulting from 2,3-DPG breakdown, which would otherwise leave red cells down its concentration gradient. Adenine is only needed for storage beyond three weeks and is an ingredient of all the additive solutions.

In emergencies in remote locations, it may be necessary to collect fresh whole blood before new supplies of fully tested components are available. Under these circumstances, drawing whole blood into the primary collection bag containing the anticoagulant–nutrient solution and holding the whole blood at room temperature for up to 24 hours is associated with good short-term preservation of function of the blood components.<sup>119</sup> The national blood services of Finland and Israel collect all of their whole blood on one day and process it on the next, holding it at 20 °C overnight in the anticoagulant–nutrient solution before separating it into components.<sup>120,121</sup> The Council of Europe Committee of Experts on Quality Assurance in Blood Transfusion Services has since 2005 advised that whole blood rapidly cooled after collection to 20–24 °C can be kept at this temperature up to 24 hours after collection before separation into red blood cells, platelets, and plasma.<sup>122</sup> A large international study has examined the metabolic trade-offs involved in the warm overnight hold of whole blood before processing.<sup>123</sup> The warm hold did reduce the initial storage pH and lead to a burst of ATP synthesis, but the differences are lost among larger individual donor differences by six weeks.

### Additive solutions

The first widely used additive solution, SAG, represented an attempt to replace the volume and sugar lost with plasma removal and add the adenine necessary for storage beyond three weeks.<sup>54</sup> It was made with normal saline supplemented with 4.5% weight/volume glucose and 2 mmol/L of adenine. Stored RBCs had good viability for five weeks, but nonetheless demonstrated 1% hemolysis. In screening a number of compounds for additives that would reduce the hemolysis, mannitol was identified as a compound that both reduced hemolysis and had an excellent safety record with IV infusion. The addition of 30 mmolar mannitol reduced hemolysis and increased the osmolarity of the solution further.<sup>55</sup> The solutions are made acidic to a pH of about 5.6 with hydrochloric acid to allow the glucose to be heat sterilized, but since the solutions have essentially no buffer capacity they do not make the RBC concentrates much more acidic than they were when separated from plasma. SAG-M and its close relatives, AS-1 and AS-5, are the most widely used additive solutions.

Other widely used first-generation additive solution systems include CP2D/AS-3 in the United States and CPD/MAP (mannitol, adenine, and phosphate) in Japan.<sup>124</sup> Table 14.2 provides a comparison of the ingredients and concentrations of the more common of these first-generation additive solutions. AS-3 is the only licensed mannitol-free additive solution. While the absence of mannitol was initially considered an advantage for transfusion of infants, studies of AS-1 in premature infant have shown it to be quite safe.<sup>125,126</sup>

Second-generation additive solutions started with attempts to rebalance the final suspending solution and to identify additional nutrients for the packed RBC concentrates. BAGP was the result of the original attempt by Beutler to preserve both ATP and 2,3-DPG by raising the pH.<sup>52</sup> However, raising the pH substantially above 7.2 led to high concentrations of 2,3-DPG at the expense of ATP and no improvement in storage function. The composition of a representative group of second-generation additive solutions is shown in Table 14.3.

As alluded to several times in this chapter, the pH of RBCs during storage is very important. Above pH 7.2, the bifunctional enzyme diphosphoglycerate mutase/phosphatase converts almost all 1,3-DPG into 2,3-DPG, depriving the cell of new ATP<sup>27</sup>. Below a pH of about 6.4, the activities of the initial enzymes of glycolysis, hexosekinase and phosphofructokinase, are too low to support ATP production. In this narrow pH range between 7.2 and 6.4, hemoglobin, the mineral salts in the suspension, and bicarbonate all serve to buffer the protons produced by glycolysis. Approximately 60 g of hemoglobin present in an RBC unit can buffer about 8 mEq of protons in that pH range.<sup>127</sup> However, conventional first-generation acidic additive solutions, which result

in an initial pH of 7.0, fail to take advantage of a quarter of that available pH range and buffer capacity. Adding 20 mmol/L of phosphate delivers 2 mmol in the 100 mL of additive solution and buffers about 1 mmol of additional protons. Adding 20 mmol/L of bicarbonate likewise delivers 2 mmol to the final RBC suspension which will be protonated to make carbonic acid, converted to CO<sub>2</sub> and water by red cell carbonic anhydrase, and buffer 2 mmol of protons as the CO<sub>2</sub> diffuses out of the plastic bag. These results demonstrate that adjusting the formulation and pH balance in the design of second-generation RBC additive solutions can almost double the amount of ATP energy available to stored red cells by depressing diphosphoglycerate mutase activity while sustaining glycolysis.

Of note, the storage of red blood cells under hypoxic and hypocapnic conditions provides similar benefits with respect to increased diphosphoglycerate mutase activity. Indeed, concomitant removal of oxygen and CO<sub>2</sub> promotes the alkalinization of the intracellular compartment through a series of compensatory events involving the activity of band 3 (exchanging chloride for bicarbonate), carbonic anhydrase (converting bicarbonate to CO<sub>2</sub>), CO<sub>2</sub> diffusion from the gas permeable plastic bag, and increased intracellular levels of hydroxide anions to achieve Donnan equilibrium. Hypoxic storage in the presence of 5% CO<sub>2</sub> still preserves the increase in glycolytic fluxes and ATP synthesis with no net benefit on the capacity to synthesize 2,3-DPG compared to conventional storage.<sup>128</sup> By decreasing the availability of O<sub>2</sub>, a substrate for Fenton chemistry, hypoxic storage mitigates the storage-induced oxidant lesion to metabolites and proteins, ultimately resulting in relative improvements in post-transfusion recoveries compared to paired normoxic counterparts in autologous transfusion studies using first-generation additive solutions.<sup>129</sup> Second-generation additive solutions achieve similar results with less disruption of blood bank processes.

Despite extensive efforts, finding additional nutrients critical to RBC viability during storage has been less successful. The only approved second-generation additive solutions are PAGGS-mannitol (phosphate, adenine, glucose, guanosine, and saline) and AS-7.<sup>130</sup> Guanosine was added because guanosine tri-phosphate was detected in red cells and known to decrease during storage. The solution worked only modestly better than first-generation additive solutions with a 74.6% 24-hour *in vivo* recovery after seven weeks in 10 units in the only published series. Experimental additive solutions with guanosine appear to improve DPG concentrations and reduce oxidative injury.<sup>131</sup>

**Table 14.2** Compositions of the Common Licensed First-Generation RBC Additive Solutions

	SAG	SAG-M	AS-1	AS-3	AS-5
NaCl	150	150	154	70	154
Phosphate				23	
Adenine	1.25	1.25	2	2	2
Glucose	45	45	111	55	45
Mannitol		30	41.2		29
Citric Acid				2	
Na <sub>3</sub> Citrate				30	

SAG is saline–adenine–glucose; SAG-M is saline–adenine–glucose–mannitol; AS-1 is a patented SAG-M variant formula sold as Adsol® by Fenwal; AS-3 is a patented formula sold as Nutracel® by Pall; and AS-5 is a patented SAG-M variant formula sold as Optisol® by Terumo. All concentrations are in mmoles/L.

**Table 14.3** Composition and Properties of Some of the Second-Generation RBC Additive Solutions

	BAGPM	PAGGS-M	PAGGG-M	ErythroSol-1	ErythroSol-2	AS-7
NaCl		72				
NaGluconate			72			
Bicarbonate	115					26
Phosphate	1	32	32	20	18	12
Adenine	1	2	2	1.5	1.3	2
Glucose	55	52	52	45	38	80
Mannitol	27	55	40	40	50	55
Guanosine		1.5	1.5			
Na <sub>3</sub> Citrate				25	21	
pH of solution		6.3	6.3	7.4	8.8	8.4
pH of RBC suspension		6.9	6.9	7.2	7.3	7.2
Volume	100	110	110	114	150	110

BAGPM is bicarbonate–adenine–glucose–phosphate–mannitol, the original additive solution developed by Beutler; PAGGS-M, which is phosphate–adenine–glucose–guanosine–saline–mannitol, is licensed in Germany; PAGGG-M is phosphate–adenine–glucose–guanosine–(sodium) gluconate–mannitol, and was developed by de Korte as a chloride-free variant of PAGGS-M; ErythroSol-1 and -2 were developed by Hogman; and AS-7 was developed by Hess and Greenwalt. All concentrations are in mmoles/L.

As ingredients were added to advanced additive solutions, the salt concentration was generally reduced to maintain osmotic balance. Reducing the chloride concentration in the suspending solutions caused intracellular chloride to passively leave the red cells, but this can only occur if other anions countered the flow, and phosphate, bicarbonate, and hydroxyl ions are the only available anions.<sup>132</sup> The influx of these anions initially increases the intracellular pH, but eventually the pH falls. PAGGG-M, in which the sodium chloride is replaced by sodium gluconate, is an example of an attempt to take advantage of the “chloride shift” phenomenon to maintain red cell 2,3-DPG.<sup>133</sup>

The ErythroSol solutions were developed based on the ideas of using the “chloride shift” to increase the intracellular pH while using a more alkaline final suspending solution and additional phosphate to simultaneously maintain ATP.<sup>134</sup> ErythroSol-1 used half-strength citrate (0.5CPD) and had problems with incomplete anticoagulation in blood collected from donors with low (but acceptable) hematocrit levels. ErythroSol-2 used full strength CPD and an alkaline additive solution made with disodium phosphate. In both systems, the glucose was placed in a separate bag from the rest of the additive solution during manufacture and sterilization and only added at the time of RBC component production. The original description of ErythroSol-2 suggested that the optimal pH had been determined to preserve both ATP and 2,3-DPG.<sup>135</sup> However, subsequent work and evaluation of ErythroSol-4 as part of an international trial showed no differences in ATP and DPG content or hemolysis between it and SAG-M or PAGGG-M.<sup>68</sup>

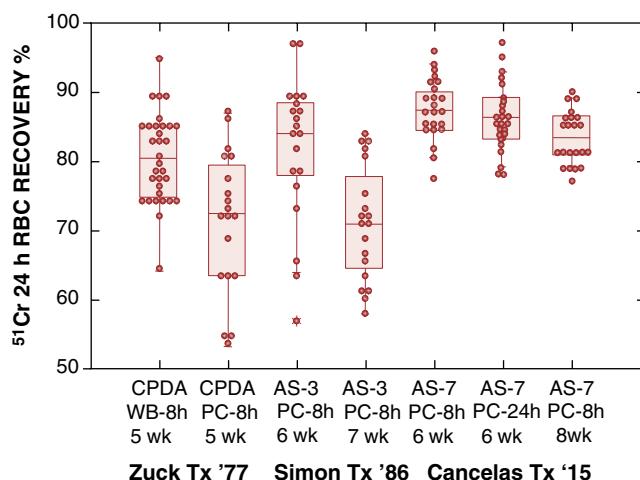
Experimental additive solution-81 (EAS-81) has now been licensed in the United States and Europe as AS-7.<sup>136</sup> It was the end result of work that re-examined the use of bicarbonate as a buffer to prevent erythro-apoptosis by maintaining high concentrations of red cell ATP and limited hemolysis with mannitol and hypotonic conditions.<sup>137</sup> Formulating the solutions to achieve a starting pH as close as possible to 7.2 preserved the 2,3-DPG concentrations for two weeks.<sup>138</sup> The buffering provided by the bicarbonate allowed RBC storage for eight weeks and storage with recoveries of 88% at six weeks based on a study involving 54 recovery measurements (Figure 14.5).<sup>139</sup> Metabolic studies have been recently performed to compare the performances of the above-mentioned alkaline storage additives. Results suggest significant improvements over conventional additives.<sup>140</sup>

Finally, increasing the volume of these advanced additive solutions increases the buffering capacity and allows greater storage time. With 300 mL additive solutions, Hess and his colleagues were able to achieve storage times of 10–12 weeks, but the higher volume and lower storage hematocrit probably make such solutions inappropriate for clinical use, especially in infants and massively transfused trauma patients with the potential to result in transfusion-associated circulatory overload (TACO) and dilutional coagulopathy.<sup>141</sup>

### Additional factors influencing RBC quality

#### *Temperature and time lapse during collection and component preparation*

Whole blood is collected at body temperature and must be maintained at room temperature if platelets are to be prepared from the collection. By present US regulations, whole blood must be separated into its components in eight hours if platelets and fresh-frozen plasma are to be manufactured. If only RBC and frozen plasma are to be made, the whole blood can be held on ice for up to 24 hours. The problems with holding blood at room temperature are two-fold.



**Figure 14.5** Chromium-51 autologous *in vivo* RBC recovery measures for RBCs stored in successive generations of storage systems. CPDA-1 shows the decrease in mean recovery from whole blood stored at a hematocrit of 38% to packed cells with a hematocrit of 80%. AS-3 is a representative first-generation additive solution showing 84% mean recovery at six weeks but failure at seven weeks. Bicarbonate buffered AS-7 allows better storage, storage for eight weeks, and storage after warm overnight hold. See Refs.<sup>53, 64, 80, 81</sup>.

Red cells metabolize glucose at higher rates when warm producing more lactate and protons that reduce the pH and further slow metabolism.<sup>70</sup> In addition, labile coagulation factors are lost. Attempts to validate the 24-hour warm hold for six-week RBC storage using first-generation additive solutions have not been successful, but in Israel and Finland, where this system is used, RBCs are stored for only five weeks because of the efficiency of their national blood services and short supply lines.<sup>142</sup> AS-7 is now licensed in Europe for eight-week storage after an overnight.

As noted above, fresh whole blood is occasionally collected in emergencies on the battlefield or in isolated locations such as Pacific Island nations and stored at room temperature for short periods. Based on the above experience and experimental data, blood probably maintains reasonable functionality for 24–72 hours if maintained between 19 and 25 °C.<sup>66</sup> However, such use can only be recommended in the most dire situations.

#### *Temperature and time lapse during storage*

RBCs that have been allowed to rewarm to greater than 10 °C during storage are considered unfit for transfusion and are destroyed according to FDA regulation. This has led to the wide use of a “30-minute rule,” which dictates that units are discarded if they have been outside of monitored refrigerated storage for more than 30 minutes. Experimental work has shown that glucose is metabolized about 10 times faster at 25 °C than at 4 °C, and that RBCs stored at 25 °C lose viability 10 times as fast. These changes can be extrapolated to suggest that a day of storage at room temperature would reduce the *in vivo* recovery to about the same extent as 10 days of 4 °C storage.<sup>143,144,145</sup> Thus, FDA regulations are very conservative with regard to temperature but are also intended to prevent bacterial overgrowth in contaminated units.

#### *Storage containers*

Polyvinyl chloride (PVC) bags plasticized with DEHP are the standard RBC storage containers. The presence of the DEHP reduces

hemolysis by four-fold during storage by intercalating into the red cell membrane.<sup>146</sup> Other plasticizers, such as butyryl-*n*-triethyl citrate, work almost as well, but are more expensive (~5% more for a multibag set with leukocyte filter), and have an unusual smell when initially unwrapped.

While questions about the safety of DEHP have been raised, they are based on very limited animal testing and must be balanced against the obvious safety value of being able to visually inspect the contents of blood bags through the clear plastic and the reduced RBC losses by extending shelf life. The trend in Europe to avoid the slowly degradable plasticizer, DEHP, has resulted in the introduction of butyryl-*n*-triethyl citrate plasticized multibag systems in Sweden, Spain, and Norway, but the use of PVC bags with DEHP remains prevalent in Europe and almost universal in the rest of the world. Other plasticizers are being explored.

### **Leukocyte reduction**

Leukocyte reduction improves RBC storage by removing highly metabolically active WBCs that make the bag more acidic sooner. WBCs also secrete cytokines, and when they die after exposure to the cold, they release proteolytic, glycolytic, and lipolytic enzymes that damage the red cell surface. On the other hand, some red cells are damaged and many red cells are lost in the leukocyte reduction filters (typically 15–35 mL of blood is lost in the filters depending on their size and whether whole blood or concentrated red cells are being filtered). Red cells from donors with sickle cell trait may also clog filters or hemolyze in them. Leukocyte reduction improves the red cell 24-hour *in vivo* recovery by several percent according to the best estimates.<sup>60</sup>

### **Washing**

Washing RBCs in saline to remove the plasma causes small losses of red cells in the bag transfers as well as loss of the supporting nutrients, glucose, phosphate and adenine. Because the cells are used soon after washing, generally within 6–24 hours, only the glucose loss is physiologically important. There is still a small amount of glucose inside the washed RBC, but it is metabolized quickly especially if they are not promptly refrigerated. In the absence of glucose, metabolism stops and the cells are very susceptible to oxidative stress and erythro-apoptotic changes. On the other hand, washing and resuspension in fresh media results in the removal of inhibitory factors of glycolysis (e.g., lactic acid) from the supernatant, promoting measurable improvement in red cell metabolism of stored cells.<sup>147</sup>

### **Irradiation**

During storage at 4 °C, red cells lose potassium. This potassium collects in the supernatant fluid in the closed storage bag at a rate of about one mEq/day until equilibrium is reached between the intra- and extracellular concentrations, usually at about 60–70 mEq/L depending on the storage hematocrit (Table 14.4). Gamma irradiation in doses of 2500 cGy, given to prevent graft vs. host disease, damages the red cell membranes and increases this rate of potassium loss to approximately 1.5 mEq/day. Irradiated red cells also show mildly reduced *in vivo* recovery.<sup>148</sup> The current FDA regulation that irradiated RBCs expire 28 days after irradiation limits the potential maximum potassium concentration by limiting the period of potassium loss. Nevertheless, care must be taken in all situations when large volumes of older, high potassium RBCs are used to prime cardiopulmonary bypass, dialysis, or apheresis circuits or infused into the central circulation and then administered at high flow rates.

**Table 14.4** Effect of Storage Duration of Characteristics of RBC Concentrates in AS-1

	<b>35-day</b> (n=25)	<b>42-day</b> (n=10)	<b>49-day</b> (n=10)	<b>56-day</b> (n=10)
% recovery	86	82	76	71
% hemolysis	0.28	0.32	0.51	0.68
ATP μM/g Hb	3.1	2.7	2.3	2.3
[K+] meq/L in plasma	45	50	52	60

Unpublished data obtained from the US FDA under the Freedom of Information Act.

### **Pathogen reduction**

Current pathogen reduction methods require additional manipulation of red cells and produce stresses on the stored RBC that are system specific. Examples from two such systems are as follows. Diethylenetriamine (DEA) was proposed as a red cell permeable nucleic acid crosslinker with broad pathogen killing potential and minimal direct red cell toxicity. However, the system required exposure of the RBC to DEA for 20 hours at room temperature and extensive secondary washing to reduce the remaining amounts of this carcinogenic chemical. When the time- and temperature-related decrease in RBC pH was added to the effects of washing the RBC with large volumes of acidic solutions and storing them in an acidic additive solution, red cell recovery suffered.<sup>149</sup> Riboflavin, a photoactive oxidizer, has also been proposed as a highly red cell permeable molecule for pathogen reduction in conjunction with UV light. However, RBCs are so optically dense that the units must be diluted and transferred to large bags only a few millimeters thick for phototreatment, and the phototreatment must be performed above a critical temperature. The bag transfers produce losses of red cells, the choice of diluent fluid is important, and the hemoglobin and other optically active molecules in the red cells are also damaged by the light exposure. The red cells need additional energy to attempt to correct the damage, and the reconstituted treated RBCs need to be stored in an additive solution that is balanced to maximize their lifespan. See also Chapter 42.

### **Effectiveness**

Since the discovery of the effect of 2,3-DPG on the oxygen affinity of hemoglobin, it has been common to discuss the functionality of stored RBCs in terms of their 2,3-DPG content.<sup>150</sup> This is an oversimplification for several reasons. First, while it is clear that low 2,3-DPG can affect oxygen delivery in animal models at critically low hemoglobin concentrations, these concentrations are well below standard transfusion triggers and may only be relevant in specialized transfusion situations such as sickle cell patients whose transfusion trigger may be set quite low to avoid alloimmunization.<sup>69</sup> Second, 2,3-DPG works in red cells by binding and stabilizing deoxyhemoglobin. This moves the base of the oxy-hemoglobin dissociation curve to the right and increases the P50, but most oxygen transport occurs at the top of the binding curve which is relatively unaffected, and even in critical situations the arterial PO<sub>2</sub> is higher than the P50. However, 2,3-DPG also plays a role in red cell membrane transport and cytoskeleton architecture because of its interaction with attachment points between the cell membrane and the cytoskeleton, and the interaction of deoxyhemoglobin with band 3. These interactions, the critical importance of oxygen transport, and the retrospective data that suggests an association between adverse patient outcomes and increased storage time of banked RBCs drive continuing efforts to reduce 2,3-DPG depletion during storage.

However, four randomized clinical trials showed no difference in the survival of critically ill patients treated with longer stored RBCs, and a fifth African child with severe malarial anemia showed no difference in the time to correction of lactic acidosis.<sup>151,152</sup>

Probably more important for normal red cell function is the loss of membrane, cell deformability, and ATP secretion that occurs with storage. Direct observation of the flow characteristics of fresh, stored, and stored rejuvenated RBCs suggests that ATP secretion in response to increased shear, by which red cells dilate small vessels to maintain their forward flow, is most important.<sup>153</sup> Membrane deformability, which is regulated by the ATP-modulated cytoskeleton, is also very important for the passage of red cells through capillaries which are half their diameter. Maintaining high ATP concentrations is therefore the critical function of modern additive solutions.

### **Rejuvenation**

Red cell loss of viability during storage is different than red cell senescence in the body. In the body, red cells undergo cumulative oxidative damage that leads to reduced enzyme activities and crosslinking of cytoskeletal components. These are essentially irreversible processes and are ultimately associated with macrophage clearance of old red cells possibly mediated by phosphatidyl serine exposure or neoantigen formation. The changes that lead to loss of viability during storage are largely reversible by a process called “rejuvenation.” Hogman showed that rejuvenating red cells at the end of six weeks of storage in SAG-M increased their 24-hour *in vivo* recovery from 77 to 89%.<sup>154</sup>

This rejuvenation is best understood as metabolic recharging of red cells at the end of their storage period. Such cells have a low pH as well as low ATP and 2,3-DPG concentrations. They can be rejuvenated by incubation in a high pH solution of phosphate, inosine, pyruvate, and adenine (PIPA, Rejuvesol®, Cytosol Labs, Braintree, MA) for two hours. Rejuvenation, through overnight incubation at 37 °C, of end-of-storage red blood cells has been associated with the restoration of energy and redox metabolism—with the caveat that irreversibly damaged erythrocytes that cannot be rejuvenated are removed at the washing step to remove excess hypoxanthine from the rejuvenated units.<sup>155,156</sup> Rejuvenation increases the RBC ATP and 2,3-DPG concentrations and *in vivo* recovery, probably by allowing them to internalize negatively charged membrane phospholipids that would otherwise signal for clearance by macrophages. Return to the normal distribution of phospholipids also prevents red cells from participating in plasma coagulation reactions. However, rejuvenation does not reverse the oxidative damage to band 3 of the cell membrane, desialylation of glycoproteins, or loss of membrane. Nonetheless, the fact that RBCs can be rejuvenated at the end of storage by increasing their pH and ATP suggests that improved storage could be achieved by a method designed to maintain pH and ATP. This was the fundamental idea behind the second-generation additive solutions.

### **Frozen storage of RBCs**

RBCs can be frozen, and in the frozen state they are stable for long periods. Valeri has reported on transfusion of RBCs stored for 37 years.<sup>157</sup> Four methods of freezing RBCs have been extensively tested, two have been developed for practical use, one of which remains in common use.<sup>158</sup>

During red cell freezing, water turns to ice and the salt concentration of the remaining intracellular water increases, drawing in more water, and expanding the cell. Under normal circumstances,

this leads to cell rupture. Rupture can be prevented by freezing the cells so rapidly that water does not have time to enter, or by diluting the total cellular water with a cryoprotectant so that not enough water enters to rupture the cell. While several cryoprotectants can work, glycerol is the standard material used because of its cost and safety.

The two systems for RBC cryopreservation that have been developed for clinical use both use glycerol. One system used a “low” glycerol concentration of about 20% and rapid cooling by plunge freezing in liquid nitrogen; the other uses a “high” glycerol concentration of about 40% and slow cooling in -80 °C freezers. The low glycerol frozen RBCs must be maintained in the vapor phase of liquid nitrogen whereas the high glycerol frozen RBCs are stable at temperatures below -65 °C. Both the high cost of maintaining liquid nitrogen freezers and the difficulty of transporting products frozen in liquid nitrogen limit the utility of the low glycerol system. High glycerol frozen RBCs, in contrast, can be transported on dry ice. When the RBCs are thawed, the glycerol must be removed promptly to prevent it from poisoning the red cell metabolism and to protect the recipient as the glycerol-loaded red cells would swell and rupture if placed directly into the bloodstream. Thus, once thawed, the RBCs must be deglycerolized by washing in a series of osmotically graded salt solutions.

In the past, the glycerolization and deglycerolization of RBCs for freezing were open manual processes, so the thawed RBCs had to be used within 24 hours or discarded. The recent partial automation of a closed system for glycerolizing and deglycerolizing RBCs now allows them to be kept post-thaw in the liquid state for 2–3 weeks. RBCs collected in any of the standard licensed systems can be frozen up to six days after collection and stored for up to 10 years. The deglycerolized RBCs are then stored in AS-3. Net losses of red cells in the freeze-thaw-wash process are of the order of 5–15%, and the 24-hour *in vivo* recovery of infused red cells is about 78% after two weeks.<sup>159</sup> Frozen RBCs entail substantial costs for processing and storage, probably four times that of a standard liquid unit, so only for the rarest blood units is there an advantage for frozen inventory.

Other systems of freezing and freeze-drying have been developed, but are associated with greater than 1% hemolysis and so would require a washing step before administration. This prevents their use in emergency medicine, and they are too costly and labor intensive to compete with glycerol frozen RBCs.<sup>90</sup>

### **Validation of red blood cell quality and *in vivo* recovery**

RBC storage systems have historically been validated by demonstrating that the stored cells do not hemolyze during storage and that they circulate normally after reinfusion. Measures of normal circulation have included increments in recipient hemoglobin, and determining the recovery and *in vivo* half-life of the transfused red cells. Transfused red cells have been counted using differential agglutination, radioactive tracer labeling, and flow-cytometric differential counting.<sup>160</sup>

Chromium-51 labeling has been the standard method used to measure RBC recovery in the United States for 70 years. Only a handful of laboratories perform the measurement, and a recent attempt to gather a decade's experience identified only 900 measurements.<sup>96</sup> Chromium-51 labeling persists in the United States because it is the only validated method compatible with autologous red cell reinfusion, and the infectious disease risks of

allogeneic RBCs are considered unacceptable for storage system development work. The methods of red cell labeling have been the subject of an excellent review.<sup>161</sup> Limitations of this approach have been noted, including (i) that these studies are performed in selected healthy autologous volunteers, and not in nonhealthy, heterologous recipients in which recoveries may be decreased by the inflammatory state of the recipient or other disease processes; (ii) early sequestration of the transfused red cells in the spleen and liver confounds the determination via regression of the baseline values, resulting in the overestimation of circulating cells at 24 hours; and (iii) the washing steps that are required to remove excess radiolabel from the transfused product may result in the lysis of the damaged end of storage red cells, resulting in an overestimate of the actual post-transfusion recovery values. These limitations and others (e.g., predicted shortages in chromium 51 supplies) have been recently reviewed by Hod and colleagues and, at least in part, can be addressed by novel approaches such as biotinylation or the use of Indium 111 or nonradioactive isotopes in place of chromium-51 labeling.

When performing chromium-51 labeling, it is important to recognize that red cells from different donors may have very different 24-hour *in vivo* recovery values. In a typical study of a modern additive solution with an 84% mean recovery, individual units of donor RBCs have recoveries as high as 95% and as low as 65%.<sup>62</sup> These differences in viability correlate with differences in the red cell ATP concentrations but poorly ( $r^2 = .4$ ).

There have been attempts to measure the functionality of red cells, as opposed to their survival. Presumably, the functionality of red cells is related to their ability to deliver oxygen to tissues and to flow in the microcirculation. However, none of these measurements, such as red cells rigidity or microcirculatory flow, is widely available or validated. The level of 2,3-DPG is just a surrogate marker for red cell function and is limited by the difficulty of performing the test. In a recent shared sample exercise, 12 of the world's premier labs could not consistently measure 2,3-DPG.<sup>162</sup> Ongoing efforts are focusing on the physiome, i.e., the comprehensive

characterization of physiological responses in the recipient with respect to tissue perfusion and oxygenation.<sup>163</sup>

Finally, in countries that do not perform chromium-51 recovery measures, there has been a trend to using the ATP concentration as a red cell quality measure. While the ATP is a poor surrogate for recovery in small clinical trials, its central role in the inhibition of erythro-apoptosis probably gives it special importance. Unfortunately, the measure is not very reproducible from laboratory to laboratory, so a wide margin of safety is required.<sup>95</sup>

## Summary

RBC storage systems work remarkably well, making red cells for transfusion available, safe, effective, and cheap for the populations who can organize effective national blood systems. Better RBC storage systems than those currently used exist, and even better ones combining buffering, chloride shift physiology, and the metabolic benefits of guanidine can be made using current understanding while maintaining the look, feel, and ease of use of current storage systems. They are, however, a product of historically slow and empiric development, and our present systems do not reflect our current understanding of the biology and physiology of red cells. We nonetheless hold out hope that this will improve in the future.

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CHAPTER 15

## Platelet production and kinetics

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### Platelet production

Platelets are essential for normal hemostasis. In adult humans, a normal circulating concentration of  $150\text{--}450 \times 10^6$  platelets per mL is maintained by the production and release of at least 75–100 billion platelets into blood per day.<sup>1</sup> This chapter uses the term “megakaryopoiesis” as synonymous with “thrombopoiesis” to denote the complete pathway of cell production from earliest progenitor to the terminal circulating platelet (Figure 15.1). “Platelet biogenesis” is used as synonymous with “terminal platelet production” and refers to the final physical stages of megakaryopoiesis which result in circulating platelets.

### Early megakaryopoiesis: proliferation and differentiation of megakaryocytes

In mammalian physiology, circulating platelets in the blood are anucleate cells derived from their precursors, megakaryocytes (MKs).<sup>2</sup> Megakaryopoiesis, under homeostatic conditions, is primarily under the control of thrombopoietin (TPO), a growth factor that acts in synergy with other cytokines such as stem cell factor (SCF) and various interleukins to regulate platelet production.<sup>3–5</sup> Megakaryocyte development can generally be considered to proceed in three stages: (a) progenitor proliferation with a DNA content of 2N; (b) megakaryocyte endomitosis; and (c) cytoplasmic maturation of megakaryocytes preceding release of platelets—each of which is detailed in this section.

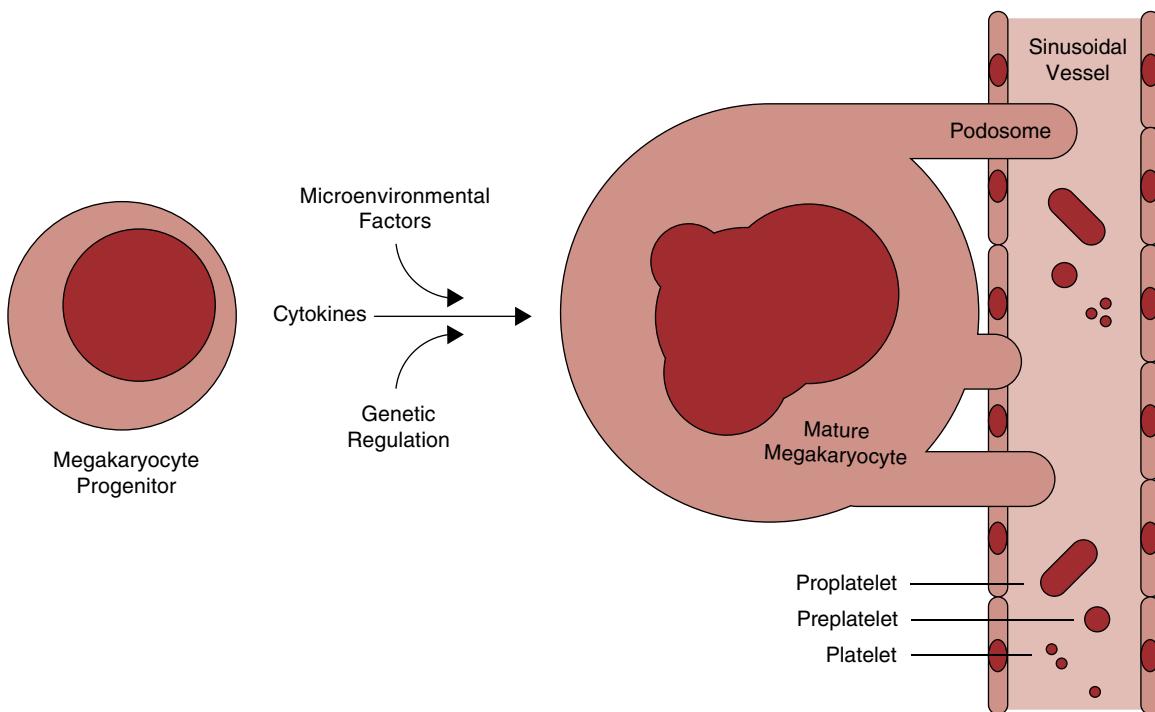
Human hematopoietic development begins in the embryonic stage and occurs in multiple locations including the yolk sac, the aorta-gonad-mesonephros region, and the fetal liver.<sup>6</sup> Bone marrow is the primary site of adult megakaryopoiesis, and terminal platelet production is thought to take place in the periphery.<sup>7</sup> Pulmonary contributions to platelet production have been historically recognized; recent studies have reinforced this concept, providing evidence that the lung-resident MKs contribute to megakaryopoiesis to a more substantial degree and may be responsible for 50% of platelet biogenesis in mice.<sup>7–10</sup> Furthermore, lung MKs and hematopoietic progenitors have the ability to migrate out of the lungs, repopulate marrow, reconstitute circulating platelets, and contribute to multiple hematopoietic lineages.<sup>8</sup> Interestingly, transcriptional and phenotypic profiling have supplied evidence that murine

lung MKs additionally play immunoregulatory roles like those of antigen-presenting cells.<sup>10–12</sup>

The classical model of MK migration within bone marrow has been challenged in recent years with the advent of imaging techniques allowing the spatiotemporal dissection of marrow hematopoietic activity. The earliest megakaryocyte progenitors have canonically been thought to proliferate near the cortical bone, i.e., in the osteoblastic or endosteal niche, where TPO acts in synergy with other cytokines to promote MK evolution from stem cell precursors and subsequent MK proliferation (discussion forthcoming, see the “Control of megakaryopoiesis: thrombopoietin and other cytokines” section).<sup>13</sup> As they mature, these MKs migrate from endosteal regions to perivascular sinusoidal regions.<sup>14</sup>

Recent *in vivo* and *in situ* imaging evidence suggests that the majority of murine marrow MKs are actually relatively sessile residents associated with sinusoids independent of their distance to endosteum.<sup>10,14–17</sup> Abundant vascular distribution seems to dictate this relationship, with the average vessel-to-vessel distance equaling approximately two average megakaryocyte diameters.<sup>14</sup> Furthermore, MKs exhibited a random distribution throughout the marrow space with respect to their maturity.<sup>10,14</sup> As such, endosteal and sinusoidal microenvironments may represent functional niches with considerable spatial overlap as opposed to niches with high spatial distinctness.<sup>14,18</sup> The degree and manner to which endosteal versus sinusoidal niches contribute to hematopoietic stem cell (HSC) derivation of MKs, MK maturation, and overall megakaryopoiesis remain to be definitively determined. Of note, this group’s findings also included a relatively low percentage of MKs observed within the vasculature, an observation potentially inconsistent with MK migration to other organs as a dominant facet of the megakaryopoiesis model.<sup>10,14</sup> Continued study of the spatiotemporal dynamics of megakaryopoiesis, as well as the interaction between marrow and extramedullary megakaryocytic compartments, is likely to offer new and clarifying insights into megakaryopoiesis, therapeutic strategies for thrombocytopenic disorders, and *ex vivo* platelet production.

On the cellular scale, megakaryopoiesis generally follows a hierarchical differentiation scheme with caveats. Canonically, HSCs give rise to a common myeloid progenitor (CMP) cell, followed by



**Figure 15.1** Canonical megakaryopoiesis overview. Megakaryocyte progenitors in the marrow differentiate and mature under the influence of factors including genetic regulatory elements, cytokines, and microenvironmental cues. Maturing megakaryocytes exhibit increased transcription of proteins that will eventually result in expression of platelet membrane components (e.g., GPIIb/IIIa) and platelet granule components (e.g., von Willebrand factor). Alpha and dense granules migrate along with the extension of podosomes and eventually into the tips of these proplatelet processes that form the preplatelet terminal ends. As these terminal ends extend into the sinusoidal vessels, shear causes release of the pro- and preplatelets into the blood circulation; mature terminal platelets are then derived from the preplatelets within the blood.

biphenotypic megakaryocyte–erythroid precursors (MEPs). TPO and other hematopoietic mediators (e.g., erythropoietin, SCF, and IL-11)<sup>19</sup> function synergistically to cause these bipotential precursors to differentiate toward the MK lineage and become promegakaryoblasts.<sup>13</sup> Promegakaryoblasts undergo endomitosis in order to increase their ploidy content (polyploidization)—a process that is probably necessary for normal maturation into MKs in the adult marrow but does not appear to be required for cord blood-derived MKs and platelet production *in utero*.<sup>20</sup>

Two major transcription factors involved in differentiation of the CMP are PU.1, which regulates granulocyte-monocyte precursors, and GATA-binding factor 1 (GATA1), which drives MEP differentiation.<sup>21</sup> The downregulation of PU.1 expression in the CMP is the first event associated with the restriction of precursor differentiation to erythroid and MK lineages.<sup>22</sup> In response to TPO, SCF, and other cytokines, the bipotential MEP can develop into the highly proliferative early MK burst-forming unit (BFU-MK), or the smaller, more mature MK colony-forming unit (CFU-MK), both of which express CD34.<sup>23</sup> GATA1 is thought to have a specific effect on fetal megakaryopoiesis; GATA1 mutations occur in trisomy 21-associated transient myeloproliferative disorder, which generally resolves spontaneously following the newborn period.<sup>24</sup> Furthermore, cell cycle activation or repression has also recently been shown to tune megakaryocytic versus erythroid differentiation of the MEP, with increased cell cycle speed being associated with erythroid differentiation and the inverse associated with megakaryocytic differentiation.<sup>25</sup>

Strict adherence to the canonical hierarchy of differentiation has been brought into question in recent years with the identification of

multipotent but megakaryocyte-biased HCSs and potentially unipotent megakaryocyte progenitors arising directly from the HSC compartment;<sup>7,10,26–32</sup> both postulated pathways would bypass the bipotent MEP defined in the classical megakaryopoiesis model. Self-renewing megakaryocyte-biased HSCs, dependent upon TPO for maintenance, have also been shown to give rise to lymphoid-biased HSCs, suggesting this HSC subset may exist at the apex of the HSC differentiation hierarchy.<sup>27</sup> However, differences between *in vitro* versus *in vivo* megakaryopoiesis, as well as interspecies differences, may influence fate-determination findings; hierarchical stages defined by clonal semisolid culture assays may also lack synonymy with murine-derived stages as defined by surface markers as well as those present in humans.<sup>10,28,32,33</sup>

The endomitotic cell cycle in MKs consists of phases G1, DNA replication in S, and then G2 but an aborted M phase. In the 2N to 4N transition, megakaryocytes fail to complete anaphase B, telophase, and cytokinesis. After the 4N stage, M phase is generally aborted prior to cytokinesis with cells demonstrating multiple pole spindles. MKs then enter a gap phase that enables reentry into the subsequent S-phase, which occurs repeatedly to form multilobulated nuclei that may reach ploidy levels as high as 128N. The mechanism of polyploidization is complex. Cyclin D3 is overexpressed (partly under the influence of TPO) in the G1-phase of maturing cells which helps overcome the normal block to polyploidization.<sup>34</sup> Aurora-B kinase (AIM-1 kinase), which mediates late anaphase and cytokinesis, had been hypothesized to be deficient during MK prophase and early anaphase, but others have found this critical kinase to be normally localized and completely functional in endomitotic MKs.<sup>35</sup> Other pathways mediating polyploidization include

MKL1-induced downregulation of GEF-H1 (discussion forthcoming, see the “Control of megakaryopoiesis: genetic regulation” section), which is critical for late cytokinesis.<sup>36</sup>

As alluded to earlier, 2N MKs can mature and produce platelets. Studies have demonstrated that neonatal MK progenitors are hyperproliferative. Low-ploidy neonatal MKs are generated at a much higher rate than their adult counterparts, likely mediated by upregulated TPO signaling. It is likely that rapid expansion of the fetal/neonatal bone marrow is the impetus for this developmental difference in megakaryopoiesis.<sup>20</sup>

Ploidy classes in MKs are identified as multiples starting with the diploid 2N baseline, i.e., 4N, 8N, etc. The ploidy distribution obtained by flow cytometry in both fractionated and unfractionated normal adult human marrow demonstrates a ploidy value of 16N in 50% of the total MK population, with the remainder of MKs equally split between cells having  $\leq$ 8N ploidy and MKs of ploidy  $\geq$ 32N.<sup>37</sup> Mature MKs eventually give rise to circulating platelets by the acquisition of cytoplasmic structural changes necessary for platelet formation and release into the circulation.<sup>38,39</sup> MKs reach cell sizes of 50–100  $\mu$ m in diameter, with peak ploidy values up to 128N.<sup>40,41</sup>

As the MK matures, the polyploid nucleus becomes eccentrically located within the cell, and the cytoplasm expands as it accommodates the formation of an extensive central invaginated membrane branching system termed the demarcation membrane system (DMS).<sup>42</sup> The DMS is similar to the open canalicular system (OCS) of platelets and is possibly the precursor of such. This DMS mass is the reserve membrane supply that will eventually form proplatelet projections in the next stage of megakaryopoiesis.<sup>43</sup>

While maturing, MKs also begin to express integrins, glycoprotein receptors, and granule components critical for eventual platelet function.<sup>7</sup> MKs synthesize some of their  $\alpha$ -granule constituents (e.g., von Willebrand factor), while other granule contents (e.g., fibrinogen) are incorporated into the granules by endocytosis and/or pinocytosis of plasma contents. There is growing evidence that  $\alpha$  granules are actually quite heterogeneous in their content and functionally have the capacity for differential release with activation.<sup>44</sup> Mature MKs will eventually exhibit three distinct cytoplasmic regions: a perinuclear zone containing the Golgi, ER, and mitochondria; a middle zone containing the DMS; and an organelle-devoid peripheral zone separated from the previous two by cytoskeletal components that must be extensively remodeled during late megakaryopoiesis.<sup>10</sup>

### **Late megakaryopoiesis: proplatelet, preplatelet, and terminal platelet production**

Mature MKs subsequently associate with the vasculature and, facilitated by matrix metalloproteinase proteins (MMPs), form extensive proplatelet processes, elongated branching projections that extend through the endothelial cells lining the sinusoidal blood vessels. Here, proplatelets are released into the circulation and fragmented by shear forces, giving rise to circulating preplatelets (sometimes termed “reticulated platelets”) and, ultimately, single platelets.<sup>10</sup>

It appears that the DMS of the differentiated MK is particularly adapted to the production of platelets via continuous directed remodeling of its underlying cytoskeletal structure. In fact, many platelet production defects have been associated with mutations in Rho-GTPases central to cytoskeletal remodeling, which include Ras homolog family member A (RhoA), Rac family small GTPases 1 and 2 (RAC1 and RAC2), and cell division cycle 42 (CDC42) as well as related proteins.<sup>10</sup> Investigations into this dynamic process

have focused on the roles of actin, microtubular rearrangements, and surface integrins. Platelet biogenesis from MKs starts with (a) microtubule reorganization into pseudopod structures; followed by (b) spreading of these structures and eventual development into proplatelet processes; accompanied by (c) organelle inclusion within the processes such that the organelles are eventually concentrated at the tips of the proplatelet branches; and finally (d) actin-dependent branching to extensively amplify proplatelet processes, with the DMS serving as the membrane reservoir for this expansion.<sup>45</sup>

Ultrastructural studies of the differentiated MK demonstrate that late stage MKs are characterized by peripheralization of the DMS membranes. This is accomplished by the membranes thinning out (evagination) such that they produce branching processes that finally extend into sinusoidal blood vessels. These processes have platelet-sized swellings all along their structure, but most importantly, the swellings at the branch tips appear to be the source of proplatelets.<sup>46</sup>

Microtubules are critical for proplatelet membrane process elongation.<sup>47</sup> Repeated rounds of microtubule extension (bundling) and bending to form proplatelet processes can be characterized by physical forces such that, on balance, final platelet size can be correctly predicted to be in the micron radius range.<sup>48,49</sup> The subsequent branching of these processes is the end result of multiple rounds of fission at both the midbody and the ends of the projections; these fission events are the result of actin-dependent bending and branching which amplifies the proplatelet ends, thereby resulting in an increased potential number of platelets to be released once membrane projections penetrate into the sinusoidal vasculature.<sup>50</sup> Live-cell imaging experiments examining MK-specific mechanisms of drug-induced thrombocytopenia in the setting of trastuzumab emtansine administration have provided insight into this process, showing that this drug is taken up by mouse MKs where it inhibits MK differentiation and disrupts proplatelet formation. The inhibitory effect of trastuzumab emtansine is mediated by its induction of abnormal tubule organization within MKs, resulting in abnormal tubule thickness and fewer branching processes.<sup>51</sup>

In vivo proplatelet extension is more polarized than that observed in vitro. F-actin is thought to play a role in megakaryocyte polarization; inactivation of various genes encoding F-actin-related proteins results in proplatelet extension defects and megakaryocyte fragmentation within the marrow space.<sup>7,52</sup> The aforementioned CDC42 is the most important Rho-GTPase for proplatelet transendothelial migration; increased activity results in not only hyperpolarization but also increased transendothelial migration of whole MKs.<sup>7,10,52</sup>

Penetration of proplatelet extensions into sinusoidal vessels precedes the release of platelet precursors and whole/partial MK fragments into blood; both processes will give rise to terminal circulating platelets.<sup>53–56</sup> Once extended into the sinusoidal vessel, vascular shear forces interact with proplatelet projections, causing the tips to separate and enter the circulation;<sup>10,46</sup> this process appears to be regulated by von Willebrand factor (vWF).<sup>57</sup> Based on ultrastructural images, barbell-shaped proplatelets and circular preplatelets are released from differentiated MKs into the blood. Thus, current studies suggest that terminal platelet formation actually occurs within the blood<sup>49</sup> and, as noted previously, within the pulmonary bed.<sup>8,10,54,58</sup> Circular preplatelets may be identical to reticulated platelets (RPs) and/or to the large platelets that are present in several of the defined macrothrombocytopenia syndromes. In studies of hemostasis in human immune thrombocytopenic purpura

(ITP), RPs have been found to contain twice as much  $\alpha$ -granule content as older circulating platelets.<sup>59</sup> RPs also have an increased density of membrane receptors than older platelet cohorts, bolstering evidence that the RP subset may represent the circular preplatelets or released proplatelets.<sup>60</sup>

There are also data suggesting that circular preplatelets can revert to a barbell proplatelet form. When barbell proplatelets divide, they form two platelets that are larger (demonstrably higher platelet volume) when compared with older circulating platelets. However, these intermediate stages of terminal platelet formation after proplatelet excision are relatively dynamic, and the exact sequence of platelet maturation has not been confirmed by independent studies.

Evidence also exists suggesting that a small fraction of in vivo circulating platelets, which are thought to be terminally differentiated, may duplicate in the bloodstream under the stimulation of thrombocytopenia;<sup>46</sup> this physiology may perhaps be analogous to circular preplatelets reverting to the barbell proplatelet form which subsequently is able to divide and form two distinct terminal platelets. Such young preplatelets should be differentiated from the majority of circulating platelets that can neither divide nor revert to a barbell shape. It is also likely that such younger platelets have greater ability for continued protein translation, and there is imaging evidence for higher content of ribosomes; hence, these platelets may also be RPs as defined by elevated RNA content in comparison to the majority of circulating, older platelets.

### **Pathological insights into megakaryopoiesis**

Much of what we know about megakaryopoiesis can be attributed to the study of clinical syndromes that decrease platelet production, often resulting in thrombocytopenia. Defects have been found in genes that affect all stages of megakaryopoiesis—from progenitor differentiation through platelet biogenesis. Some of these mutations have prognostic implications involving predisposition to hematologic malignancies, further supporting investigation of the molecular etiologies of thrombocytopenic syndromes.<sup>61</sup>

Defects in the TPO receptor gene, *MPL*, result in impaired fate differentiation of megakaryocyte progenitors as well as the maintenance of self-renewal in the HSC-compartment leading to congenital amegakaryocytic thrombocytopenia.<sup>61</sup> Defects in *RBM8A*, implicated in thrombocytopenia-absent radius syndrome, are hypothesized to affect MK differentiation by impeding TPO signaling downstream from the MPL-binding event.<sup>61</sup> Additional defects in transcription factor genes *HOXA11* and *MECOM* (encoding for EVI1) have been implicated in the development of radioulnar synostosis with amegakaryocytic thrombocytopenia.<sup>61</sup> All of these defects impair early megakaryopoiesis via disruption of multipotent progenitor differentiation.

Many genetic defects have been identified in relation to abnormal megakaryocyte maturation. Some of these include *RUNX1* defect, which results in familial platelet disorder with propensity to acute myelogenous leukemia; 11q23 deletion or *FLI1* missense mutation, both defects involving *FLI1* resulting in Paris–Trousseau thrombocytopenia/Jacobsen syndrome or *FLI1*-related thrombocytopenia respectively; and *NBEAL2* mutation, resulting in impaired membrane dynamics and vesicle trafficking seen in gray platelet syndrome. Other important loci with associated inherited thrombocytopenia include *ANKRD26*, *ETV6*, *GATA1*, *GFI1B*, *SLNF14*, *FYB*, and *SRC*.<sup>61</sup>

Defects in terminal platelet production (i.e., regulation of proplatelet formation, release, and/or maturation to preplatelets) often result in macrothrombocytopenia syndromes. There is evidence that  $\beta 3$  integrin plays a role in proplatelet formation and platelet

size. In Glanzmann thrombasthenia where there is a  $\beta 3$  integrin defect producing larger platelets in some instances, there appears to be inability to constrict the ends of the proplatelets.<sup>62,63</sup>

Proplatelet extension is driven by extension and bending of microtubules which, in turn, is dependent on actin motility and actin filament turnover. Filamin A anchors receptors (e.g.,  $\beta 3$  integrin and GPIb) to the cytoskeleton,<sup>50,64,65</sup> and human defects in filamin A result in macrothrombocytopenia.<sup>66</sup> Although MKs are normal in number in Bernard Soulier syndrome (BSS), there is a disordered central membrane invagination system that appears to be dependent on the normal expression of GPIb $\alpha$ .<sup>67,68</sup> Knockout mice lacking GPIb $\alpha$ , but not GPV, have an identical macrothrombocytopenic phenotype as in BSS, making this a possible pathway defect for terminal platelet production.

Another macrothrombocytopenia syndrome, May–Hegglin anomaly, appears to have defective microtubule extension based on abnormal thickness of the packed microtubule layers.<sup>46</sup> This defect results in larger proplatelet formation and release, but there is evidence that microtubule band thinning, and therefore normal extension, may also be inhibited through a distinct nontubule pathway. May–Hegglin anomaly is caused by an *MYH9* mutational defect in nonmuscle myosin IIA heavy chain<sup>69</sup> and produces an autosomal dominant macrothrombocytopenia in humans. Similar macrothrombocytopenia syndromes with varying associated phenotypes (e.g., Fechtner syndrome, Sebastian platelet syndrome, and Epstein's syndrome) have also been attributed to *MYH9* defects and are now all recognized as a single entity, *MYH9*-related disease.<sup>70–72</sup> The *MYH9* defect has been shown to have normal MK ultrastructure by EM, i.e., there is no early differentiation defect that affects the central membranes (DMS) nor is there disruption of internal granule structure.<sup>73</sup>

In vivo actin filament turnover is critical for the terminal processes of proplatelet formation and subsequent normal platelet sizing; actin filament turnover does not appear to have a prominent effect on MK maturation.<sup>74</sup> Actin depolymerizing factor (ADF)/cofilin severing proteins regulate actin turnover. Using murine knockouts, cofilin, but not ADF, was shown to be essential for normal platelet size and shape. Yet, if both proteins are knocked out, platelet formation is nearly absent; the latter is characterized by severe disruption of the MK central membrane system and disorganization and diminution of proplatelet processes. Thus, actin has a major role in proplatelet formation and preplatelet conversion to the terminal platelet with normal maturation/sizing.

MK motility at the vascular niche and subsequent proplatelet budding require rearrangement of the actin cytoskeleton such that podosomes form dynamic contacts with the extracellular matrix (ECM).<sup>75</sup> In the context of recent insights into the limited role of megakaryocyte migration and the spatial relatedness of the endosteal versus vascular niche, it may be that podosomes act primarily to probe their surroundings for regions of transendothelial permissibility<sup>76</sup> as opposed to facilitating transmarrow migration. This hypothesis is supported by the observation that megakaryocytes can shift their center of mass despite exhibiting low motility in vivo.<sup>14</sup> MKs from Wiskott Aldrich syndrome (WAS) cannot form podosomes, and WAS MKs prematurely release proplatelets into the marrow, not blood.<sup>77</sup> MK podosomes have a role in degrading ECM to enhance and subsequently promote extension of proplatelets processes into sinusoidal vessels. Normal MK formation of podosomes and their associated actin-rich protrusions across the sinusoidal basement membrane have been shown to be dependent on MMP function; both of these abilities are lost in WAS.<sup>75</sup>

Numerous additional clinical syndromes attributed to defects in platelet biogenesis have been documented.<sup>61</sup> Recently, large correlative human genetic studies utilizing strategies such as genome-wide association, whole exome sequencing, and whole genome sequencing in patients with inherited thrombocytopenia disorders have enabled identification of additional genes correlated with platelet production and kinetics and have also identified loci associated with other clinical traits and diseases.<sup>61,78</sup> For example, genetic correlations with platelet count and mean platelet volume (MPV) have been found between genes influencing lipid biology (e.g., *APOH*, *LDLRAP1*, *FADS1/2*, *MAP1A*, *PNPLA3*, and *GCKR*), G protein signaling (*RGS10* and *RGS18*), and even height (e.g., *CABLES1*, *DNM3*, and *MICA*). Techniques such as Mendelian randomization, which can shed insight into causative associations between MPV and various genes, identified associations with *SH2B3* and *MYL2*, genes associated with coronary heart disease. However, observational studies examining this association have provided mixed results, and systematic meta-analyses found a positive (opposite) association relationship.<sup>61,79–81</sup> Regardless, these strategies may continue to uncover genetic loci vital to platelet production and kinetics as sample size, depth, and diversity increase.

### Control of megakaryopoiesis

It is important to study megakaryopoiesis not only for physiological and pathological insights but also because of the hoped-for prospect of ex vivo production. Donation of blood or apheresis for platelet units that are eventually to be transfused is a significant undertaking, and the short shelf life of donor platelets is a major impediment to medical care. Hence, there is some impetus for developing alternatives to platelet products or artificial sources of actual platelet production.<sup>82</sup> For the latter, human CD34+ stem cell populations have been described that can generate ex vivo functional MKs with ploidy levels up to 32N.<sup>83–85</sup> However, the generation of sufficient MK numbers in culture and ensuring the subsequent production of adequate platelets per MK in a cost-efficient manner are still challenging goals. Studies that examine the regulatory layers of control over megakaryopoiesis improve not only our understanding of physiology but also our ability to manipulate such factors for the goal of therapeutic intervention or the eventual goal of ex vivo production of platelets.

### Control of megakaryopoiesis: thrombopoietin and other cytokines

Multiple cytokines are implicated in the control of megakaryopoiesis, but TPO is the primary factor stimulating the proliferation and maturation of MK progenitor cells into their terminally differentiated form.<sup>4</sup> TPO is encoded by the *THPO* gene, located on chromosome 3q26.3-3q27, which encodes a 30 kDa, 353-amino-acid precursor protein. The mature molecule, composed of 332 amino acids, is acidic and heavily glycosylated.<sup>5</sup> TPO shares high homology with erythropoietin (EPO) at its N-terminus.<sup>86</sup> TPO is produced in the liver and, to some extent, the kidney and marrow stromal cells.<sup>7</sup>

TPO initiates its effects via binding to its cognate receptor, MPL (encoded by *MPL* on chromosome 1p34).<sup>5,87,88</sup> MPL exists in both monomer and homodimer forms in its unbound state and possesses no intrinsic kinase activity. Upon TPO binding, MPL undergoes conformational changes that induce transphosphorylation, associated Janus kinase 2 (JAK2) activation, and subsequent phosphorylation of receptor tyrosine residues—facilitating the initiation of multiple signal transduction pathways including signal

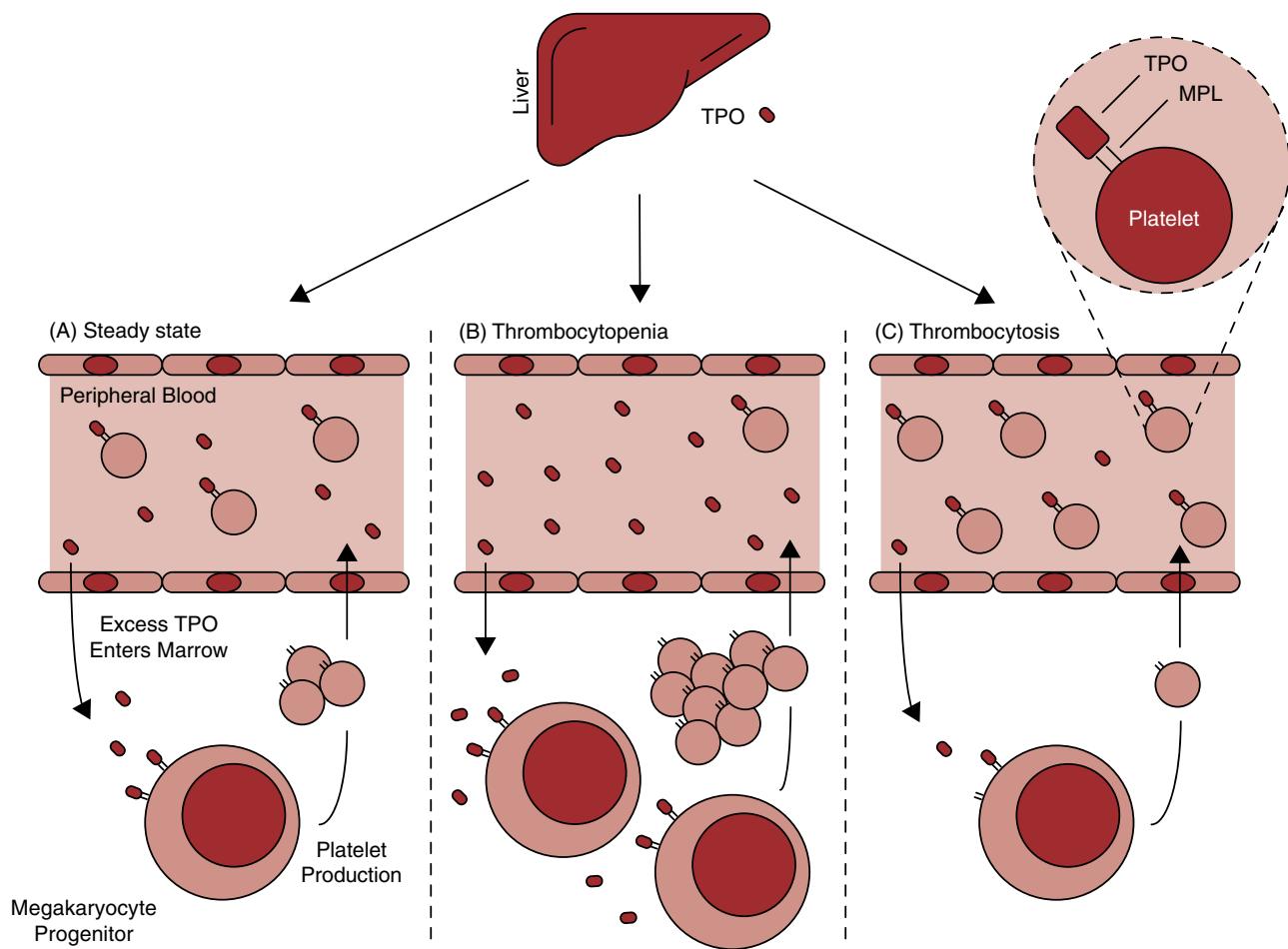
transducer and activator of transcription (STAT)-, phosphatidylinositol 3-kinase (PI3K)-, and mitogen-activated protein kinase (MAPK)-dependent processes.<sup>5,7</sup>

As noted previously, megakaryopoiesis is the process of differentiation of hematopoietic stem cells leading to platelet production; this is characterized by an initial phase of proliferation of MK progenitors and subsequent differentiation of maturing MKs that do not proliferate. Since both processes are mediated largely by TPO, this suggests that there is a physiologic balance in TPO's ability to both enhance and inhibit proliferation.<sup>89</sup> Regulation of this balance is important for timing and adequacy of platelet production. TPO has been shown to act via MAPK signaling to halt MK proliferation and induce the same MKs to undergo differentiation.<sup>90</sup> Besides MAPK, this balance of TPO-induced proliferation versus differentiation is also dependent upon JAK2 and MPL protein levels.<sup>89</sup> When either one of the JAK2 or MPL proteins is expressed at relatively lower levels, TPO exposure will induce preferential MK proliferation via weak MAPK signaling; by contrast, when both proteins are highly expressed and MAPK signaling is strongly activated, MK cell cycle arrest occurs,<sup>39–41</sup> which is accompanied by MK differentiation.<sup>91,92</sup>

Steady-state megakaryopoiesis supplies about  $10^{11}$  platelets every day into the circulation. MKs will respond to changes in platelet lifespan, increasing by 10-fold with destructive thrombocytopenic conditions,<sup>4,93</sup> and the proportion of higher ploidy MKs also increases.<sup>37</sup> Compensatory responses of marrow MKs are evident within 24–48 hours after inducing thrombocytopenia; in response to thrombocytapheresis and in thrombocytopenia caused by immune destruction, there is a marked increase in MK number and size.<sup>41,94</sup> Reciprocal decreases in MK size, ploidy, and volume occur with experimentally induced thrombocytosis.<sup>95,96</sup> These alterations, found in both experimental animals and human subjects, are primarily mediated by TPO.<sup>4</sup>

A large percentage of circulating TPO is produced constitutively by the liver, and its levels are partly regulated by the extent of TPO binding to MPL on circulating platelets and marrow MKs, which results in the internalization and elimination of TPO-MPL complexes.<sup>87,97</sup> However, data suggest that hepatic secretion of TPO is only partially constitutive, and significant regulation of TPO production occurs via the senescent platelet mass interacting with hepatocyte receptors (discussion forthcoming; see the “Platelet lifespan and its regulation” section).<sup>98</sup> Blood and marrow levels of TPO are usually inversely related to marrow MK mass and peripheral platelet counts;<sup>4</sup> hence, regulatory aspects of the platelet mass by circulating TPO levels is sometimes termed the “sponge model.” High-affinity MPL expressed on platelets binds TPO, which is then internalized and degraded, allowing the circulating platelet mass to affect, at least in part, the amount of TPO available to MKs for stimulation; low platelet counts allow more available TPO to induce megakaryopoiesis. In the sponge model of TPO regulation, as long as constitutive liver production of TPO occurs, the total platelet mass is preserved (Figure 15.2).

Accordingly, the elimination of a functional *MPL* gene, or its congenital absence, results in severe thrombocytopenia accompanied by decreased hematopoietic stem and lineage-committed progenitor cells.<sup>4</sup> Studies in mice lacking MPL expression in only MKs and platelets resulted in thrombocytosis with increased megakaryocytic ploidy and progenitor counts, further suggesting that TPO maintains circulating platelet mass primarily via stimulation of MPL-expressing progenitors and that megakaryocytic and platelet MPL expression primarily serves the role of restricting the amount



**Figure 15.2** The “sponge” model of megakaryopoiesis. The liver constitutively secretes thrombopoietin (TPO) into the blood; MPL receptors on platelets bind, internalize, and degrade TPO. In this model, the platelet mass determines the amount of free TPO in the circulation. At steady state (A), the normal platelet count reflects balanced platelet production and destruction; free TPO in blood moves to the marrow to stimulate megakaryocyte progenitors and normal ploidy distribution. When thrombocytopenia occurs (B), free TPO is increased because there are too few platelets available for binding; a larger amount of TPO is available to the marrow, which results in an increased number of megakaryocytes with higher average ploidy. In contrast, reactive thrombocytosis (C; e.g., with chronic inflammation) results in less free TPO available to transit to the marrow; decreased megakaryopoiesis results in fewer megakaryocytes with lower average ploidy levels.

of TPO available to progenitors,<sup>5,99,100</sup> despite evidence of TPO involvement in megakaryocytic maturation.<sup>20</sup>

The sponge model does well to explain why plasma levels of TPO are increased by several orders of magnitude in patients with hypoproliferative thrombocytopenia, and why TPO levels decline after recovery of hematopoiesis.<sup>87,101</sup> However, when severe platelet destruction via Fc $\gamma$ R binding causes thrombocytopenia, such as in ITP, TPO levels are not as escalated as might be expected from the degree of thrombocytopenia. This was thought to be due to the markedly increased platelet turnover rate,<sup>102</sup> but data on platelet clearance via hepatic interaction suggest otherwise, i.e., that ITP bypasses normal senescent mechanisms of platelet loss that are partly responsible for upregulating TPO production.<sup>98</sup> Thus, megakaryopoiesis appears to be regulated by plasma levels of unbound TPO, which reflects the balance between constitutive TPO production, the rate of platelet and MK binding dictated by overall platelet levels, and TPO production regulation by senescent platelet clearance by the liver.

While TPO is a major physiologic driver of megakaryopoiesis, other cytokines have been shown to potentiate or promote megakaryopoiesis. Stem cell factor (SCF) is one such cytokine thought to

augment TPO's effects through JAK2 and Src kinase signaling and may facilitate important cell-to-cell relationships within the marrow.<sup>103</sup> Additional cytokines including IL-3, IL-6, IL-9, IL-11, GM-CSF, Flt3-ligand (FL), and leukemia inhibitory factor (LIF) have been shown to potentiate megakaryopoiesis in vitro, though many appear dispensable for steady-state megakaryopoiesis in murine studies.<sup>5</sup> Note should also be made of additional TPO-independent pathways including IGF1 and YRS<sup>ACT</sup>-, CCL5-, and IL1 $\alpha$ -dependent pathways.<sup>7,31,104</sup>

The CXCR4-CXCL12 axis has also been identified as influential upon megakaryopoiesis regulation. Upregulation of the chemokine receptor CXCR4 is associated with MK maturation and differentiation.<sup>105</sup> Although its ligand CXCL12 (a.k.a. stromal cell-derived factor 1) by itself cannot stimulate MK growth and differentiation, this finding has importance for nonhomeostatic regulatory mechanisms of platelet production since CXCL12 acts synergistically with TPO both in vitro and in vivo.<sup>7,106-108</sup> Deregulation of the CXCL12-CXCR4 axis is associated with human thrombocytopenia.<sup>5</sup> Binding of chemokine CXCL12 to CXCR4 stimulates secretion of matrix metalloprotease 9 (MMP-9), enabling motility along the CXCL12

gradient.<sup>5,109</sup> While megakaryocyte migration from spatially distinct niches may not be as important as initially thought,<sup>14,15</sup> podosome extension and subsequent vascular interaction may be affected by CXCL12-mediated signaling, either through interaction with CXCR4 or another pathway such as the Src–Syk–PLC $\gamma$ 2 axis.<sup>14,110</sup>

Other megakaryopoiesis-mediating cytokines include FGF4, which has been shown to mediate megakaryocyte adhesion to endothelial cells.<sup>5,107</sup> Bioactive lipid sphingosine 1-phosphate (S1P) and its receptor, S1pr1, are important for directing proplatelet extensions, with deficiency of either resulting in extension in random directions without vascular directional bias.<sup>111,112</sup>

Inflammation is a significant and frequent cause of thrombocytosis, perhaps as a compensatory process to promote angiogenesis and wound healing. The proinflammatory VEGFR1-mediated pathway has now been shown to stimulate further upregulation of CXCR4 on megakaryocytes in a murine model, and the VEGFR1 pathway additionally stimulates MK endomitosis; hence, inflammation mediated by VEGFR1 enhances platelet production on multiple levels.<sup>113</sup> IL-1 $\alpha$  has also been shown to promote platelet production under inflammatory conditions leading to rapid megakaryocyte fragmentation, termed megakaryocyte rupture, to release large numbers of platelets into circulation.<sup>5,114</sup> Other inflammatory agents and mediators including lipopolysaccharide (LPS) and tumor necrosis factor alpha (TNF $\alpha$ ) have also been shown to influence megakaryopoiesis by promoting megakaryocytic protein expression within the HSC compartment.<sup>5,115</sup>

### Control of megakaryopoiesis: genetic regulation

Genetic regulation of megakaryopoiesis takes place on multiple levels and includes involvement of transcription factors, noncoding/regulatory RNA, and epigenetic effectors. GATA1 is a transcription factor mentioned previously that drives MEP differentiation. While largely studied for its association with erythropoiesis, GATA1 is also necessary for megakaryopoiesis and the expression of MK-specific glycoprotein IIb. GATA2 has been shown to promote megakaryopoiesis at the expense of erythropoiesis.<sup>31</sup> MDS1 and EV11 complex locus protein (MECOM); nuclear factor, erythroid 2 (NFE2); and ETV6 are additional transcription factors involved in megakaryocyte maturation.<sup>7,116</sup> Patients possessing mutations in gene ETV6, a tumor suppressor that interacts with FLI1 and regulates CDC42 and RhoA, have been shown to exhibit increased megakaryocyte proliferation along with various cytoskeletal defects that lead to decreased proplatelet formation and altered platelet shape and spreading. ETV6 variants have also been associated with increased circulating CD34+ progenitors and predisposition to myelodysplastic syndrome and leukemia.<sup>116</sup>

Myocardin-like transcription factors A and B (a.k.a. MKL1 and MKL2) have also been identified as important regulators of megakaryopoiesis. Mutation of the *MKL1* gene is part of the t(1;22) translocation found in some infantile AMKLs, where *MKL1* is fused to the RNA binding motif protein 15 gene (*RBM15*).<sup>47</sup> MKL1 is known to act as a cofactor for serum response factor (SRF) to induce muscle differentiation.<sup>117,118</sup> However, SRF can also regulate cytoskeletal genes critical for MK differentiation, and it has a role in the actin cytoskeleton development of stem cells.<sup>119</sup> SRF, in association with cofactors other than MKL1, is also a downstream target for MAPK signaling.<sup>120-124</sup>

In murine models, hematopoietic-specific SRF knockouts demonstrated decreased platelet counts, concomitant with a reduction in polyploid MKs and an increase in immature MKs with abnormal morphology.<sup>47</sup> The morphologic dysplasia and macrothrombocytopenia

induced by SRF deletion is accompanied by the downregulation of actin cytoskeletal regulatory proteins in the MKs, clearly disruptive of essential MK mechanisms for megakaryopoiesis.<sup>125</sup> Additional studies found that SRF is required with MKL1 to produce polyploidization during MK differentiation. The effects of SRF depletion are more extreme than MKL1 deletion, which also causes a decrease in platelet counts and an increase in MK progenitors in the bone marrow. These findings suggest that (a) SRF-transcription is critical for terminal MK processes that lead to proplatelet formation and platelet release and (b) there are additional factors besides MKL1 that promote SRF-mediated megakaryopoiesis.<sup>47,126</sup> The authors suggested that MKL2 expressed in MKs might redundantly function with MKL1 in murine MK maturation.<sup>126</sup>

When both MKL1 and MKL2 are absent, the arrest of MK polyploidization is more dramatic than with MKL1 alone (a majority of MKs exhibit ploidy of 4N or less).<sup>126</sup> Expression of guanine nucleotide exchange factor GEF-H1, which promotes RhoA activity to complete cytokinesis,<sup>36</sup> is linked to MKL1 regulation of MK maturation.<sup>10</sup> GEF-H1 expression is normally decreased early in MK endomitosis and subsequently rises with maturation, and SRF is known to bind to the GEF-H1 promoter in hematopoietic cells.<sup>127</sup> By contrast, GEF-H1 is increased when MKL1 is absent. Hence, MKL1 regulation of SRF-influenced transcription is probably most relevant to early MK maturation. RUNX1 also plays a role in early MK maturation since it silences the nonmuscle myosin IIB heavy chain, MYH10, a required event for the transition from mitosis to endomitosis.<sup>128</sup>

While transcription factor mediated regulation is one mechanism of genetic control, other layers of regulation are also important. Post-transcriptional regulation via noncoding RNA has also been explored, with the silencing of growth factor interdependent 1 (GFI1) expression via *miR-22* identified as a promoter of megakaryocyte differentiation.<sup>7,129</sup> Other megakaryopoiesis-regulating miRNAs identified include *miR-34a*, *miR-105*, *miR-125a-5p*, *miR-142*, *miR-146a*, *miR-146b*, *miR-150*, *miR-155*, and *miR-486*.<sup>130,131</sup> Long noncoding RNAs (lncRNAs) such as AS-RBM15 have also been demonstrated to influence megakaryopoiesis, in this case via regulation of transcription and translation of *RBM15*.<sup>132,133</sup> Furthermore, extensive de novo methylation is thought to be an important component of the epigenetic regulation of megakaryopoiesis.<sup>134</sup>

### Control of megakaryopoiesis: biophysical niche forces

The hematopoietic niche encompasses the various autocrine, paracrine, juxtacrine, and endocrine signals to which megakaryocytes and their progenitors are exposed. Neighboring cell types, cellular density, ECM composition, and diffusibility further influence these dynamics. Recent work has been carried out to dissect the contribution that ECM components and associated biophysical cues (e.g., stiffness) of the microenvironment have upon megakaryopoiesis.

Marrow is among the softest tissues of the body, with a Young's modulus ranging from approximately 0.3 kPa in medullary regions<sup>7,135</sup> to approximately 27 kPa in osteoblastic endosteal regions.<sup>7,136</sup> Stiffer type I collagen fibrils have been found to promote HSC differentiation through the megakaryocytic lineage. However, collagen I inhibits proplatelet formation via integrin  $\alpha 2\beta 1$  activation and subsequent Rho-ROCK axis signaling, while less stiff type III and IV collagens promote proplatelet formation through PI3K/Akt signaling.<sup>7,137</sup> Other ECM components including fibronectin, vitronectin, fibrinogen, plasmin, PAI-1, and thrombospondin-2 have also been shown to influence megakaryopoiesis.<sup>137</sup> Furthermore, murine platelet production has been increased by

limiting marrow stiffness via the inhibition of lysyl-oxydase (LOX)-mediated collagen crosslinking, further suggesting the importance of niche biophysical cues for megakaryopoiesis.<sup>7</sup>

Additional work has been carried out in evaluating the effects of microenvironmental stiffness upon *in vitro* megakaryopoiesis using various methylcellulose substrates as well as two- and three-dimensional substrates.<sup>138</sup> Cytoskeletal characteristics are modulated by microenvironment mechanical properties, as megakaryocytes confined within soft hydrogels have been shown to influence MYH9 distribution via promoting activation of the mechanosensitive transcription factor MKL1.<sup>7</sup> Potential mechano-sensing pathways proposed include initiation of calcium influx leading to subsequent PI3K/Akt signaling via a TRPV4-dependent (transient receptor potential cation channel subfamily V member 4) mechanism.<sup>7,137</sup>

As mentioned previously, vascular niche shear forces facilitate the extension and subsequent release of proplatelets. High shear stress is known to enhance MK fragmentation and promote higher proplatelet formation via cytoskeletal remodeling.<sup>53</sup> It is possible that shear forces act physiologically since hemodynamic stress in humans (e.g., exercise) has been shown to elevate platelet counts.<sup>139,140</sup> Interestingly, DNA synthesis in immature MKs can also be enhanced via exposure to shear stress.<sup>141</sup>

Exact mechanisms by which megakaryocytes sense various biophysical cues are still largely unknown;<sup>7</sup> dissection of the roles of ECM composition and mechano-sensing may have implications for the study of mechano-altering disease processes such as myelofibrosis, as well as the *ex vivo* production of platelets.

## Platelet kinetics

### Platelet production as a function of platelet circulatory survival

The kinetics of normal platelet production have been measured indirectly by determining the turnover rate of circulating platelets (platelet count divided by platelet survival corrected for recovery). The median overall rate of platelet production under steady-state conditions (platelet removal is equivalent to platelet production when the platelet count is constant) in healthy humans ranges from 35,000 to 44,000 platelets/ $\mu\text{L}/\text{day}$ .<sup>142–145</sup> The reliability of platelet turnover estimates depends on the accuracy with which each of the three variables used in calculating platelet turnover can be determined—mean platelet lifespan, recovery of platelets in the circulation, and blood platelet count. The summation error may be considerable, as occurs among patients with severely enhanced platelet destruction (e.g., ITP).<sup>145</sup>

### Platelet lifespan and its regulation

The human platelet lifespan is  $9.5 \pm 0.6$  days,<sup>142</sup> and healthy platelet disappearance is generally linear and thought to mostly reflect platelet senescence.<sup>144</sup> With consumptive thrombocytopenia, the platelet lifespan is shortened, with exponential, likely random, platelet removal from circulation.<sup>146</sup> Platelet survival time has also been shown to shorten progressively as the platelet count decreases to  $<100,000/\mu\text{L}$ .<sup>144,147</sup> Therefore, a shortened platelet survival time in patients with thrombocytopenia does not necessarily indicate a destructive process.

Hanson and Slichter proposed a model for platelet removal that predicted shortening of platelet lifespan in relation to the level of thrombocytopenia.<sup>144</sup> The analysis indicated that 82% of normal platelet turnover is caused by platelet senescence; only 18% of

platelet removal is due to the requirement of platelets to support vascular integrity. In this model, healthy hemostatic platelet loss represents about 7000 to 10,000 platelets/ $\mu\text{L}/\text{day}$ . Since there is evidence that clearance of platelets via hemostatic integrity is a fixed absolute requirement, thrombocytopenia caused by marrow hypoplasia causes the daily proportion of platelets removed by hemostatic consumption to rise significantly.<sup>148</sup> Hemostatic clearance in patients with megakaryocytic hypoplasia can actually be predicted by the platelet count (15.1–28.0% of overall platelet turnover).<sup>148</sup>

Kinetic modeling of platelet lifespan using labeling in murine populations suggests that, at steady state, circulating platelet senescence is internally programmed.<sup>149</sup> Since platelets contain the key components of a critical apoptotic pathway that regulates lifespan,<sup>150</sup> it may well be that platelet lifespan and the induction of senescent platelet death are regulated by internal, programmed functions rather than external injuries or “hits.” Antiapoptotic Bcl-2 family proteins restrain proapoptotic proteins Bak and Bax. Knockout and pharmacological inhibition studies suggest that Bcl-xL and Bak are likely the most important regulators of apoptotic-mediated platelet clearance, with induced deficiency of either’s activity producing thrombocytopenia or prolonged platelet lifespan, respectively.<sup>151,152</sup> Platelet-specific triggers that commence apoptosis have yet to be established. Of note, platelets seem to lack the Fas receptor central to the extrinsic apoptotic pathway. It remains to be determined how platelets integrate intrinsic and extrinsic cues that push them toward senescence.<sup>153</sup>

When platelets become senescent, the physiology of their removal is postulated to include (a) irreversible changes in membrane glycoproteins; (b) increased platelet-associated immunoglobulin with subsequent clearance of high-immunoglobulin-expressing platelets by the reticuloendothelial system; (c) increased exposure of the negatively charged procoagulant lipid, phosphatidylserine, on the external platelet membrane; and (d) decreased levels of surface sialic acid.<sup>154</sup> Mechanisms of platelet clearance have been examined with particular relevance to platelet storage; such studies may shed light on *in vivo* platelet circulation. Desialylation of platelet receptors occurs when platelets are warmed after cold exposure,<sup>155</sup> warming activates sialidases in plasma that target the sialic acid residues on platelet glycoproteins. Interestingly, oseltamivir phosphate, a sialidase inhibitor used clinically to treat influenza infection, has been shown to increase platelet counts in patients with ITP independent of influenza infection.<sup>156–158</sup> Cold exposure also primes matrix metalloproteinase (MMP) cleavage of GPIb $\alpha$ , and the end result of both pathways is increased platelet clearance by the liver.

The association between loss of sialic acid and platelet senescence has recently reinforced the possibility that platelets themselves act directly on TPO mRNA expression<sup>159–163</sup> rather than MPL uptake of TPO as the exclusive model for the regulation of hepatic production.<sup>101,164–167</sup> As platelets age in the circulation, they lose  $\alpha$ 2,3-linked sialic acid from surface glycoproteins; these desialylated platelets can then be cleared by binding to the hepatocyte Ashwell–Morell receptor (AMR), a heteromeric complex of asialoglycoprotein receptors 1 and 2.<sup>98</sup> Binding of platelets to the AMR induces hepatocyte TPO mRNA via JAK2-STAT3 signaling, and this may partly explain why TPO levels are higher than expected in essential thrombocythemia (ET) and lower than expected in ITP. In ET where platelet lifespan is unchanged, one would expect a higher daily number of senescent platelets to be cleared by the liver, thereby inducing more TPO; by contrast, since platelets in ITP are primarily cleared by macrophage Fc $\gamma$ R binding and would bypass the hepatocyte AMR, lower TPO production would be the result. In this

model, senescent platelet regulation of TPO production accounted for up to 50% of secretion, suggesting that this pathway may even be equivalent to constitutive TPO production for regulation of platelet mass (Figure 15.3). It is unclear if the apoptotic pathway intrinsic to platelets initiates these sialylation changes, but the apoptotic mechanism does affect platelet survival,<sup>150,168,169</sup> including proapoptotic platelet clearance via the liver.<sup>170</sup>

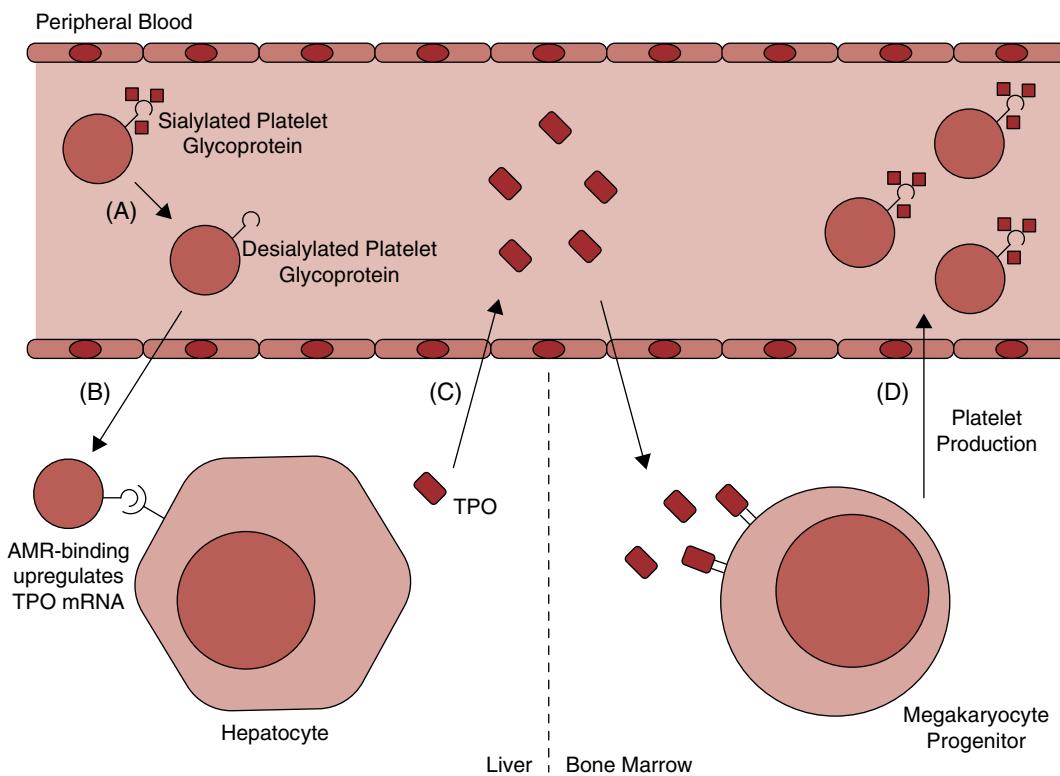
As noted in the “Platelet production” section, neonatal and adult MKs differ in their maturational abilities, and similarly, studies of neonatal platelets suggest kinetic differences from their adult counterparts. Murine studies suggest that platelets survive 1 day longer in neonatal mice when compared to platelets in the circulation of adult mice of the same genotype.<sup>171</sup> The antiapoptotic protein Bcl-2 was much higher in human neonatal platelets compared with adult platelets, suggesting relatively increased resistance to apoptosis.<sup>150,170</sup> These findings provide additional support for the concept of programmed cellular senescence via apoptosis, but they do not rule out additional processes governing nonhemostatic platelet clearance.

Newly released reticulated platelets (RPs) are identified by their increased RNA content;<sup>172</sup> murine studies have directly confirmed that RPs are the platelet subset most recently released from megakaryocytes into the blood.<sup>173,174</sup> The RP% in humans correlates with thrombopoietic activity.<sup>175</sup> Analogous to red cell reticulocytes, RPs increase in response to destructive thrombocytopenia and decrease when megakaryocyte hypoproliferation occurs with chemotherapy or intrinsic marrow aplasia.<sup>176</sup> The absolute RP count in humans

with normal platelet counts ranges from 15,000 to 48,000/ $\mu\text{L}$ ; this average platelet production agrees with isotopic labeling studies.

The RP% has proven valuable for detecting changes in overall platelet turnover. The RP% has been applied for predicting thrombopoietic recovery, transfusion independence, and thrombotic risk. An increase in the RP% was associated with subsequent graft-vs.-host disease after allogeneic hematopoietic stem cell transplantation.<sup>177</sup> Similarly, an asymptomatic increase in the RP%, with no change in platelet counts, from the first to second trimester in pregnancy was associated with subsequent preeclampsia.<sup>178</sup> Asymptomatic patients with steady-state thrombocytosis and an elevated RP% or absolute RP count were at increased risk for subsequent thrombosis, and their relative risk was higher than the risk associated with a high platelet count alone.<sup>179</sup> The RP% and absolute RP count in thrombocytosis decrease with either successful aspirin therapy or hydroxyurea.<sup>180,181</sup>

A compensatory increase in platelet production and the corresponding rise in circulating RP% are well established in patients with ITP.<sup>182,183</sup> Platelet lifespan in severe ITP can be shortened to hours. This increase in peripheral platelet destruction is so significant that platelet lifespans are shorter than predicted by Hanson and Slichter’s model. Hence, post-transfusion platelet counts are probably the only way to provide the clinician with the necessary platelet survival information for the management of such patients. ITP antibodies are commonly directed against the two most abundant platelet glycoprotein complexes, GPIIb-IIIa and GPIb-IX. While these antibodies are thought to facilitate platelet clearance



**Figure 15.3** The “platelet feedback” model of megakaryopoiesis. Liver secretion of thrombopoietin (TPO) into the blood is regulated by platelet-hepatocyte binding. Circulating platelets have  $\alpha 2,3$ -linked sialic acid on their surface glycoproteins. Over time in the circulation, platelet desialylation (A) occurs. When aged, desialylated platelets transit the liver (B) where the desialylated platelet glycoproteins bind to their receptor, the hepatocyte Ashwell-Morell receptor (AMR). This ligand-receptor binding event upregulates hepatocyte TPO mRNA levels and increases TPO production. TPO secreted from the liver into the blood (C) can either bind to platelet MPL or remain free in the circulation. Free TPO is then able to transit to the marrow where it is available to stimulate megakaryopoiesis (D); fully sialylated platelets are then released into circulation.

via Fc $\gamma$ R-mediated uptake by splenic macrophages, hepatic uptake is thought to be the predominant mechanism in cases associated with anti-GPIb $\alpha$  antibodies.<sup>158</sup> Mixed splenic and hepatic uptake patterns have also been observed.<sup>184</sup>

Although the marrow can increase megakaryocyte mass by 10-fold, this response is insufficient to maintain a normal platelet count with ITP; the increased RP% confirms the compensatory marrow production response.<sup>185</sup> The RP% is very sensitive to megakaryocyte production, and RP changes may precede changes in platelet counts; in fact, the RP% has been shown to decline about 24 hours before platelet count recovery in ITP<sup>59</sup> and to similarly rebound prior to marrow recovery from chemotherapy-induced hypoplasia.<sup>186</sup> The RP% in both situations will eventually normalize as the platelet count approaches the normal range, indicating that the restoration of a normal platelet lifespan rapidly downregulates MK platelet release.

Automated hematology analyzers have since incorporated methods for the measurement of young platelet subsets utilizing a combination of opening impedance, optical scattering, and fluorescence modalities. Immature platelet fraction (IPF) is one such parameter that correlates decently with reticulated platelet count as defined by flow cytometry in the settings of peripheral platelet destruction and nonimmune thrombocytopenia associated with hypersplenism, though not necessarily in the setting of defective platelet production or steady-state production.<sup>187</sup> IPF% has been shown to predict some etiologies of thrombocytopenia, but not others, e.g., myelodysplastic syndrome and *MYH9*-related congenital thrombocytopenia. Like RPs, the IPF% is sensitive to MK regulation and has been shown to increase in advance of platelet recovery after chemotherapy.<sup>188,189</sup> Higher IPF values may predict the preservation of a normal platelet count in patients with preeclampsia.<sup>189</sup> Prediction of thrombotic risk in thrombocytosis, sepsis development and severity, and outcomes in cardiovascular disease have also been explored with IPF.<sup>189</sup> One challenge that affects the utility of this particular platelet metric is the lack of method standardization among manufacturers, which consequently inhibits studies validating their use.<sup>189,190</sup>

### Platelet sequestration

Approximately two-thirds of human platelets circulate while the remaining are reversibly sequestered, primarily in the spleen.<sup>191</sup> Massive splenomegaly can cause severe thrombocytopenia (counts <50,000/ $\mu$ L) when as much as 90% of total-body platelet mass is sequestered in the spleen.<sup>145,191</sup>

Platelet accumulation in the spleen reaches 90% of maximum activity within 12.5 minutes after injection of labeled platelets.<sup>191</sup> Splenic pooling of platelets is reversible; intravenous epinephrine, which reduces blood flow to the spleen and causes the organ to empty passively into the circulation, will cause the blood platelet count to increase by 30–50%, and epinephrine does not affect platelet count in asplenic individuals.<sup>192</sup>

Splenectomy in humans may or may not affect platelet survival.<sup>193</sup> An animal model has demonstrated a significant (>40%) increase

in platelet lifespan after splenectomy.<sup>194</sup> Patients with moderate splenomegaly, such as seen with cirrhosis and portal hypertension, have platelet counts in the range of 60,000–100,000/ $\mu$ L. Hepatic pooling of platelets probably accounts for 10% of total-body platelets, but this fraction may increase after splenectomy.<sup>143,145</sup>

### Summary

Megakaryopoiesis is a complex biological process encompassing events from megakaryocyte differentiation and maturation to pro-platelet release and terminal platelet biogenesis. A milieu of cytokines, cellular interactions, and biophysical cues drives its progression and regulation on genetic, epigenetic, post-transcriptional, and post-translational levels. The resulting platelets carry out hemostatic functions and have lifespans that are normally dictated by their own varied processes that feed back to influence platelet production; an interconnected cycle of production and destruction ensues. Through the continued study of megakaryopoiesis and platelet kinetics, new insights regarding human physiology, pathophysiology, and improved clinical technologies can and will be elucidated.

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## CHAPTER 16

# Platelet immunology and alloimmunization

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Platelets are critical mediators of hemostasis, and platelet transfusion remains an essential therapy for the treatment and prevention of bleeding. Platelets express a variety of immunogenic proteins, either unique to platelets themselves or shared with other cell types, and alloimmunization to platelet antigens can render patients refractory to transfusion, with important consequences on clinical outcomes.<sup>1,2</sup> Indeed, platelet refractoriness represents a major barrier to efficacious platelet transfusion and is associated with increased risk of bleeding, longer hospital stays, higher inpatient costs, and decreased overall survival.<sup>2–4</sup> This chapter reviews the antigens most relevant to platelet refractoriness, the impact of alloimmunization on transfusion responses, and finally the strategies employed to mitigate or prevent alloimmunization and immune-mediated platelet refractoriness.

### Platelet antigens and alloimmunization

Platelets express a number of antigens relevant to alloimmunization and platelet refractoriness, including human leukocyte antigens (HLA) and human platelet antigens (HPAs) (Figure 16.1). Platelet alloimmunization refers to the de novo development of alloantibodies capable of binding platelet antigens, which may or may not affect the survival of transfused platelets. In addition, in the context of efficacious platelet transfusion, it may be important to consider ABO blood group antigens expressed on platelets as patients may harbor naturally occurring alloantibodies against these antigens. Finally, it is important to note that drug-dependent antibodies can also contribute to platelet refractoriness (discussed below), though the mechanism by which these antibodies are generated is distinct from alloimmunization.

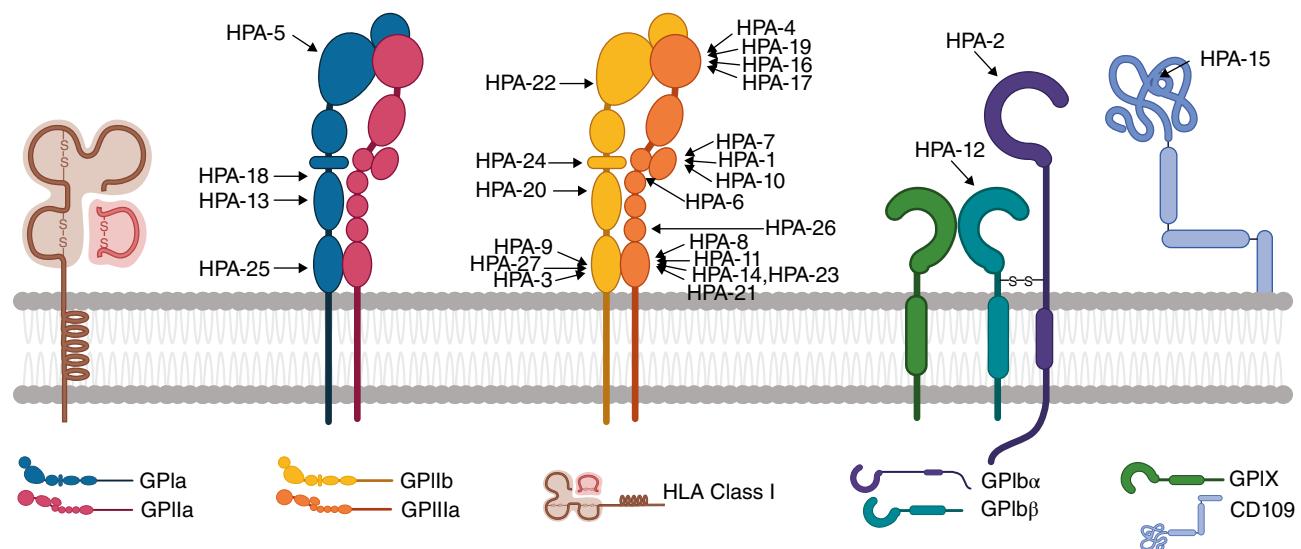
### Human leukocyte antigens

Human leukocyte antigens (HLAs) are highly polymorphic glycoproteins representing the major histocompatibility complexes (MHCs). As of December 2020, over 29,000 HLA allelic variants are reported in the IMGT/HLA database (<https://www.ebi.ac.uk/ipd/imgt/hla/intro.html>), making HLA one of the most significant immunological barriers for the management of patients requiring

life-long transfusion support. The majority of these allelic variants are found within the two major classes of HLA molecules: Class I (HLA-A, HLA-B, and HLA-C) and Class II (HLA-DR, HLA-DP, and HLA-DQ). HLA Class I molecules are expressed on platelets and the vast majority of nucleated cells in the body, whereas only a restricted group of specialized leukocytes involved in antigen presentation (e.g., macrophages, dendritic cells, and B cells) expresses HLA Class II molecules. It is worth noting that platelets from patients with idiopathic autoimmune thrombocytopenic purpura may transiently express HLA-DR.<sup>5,6</sup> Given the broad expression profile of HLA Class I, there is a significant risk of exposure to allogeneic HLA Class I during platelet transfusion, pregnancy, and solid-organ transplantation. HLA alloimmunization in these contexts can result in the formation of an immune response that renders subsequent transfusions ineffective, and patients may be classified as having platelet refractoriness as discussed below.

Platelets express approximately 50,000–120,000 HLA Class I molecules per cell.<sup>7</sup> Although all three types of HLA Class I molecules are detected on platelets, the degree of expression varies. HLA-A and HLA-B are predominantly found on the surface of platelet membranes and are the primary targets for alloantibodies implicated in platelet refractoriness.<sup>8</sup> The level of HLA-B8, HLAB44, HLA-B45 and HLA-B35 has been reported to vary between donors, with some expressing low to undetectable levels.<sup>9–12</sup> HLA-C are also present,<sup>13</sup> but with rare exception,<sup>14</sup> alloantibodies to HLA-C do not appear to significantly impact transfused platelets. Despite having less HLA Class I molecules on their surface compared to leukocytes, platelets represent a major source of circulating HLA Class I given their very large numbers present in the circulation.

HLA alloimmunization may result from pregnancy or prior transfusions, with exposure to leukocyte-containing blood components resulting in high rates of alloantibody formation in patients receiving multiple transfusions. In the context of prior platelet transfusions, passenger donor leukocytes are implicated as a major cause of HLA alloimmunization. Studies in humans show that when leukocyte-reduced platelet concentrates are transfused, primary alloimmunization to HLA is delayed or even absent,<sup>15–17</sup> whereas unmodified platelet concentrates are associated with a rate of HLA alloimmunization ranging from 19% to 71% (Table 16.1).<sup>18–20</sup>



**Figure 16.1** Alloantigens expressed on platelets.

**Table 16.1** Platelet Alloimmunization in Multitransfused Patients Receiving Nonleukocyte-Reduced Blood Components\*

Study	No. of Patients	Anti-HLA†	Anti-PSA‡
Seftel <i>et al.</i> (2004) <sup>21</sup>	315	61/315 (19%)	
TRAP (1997) <sup>20</sup>	131	59/131 (45%)	11/131 (8%)
Atlas <i>et al.</i> (1993) <sup>22</sup>	134	95/134 (71%)	
Meenaghan <i>et al.</i> (1993) <sup>23</sup>	106	37/106 (35%)	45/106 (42%)
Godeau <i>et al.</i> (1992) <sup>24</sup>	50	13/50 (26%)	4/50 (8%)
Pamphilon <i>et al.</i> (1989) <sup>25</sup>	49	20/49 (41%)	11/49 (22%)
Murphy <i>et al.</i> (1987) <sup>26</sup>	154	55/154 (36%)	5/154 (3%)

\* Frequency of HLA alloantibody and platelet-specific alloantibody formation in studies of patients with hematologic and oncologic diagnoses requiring repeated platelet transfusion.

† HLA alloantibodies determined by lymphocytotoxicity testing.

‡ Platelet-specific alloantibodies (PSA) determined by a variety of methods.

The risk of HLA alloimmunization appears to be influenced by several patient and blood component factors. Transfused patients previously exposed to allogeneic HLA via transfusion or pregnancy develop HLA alloantibodies sooner—and, in many studies—more often than patients without a previous exposure.<sup>20,25,27,28</sup> In the Trial to Reduce Alloimmunization to Platelets (TRAP) study,<sup>20</sup> 62% of previously pregnant women with AML receiving untreated blood components (control product) developed alloantibodies compared to 33% of those who had not been pregnant or transfused previously. Furthermore, the underlying disease for which patients require platelet transfusion also appears to influence the rate of HLA alloimmunization. Studies have shown that patients undergoing induction chemotherapy for AML are more likely to become alloimmunized than patients being treated for acute lymphoblastic leukemia (ALL) (44 vs. 18%;  $p < 0.001$ ).<sup>29</sup> Others have corroborated this finding and noted that alloimmunization may occur sooner in patients with AML than in those with ALL.<sup>25,30</sup> In addition, a study of chronically transfused sickle cell disease patients determined that 85% of those with  $\geq 50$  past red cell transfusions had evidence of platelet-reactive alloantibodies, whereas 48% of those with  $< 50$  past exposures were sensitized and no HLA alloantibodies were detected in patients with no past transfusions.<sup>31</sup>

### Human platelet antigens (HPAs)

Human platelet antigens (HPAs) are platelet-specific antigens with single nucleotide polymorphisms (Figure 16.1). Although these amino acid substitutions do not appear to generate functional defects, exposure to these alloantigens during pregnancy or transfusion can result in the formation of alloantibodies and result in (1) fetal and neonatal alloimmune thrombocytopenia (FNAIT),<sup>32</sup> (2) post-transfusion purpura (PTP)<sup>33</sup> or (3) platelet refractoriness.<sup>34</sup> To date, 35 types of HPAs have been serologically identified either in women with infants affected by FNAIT or in patients refractory to platelet transfusion. Characteristics of these HPAs are listed in Table 16.2 (modified from the HPA Database, <https://www.versiti.org/hpa>).<sup>35</sup> The HPAs identified thus far are located on one of the seven glycoproteins, with the vast majority residing on GPIIIa (CD61). While 6 HPA systems are paired with either one or two other alleles, the remaining 29 HPAs do not appear to correspond with pairs of alleles and are designated with a “w” for workshop (e.g., HPA-8bw). Alloantigens that do not correspond with pairs of alleles occur at a low frequency (<1%) and have been discovered in mothers who have given birth to infants affected by FNAIT.<sup>36</sup> Of the multiallelic HPA systems, HPA-1 is the only alloantigen that is a triallelic,<sup>37</sup> while HPA-2, HPA-3, HPA-4, HPA-5, and HPA-15 are biallelic. As HPAs are inherited in a codominant autosomal manner, the most frequent allele is designated with an “a,”<sup>38,39</sup> while “b” identifies the less frequent allele.

HPA frequencies have been best studied in individuals of Caucasian descent, and it is important to note that the frequency varies significantly by racial and ethnic group. For example, approximately 15% of European descendants but <1% of individuals of Asian ancestry express HPA-1b. Alloimmunization to high-frequency platelet-specific antigens can thus present a major challenge in finding compatible platelets to support a patient requiring multiple platelet transfusions. Fortunately, these cases are extremely rare.<sup>40–42</sup> However, knowledge of HPA frequencies among different populations is essential to reduce antigenic differences and thereby diminish the risk of alloimmunization.

It is important to note that the majority of refractory patients with platelet-specific alloantibodies also have HLA alloantibodies.<sup>43</sup> Although platelet-specific antibodies against defined HPAs can

**Table 16.2** Human Platelet Antigens.

System	Alloantigen	Original Name	Frequency*	GP/Substitution	Gene	Nucleotide Change
HPA-1	HPA-1a	Zw <sup>a</sup> , PI <sup>A1</sup>	72% a/a	GPIIIa/L33P	<i>ITGB3</i>	T176C
	HPA-1b	Zw <sup>b</sup> , PI <sup>A2</sup>	26% a/b			
	HPA-1c <sup>37</sup>		2% b/b			
HPA-2	HPA-2a	Ko <sup>b</sup> Ko <sup>a</sup> , Sib <sup>a</sup>	< 1 % a/c	L33V		
	HPA-2b		85% a/a	GPIba/T145M	<i>GPIBA</i>	C175G
			14% a/b			C482T
HPA-3	HPA-3a	Bak <sup>a</sup> , Lek <sup>a</sup> Bak <sup>b</sup>	37% a/a	GPIIb/I843S	<i>ITGA2B</i>	T2621G
	HPA-3b		48% a/b			
			15% b/b			
HPA-4	HPA-4a	Yuk <sup>b</sup> , Pen <sup>a</sup>	>99.9% a/a	GPIIIa/R143Q	<i>ITGB3</i>	G506A
	HPA-4b	Yuk <sup>a</sup> , Pen <sup>b</sup>	<0.1% a/b			
			<0.1% b/b			
HPA-5	HPA-5a (Brb)	Br <sup>b</sup> , Zav <sup>b</sup>	80% a/a	GPIa/E505K	<i>ITGA2</i>	G1600A
	HPA-5b (Bra)	Br <sup>a</sup> , Zav <sup>a</sup> , Hc <sup>a</sup>	19% a/b			
			1% b/b			
	HPA-6bw	Ca <sup>a</sup> , Tu <sup>a</sup>	<1%	GPIIIa/R489Q	<i>ITGB3</i>	G1544A
	HPA-7bw	Mo <sup>a</sup>	<1%	GPIIIa/P407A	<i>ITGB3</i>	C1297G
	HPA-8bw	Sr <sup>a</sup>	<0.1%	GPIIIa/R636C	<i>ITGB3</i>	C1984T
	HPA-9bw	Max <sup>a</sup>	<1%	GPIIb/V837M	<i>ITGA2B</i>	G2602A
	HPA-10bw	La <sup>a</sup>	1%	GPIIIa/R62Q	<i>ITGB3</i>	G263A
	HPA-11bw	Gro <sup>a</sup>	<0.5%	GPIIIa/R633H	<i>ITGB3</i>	G1976A
	HPA-12bw	Iy <sup>a</sup>	1%	GPIb $\beta$ /G15E	<i>GPIBB</i>	G119A
	HPA-13bw	Sit <sup>a</sup>	<1%	GPIa/T799M	<i>ITGA2</i>	C2483T
	HPA-14bw	Oe <sup>a</sup>	1%	GPIIIa/G611del	<i>ITGB3</i>	1909_1911delAAG
	HPA-15a	Gov <sup>b</sup>	35% a/a	CD109/S682Y	<i>CD109</i>	C2108A
	HPA-15b	Gov <sup>a</sup>	42% a/b			
			23% b/b			
	HPA-16bw	Duv <sup>a</sup>	<1%	GPIIIa/T140I	<i>ITGB3</i>	C497T
	HPA-17bw	Va <sup>a</sup>	<1%	GPIIIa/T195M	<i>ITGB3</i>	C662T
	HPA-18bw	Cab <sup>a</sup>	<1%	GPIa/Q716H	<i>ITGA2</i>	G2235T
	HPA-19bw	Sta <sup>a</sup>	<1%	GPIIIa/K137Q	<i>ITGB3</i>	A487C
	HPA-20bw	Kno <sup>a</sup>	<1%	GPIIb/T619M	<i>ITGA2B</i>	C1949T
	HPA-21bw	Nos <sup>a</sup>	<1%	GPIIIa/E628K	<i>ITGB3</i>	G1960A
	HPA-22bw	Sey <sup>a</sup>	<1%	GPIIb/K164T	<i>ITGA2B</i>	A584C
	HPA-23bw	Hug <sup>a</sup>	<1%	GPIIIa/R622W	<i>ITGB3</i>	C1942T
	HPA-24bw	Cab2 <sup>a+</sup>	<1%	GPIIb/S472N	<i>ITGA2B</i>	G1508A
	HPA-25bw	Swi <sup>a</sup>	<1%	GPIa/T1087M	<i>ITGA2</i>	C3347T
	HPA-26bw	Sec <sup>a</sup>	<1%	GPIIIa/K580N	<i>ITGB3</i>	G1818T
	HPA-27bw	Cab <sup>3++</sup>	<1%	GPIIb/L841M	<i>ITGA2B</i>	C2614A
	HPA-28bw	War <sup>a</sup>	<1%	GPIIb/V740L	<i>ITGA2B</i>	G2311T
	HPA-29b	Kha <sup>b</sup>		GPIIIa/T33M	<i>ITGB3</i>	C98T
	HPA-30b	Lap <sup>a</sup>		GPIIb/Q806H	<i>ITGA2B</i>	G2551C
	HPA-31b	Cab4 <sup>b+</sup>	<1%	GPIIX/P123L	<i>GP9</i>	C368T
	HPA-32b	Dom <sup>b</sup>		GPIIIa/N174S	<i>ITGB3</i>	A521G
	HPA-33b	Bl <sup>a</sup>		GPIIIa/D458G	<i>ITGB3</i>	A1373G
	HPA-34b	Bzh <sup>a</sup>		GPIIIa/R91W	<i>ITGB3</i>	C349T
	HPA-35b	Efs <sup>a</sup>		GPIIIa/R479H	<i>ITGB3</i>	A1514G

\* Phenotypic frequencies for each HPA shown are for the Caucasian population. Significant differences in gene frequencies may be found in African and Asian populations. Nucleotide and protein substitutions are demonstrated as changes from the most frequency allele (a) to the least common allele (b).

Source: Modified from Versiti.<sup>35</sup>

result in transfusion failure,<sup>44–46</sup> most platelet reactivity lacking specificity (e.g., nonspecific reactivity) does not seem to influence transfusion response.<sup>20,24,47</sup> Evidence implicating HPA or platelet-specific antibodies in the destruction of transfused platelets comes from the failure of some HLA-matched platelet transfusions given to patients who are refractory to random whole-blood-derived platelets.<sup>15</sup> The poor responses to these platelet transfusions, despite HLA matching, suggests that other antigens may be involved. Assays detecting platelet glycoprotein-specific reactions allow for the identification of platelet-specific non-HLA antibodies. Using one such method, the monoclonal antibody-specific immobilization of platelet antigens (MAIPA) assay, a group documented that among 252 patients with hematologic-oncologic diagnoses receiving platelet transfusions, 20 (8%) developed platelet-specific alloantibodies with clear-cut specificity. The most common specificity in these patients was for HPA-5b in 10 of the 20; followed by HPA-1b

in 4; HPA-5a in 2; and 1 each with HPA-2b, HPA-1a, HPA-1b plus HPA-5b, and HPA-1b plus HPA-2b.<sup>48</sup> This study confirmed earlier findings in the TRAP study where 8% of the 530 patients in the trial formed platelet-specific alloantibodies, the most common being anti-HPA-1b.<sup>20</sup>

### ABO blood group antigens

Platelets express low levels of blood group antigens I,<sup>49</sup> P,<sup>49,50</sup> and ABO.<sup>51</sup> While antibodies against blood group antigens I and P do not appear to impact the survival of transfused platelets, antibodies against ABO can contribute to accelerated platelet clearance<sup>52–54</sup> and are associated with an increased incidence of HLA alloimmunization.<sup>55,56</sup> Notably, platelets derived from individuals with an A<sub>2</sub> blood group subtype do not express detectable A antigens.<sup>57,58</sup> As the ABO blood group antigens are terminal carbohydrate determinants, these antigens are expressed by a variety of glycosphingolipids and

glycoproteins on platelets.<sup>59–63</sup> Of the glycoproteins, GPIb, GPIIb, and GPIIa account for the highest degree of ABO motifs per molecule.<sup>59,64</sup> However, ABO expression levels have been reported to vary on platelets within a given individual.<sup>49,51,58,65,66</sup> While not all groups have found this difference to be significant,<sup>52</sup> to the extent that intra-platelet variability exist, these data may provide insight into why early studies demonstrate a rapid destruction of a subset of transfused ABO-incompatible platelets, followed by near to normal survival of the remaining cells.<sup>53,67</sup>

Platelet destruction in ABO-incompatible platelet transfusions is thought to result from recipient anti-A or anti-B IgM and IgG. These naturally occurring isoagglutinins can interact with A and B motifs on transfused platelets, resulting in their destruction. Explanations offered for the aforementioned biphasic survival curves of ABO-incompatible platelets include (1) the elution of a portion of group A motifs from the platelet surface; (2) the nonhomogeneous distribution of group A motifs on donor platelets, with resultant rapid destruction of the subpopulation with the highest expression; and (3) secondary injury to a subset of transfused platelets caused by the reaction between anti-A isoagglutinins and A-positive red cells in the platelet concentrate.<sup>53,58</sup>

Interindividual variability in the level of blood group antigen A and B expression on platelets has also been reported in healthy individuals.<sup>52,58,66,68</sup> In particular, a study found that the level of group A on platelets from individuals with an A<sub>1</sub> blood group subtype can range from 2100 to 16,000 molecules per platelet.<sup>58</sup> This was attributed to the roughly 7% of group A individuals that demonstrate strong expression of blood group antigen A.<sup>58</sup> Similarly, 4–7% of group B donors present with up to 20 times greater group B expression than normal.<sup>51,58</sup> Healthy donors with platelets expressing these unexpectedly high levels of blood group antigens A and/or B are described as having a “high expresser phenotype” trait, termed *type II high expressers*.<sup>58</sup> It is worth noting that in addition to high and normal expressers, there is an additional group of individuals that illustrate a similar pattern of blood group antigen A expression on platelets from normal expressers but have an overall higher level of expression (termed *type I high expressers*). Although the high expresser trait was first identified in Japanese blood donors,<sup>51</sup> recent studies have observed a similar phenotype in other ethnic groups (e.g., Caucasians).<sup>58,66</sup> Despite the prevalence of “high expresser phenotype,” the mechanism(s) driving increased blood group antigen A and/or B expression on platelets remains poorly defined. However, the “high expresser phenotype” has been reported to correlate with increased glycosyltransferase activity and was found to be inherited as an autosomal dominant trait,<sup>51,58</sup> suggesting that inherited factors may contribute to its etiology. Nevertheless, the “high expresser phenotype” trait is of significant clinical relevance as the transfusion of high expresser platelets into ABO-incompatible recipients has been implicated in FNAIT<sup>69</sup> and platelet transfusion refractoriness.<sup>51</sup>

Several studies have documented the impact of ABO incompatibility on platelet recovery. One study demonstrated a reduced recovery of 23% in ABO-incompatible donor-recipient pairs, while another reported an increased frequency of refractoriness in patients receiving ABO-unmatched platelet transfusions (69% vs. 58%;  $p = 0.001$ ).<sup>56,70</sup> In addition, others have shown that as many as 20% of group O patients develop severe refractoriness to group A platelets.<sup>51,57,71</sup> Another study examined the impact of ABO in transfusion responses by randomly assigning forty patients with hematologic diseases to receive either ABO-identical or ABO-unmatched platelets.<sup>72</sup> The responses in the group of patients receiving

ABO-unmatched transfusions (i.e., when either the recipient would be expected to have isoagglutinins to the antigens on transfused platelets or the donor had such antibodies directed at the recipient's blood type) were significantly worse than those observed in patients receiving ABO-identical platelet transfusions. Analysis of the first 25 transfusions in each group showed a significantly better response in the ABO-identical arm (mean corrected count increment [CCI], 6600 vs. 5200;  $p < 0.01$ ). This effect was most important in the first 10 transfusion episodes and tended to predict subsequent alloimmunization and refractoriness to platelet transfusions.

It is important to note that adverse effects of ABO-incompatible platelet transfusion extend beyond platelet alloimmunization and refractoriness. For example, a retrospective cohort study of cardiac patients significantly demonstrated more red cell transfusions, longer hospital stays, more days of fever, and increased healthcare costs in patients who received ABO-mismatched platelets compared to those who received ABO-identical platelets.<sup>73</sup> Indeed, a later study failed to identify negative effects of ABO-mismatched platelet transfusions in a larger cohort of cardiac patients;<sup>74</sup> nevertheless, there remains the possibility that ABO-mismatched platelet transfusions may be associated with increased inflammation. Finally, ABO plasma-incompatible transfusions, such as the transfusion of products from group O donors into non-group O recipients, carry a risk of acute hemolytic transfusion reactions caused by high titer isoagglutinins. This risk has increased with the shift from pooled whole-blood-derived platelet concentrates to apheresis platelets as some donors have potent ABO isoagglutinins; therefore, many blood product providers have taken steps to alleviate this risk by reducing the volume of incompatible plasma or screening group O donors for high-titer isoagglutinins.<sup>75</sup>

## Transfusion refractoriness

*Alloimmunization* is an immune response in a recipient stimulated by foreign donor antigens. In the setting of platelet transfusion, these responses involve the production of alloantibodies against donor platelets. *Platelet refractoriness* describes the clinical condition in which patients do not achieve the anticipated platelet count increment following platelet transfusion. It is possible to be alloimmunized to platelet antigens without being refractory to platelet transfusions and also to be refractory to platelet transfusions without being alloimmunized. *Alloimmune or antibody-mediated platelet refractoriness* occurs when the level of alloimmunization, as measured by the breadth of antibody response to platelet antigens, is sufficient to affect the majority of randomly selected platelet products.

The detection of alloimmunization is performed using laboratory techniques to detect antibodies in the patient's serum that are reactive with allogeneic HLA or platelet-specific antigens. In contrast, the definition of the refractory state is less precise.<sup>8,76</sup> A standard dose of platelets (six units of pooled whole-blood-derived platelet concentrates or one apheresis platelet unit) generally results in a post-transfusion increment of about 30,000–40,000 platelets/ $\mu$ L one hour after platelet transfusion.<sup>76</sup> The TRAP study, mentioned previously, defined the refractory state as a corrected count increment (CCI; Table 16.3), which normalizes transfusion responses for the patient's blood volume estimated using body surface area and platelet dose, of <5000 after two sequential ABO-compatible platelet transfusions.<sup>20</sup> Clinically, some institutions use a CCI cutoff of <7500 to define platelet refractoriness.<sup>34,77</sup> The age of the transfused platelet unit should also be considered when

assessing CCI, which is expected to increase more when using platelets stored for less than 48 hours compared to longer storage times.<sup>78</sup> Post-transfusion platelet counts should be measured 10 minutes to 1 hour after completion of the platelet unit. Low CCI values observed at 1 hour are indicative of immune-mediated clearance, while low CCI observed at 24 hours following acceptable CCI values at 1 hour is more indicative of nonimmune-mediated platelet clearance,<sup>77,79,80</sup> although recent data do suggest a potential role for antibody-independent immune mediated refractoriness involving recipient T cells.<sup>81</sup> An additional measure of platelet transfusion response is the percent platelet recovery (PPR; Table 16.3). Similar to the CCI, the PPR uses platelet dose and patient blood volume; the latter is estimated using the patient's body weight in kilograms. Studies in normal autologous platelet donors show an average one-hour PPR of approximately 66%.<sup>77</sup> Recovery less than 20–30% at one-hour post-transfusion indicates a refractory response.<sup>82</sup>

It is important to recognize that refractoriness does not necessarily imply alloimmunization. Indeed, only about 30% of refractory responses to platelet transfusion are attributable to alloimmunization. The remainder are due to nonimmune factors that result in shortened platelet survival and/or markedly decreased platelet recoveries in patients who receive transfusions.<sup>77,83,84</sup> Table 16.4 provides a list of nonimmune causes of poor response to platelet transfusion that should be considered when evaluating a patient for

**Table 16.3** Calculations Determining Platelet Count Increment in the Evaluation of Platelet Refractory Patients

Methods to Calculate Response to Platelet Transfusion	Equation
Corrected count increment (CCI)	$= (\Delta \text{ platelet count} \times \text{BSA}) / \text{platelet count of unit(s)}$
Percentage platelet recovery (PPR)	$= (\Delta \text{ platelet count} \times \text{TBV}) / \text{platelet count of unit(s)}$

$\Delta$  platelet count = post-transfusion platelet count – pretransfusion platelet count;  
BSA: body surface area; TBV: total blood volume; platelet count of unit(s): generally  $3 \times 10^{11}$  is used per unit unless the exact count of unit is available.

**Table 16.4** Nonimmune Etiologies of Platelet Refractoriness

Splenic sequestration
Bleeding
Increased consumption
• Disseminated intravascular coagulopathy
• Microangiopathic hemolytic anemia
Fever
Infection
Sepsis
Graft-versus-host disease
Chemotherapy
Total body irradiation
Liver disease/injury
Drugs
• Vancomycin
• Amphotericin B
• Metronidazole
• Ranitidine
• Interferon
• Antithymocyte globulin
• Ibuprofen
Transfused platelet characteristics
• Storage age (e.g., 4–5-day-old platelet)
• Low platelet count

platelet refractoriness. Indeed, prior to performing time-consuming and resource-rich antibody and molecular typing tests, nonimmune causes of platelet refractoriness should be ruled out. Individual patients may have significantly different responses to the same nonimmune causes of refractoriness, with some patients experiencing minimal impact and others having a markedly impaired response to platelet transfusions. Some patients, particularly those with multiple clinical complications (e.g., sepsis and fever), appear to respond particularly poorly to platelets approaching the end of the recommended storage interval (five days). Such patients may experience an improvement in their platelet response after receiving “fresh platelets”—platelets collected less than 48 hours earlier.<sup>76,85</sup>

### Treatment of the alloimmunized platelet-refractory patient

In the modern era, wherein universal leukocyte reduction of blood products for patients receiving multiple platelet transfusions has become standard practice, the rate of alloimmunization (primarily to HLA class I antigens) remains about 20%,<sup>20,84</sup> with the rate of refractoriness due to alloimmunization being about half of that. As such, not all patients who develop HLA alloantibodies will become platelet refractory.<sup>20</sup> The refractoriness, if related to HLA alloimmunization, can be transient or persistent. Generally, only sensitization to HLA-A and HLA-B is considered clinically significant as expression of HLA-C is low; however, sensitization to HLA-C has been implicated in some reports of platelet refractoriness.<sup>14</sup> Several approaches can be considered to provide such patients adequate platelet support: (1) HLA-matched platelets, (2) antigen specificity prediction (ASP, i.e., antigen negative platelets), (3) and cross-matched platelets.

As the majority of immune-mediated platelet refractoriness is due to HLA alloantibodies, the remainder of this section focuses on HLA alloimmunization-mediated platelet refractoriness with references to platelet-specific alloantibodies as appropriate. Clinically, it is also important to consider ABO antibodies, as discussed previously, which can lead to increased platelet clearance in the setting of ABO-incompatible platelet transfusion. A number of studies have demonstrated superior CCI with ABO-identical platelet transfusions compared to ABO-nonidentical transfusions.<sup>86</sup> Another immune-mediated cause of platelet refractoriness is drug-induced antibody, which are hypothesized to develop from drug and platelet surface glycoprotein interactions.<sup>34</sup> Implicated drugs include ceftriaxone, sulfamethoxazole/trimethoprim, quinine, piperacillin, and carbamazepine.

### HLA-matched platelet transfusions

Given that the primary cause of immune refractoriness to platelet transfusion is alloimmunization to HLA Class I, it is reasonable that the avoidance of incompatible HLA specificities should result in a more successful platelet transfusion response. This was first demonstrated in a study that showed that patients who were refractory to platelets from unselected donors could be successfully supported by the transfusion of platelet concentrates from an HLA-matched family member.<sup>87</sup>

Certain “private” HLA can be segregated into so-called cross-reactive groups (CREGs), defined by antibodies directed against shared “public” determinants.<sup>88</sup> These shared determinants are the basis for crossreactivity and are different from private determinants, which account for the highly polymorphic HLA system. Selection of platelet donors with antigens in the same CREGs as

the antigens in the patient, so-called crossreactive antigens, was demonstrated to be nearly as successful in supporting alloimmune platelet refractoriness in patients as that of HLA-identical transfusions.<sup>89</sup> This appeared to be due to the relative inability of the patient's immune system to recognize these crossreactive antigens as different, thereby greatly increasing the number of potentially compatible platelet donors in a given pool.<sup>90</sup> However, it is important to bear in mind that a patient can make antibody to more private determinants within a CREG group. Hence, just because a patient receives a crossreactive antigen platelet does not equate to compatibility, especially in highly sensitized patients.

In alloimmune refractory patients, the best increases in CCI occur with the subset of grade A and B1U or B2U HLA-matched platelets, but platelets mismatched for some antigens (e.g., B44 and B45) that are poorly expressed on platelets can also be successful.<sup>9</sup> Although alloimmunized patients with high percent reactive (HLA) antibody (PRA) values benefit only from platelet products that lack any incompatible HLA Class I (match grades A, B1U, B1X, B2U, and B2UX; Table 16.5), patients with lesser degrees of sensitization can sometimes benefit from less well-matched platelets.<sup>91,92</sup> However, in cases in which patients are highly sensitized (e.g., PRA > 90%), HLA-matched platelets may be a patient's best and only option.

Disadvantages of HLA-matched platelets include the need to HLA type patients and prospective donors. HLA typing is both time intensive and expensive. See Chapter 54 for details on HLA molecular typing methods. Depending on the method employed

and location of testing (in-house vs. reference laboratory), HLA typing can take anywhere from 24 to 72 hours. As such, providing HLA-matched platelets may delay the provision of compatible platelets. However, given that many platelet refractory patients are those who are being evaluated for or have already undergone hematopoietic stem cell transplantation, they may have already been HLA typed. In such cases, HLA matching would only entail an inventory search of HLA-typed donors. That being said, in order to find sufficient HLA-compatible matches to support a typical patient, it is estimated that a pool of 1000–3000 or more HLA-typed potential apheresis donors is generally needed.<sup>93</sup> Moreover, donor selection on the basis of HLA type can lead to the exclusion of donors whose HLA types, although different from that of the recipient, may still be effective. HLA-matched platelets can also be exceedingly difficult to procure for patients with rarer HLA haplotypes. In these cases, searching among family members for HLA identical/similar individuals may help identify appropriate donors.

Epitope-based matching, which is a current research focus in the HLA and solid organ transplant communities, has made its way into platelet matching. Epitopes can be short linear amino acid sequences or discontinuous amino acids that are spatially brought in proximity by the tertiary folding of the protein.<sup>94</sup> HLA molecules are composed of numerous epitopes, which can themselves be shared amongst numerous HLA molecules. Consequently, patients can be matched based on epitope profiles in lieu of antigen matching; and software has been developed to assist in epitope matching using recipient and donor HLA typing.<sup>95</sup> Epitope matching is well represented in solid organ transplantation literature. More recently, it has been employed in platelet matching. Studies have demonstrated that HLA epitope-based matching results in similar CCI increases when compared to traditional HLA matching.<sup>96,97</sup>

**Table 16.5** Classification of Donor/Recipient Pairs on the Basis of HLA Match

Match Grade	Definition	Example	
		Recipient	Donor
A	All four antigens* in donor are identical to those in recipient.	A*02, A*29 B*08, B*44	A*02, A*29 B*08, B*44
B1U**	Only three antigens are detected in donor; all are present and identical in recipient.	A*02, A*29 B*08, B*44	A*02, A*29 B*08, XX
B1X	Three donor antigens are identical to recipient; the fourth antigen is crossreactive with recipient.	A*02, A*29 B*08, B*44	A*02, A*29 B*08, B*45
B2U	Only two antigens are detected in donor; both are present and identical in recipient.	A*02, A*29 B*08, B*44	A*02, XX B*08, XX
B2UX	Only three antigens are detected in donor; two are identical with recipient, and the third is crossreactive.	A*02, A*29 B*08, B*44	A*02, A*30 B*08, XX
B2X	Two donor antigens are identical to recipient; the third and fourth antigens are crossreactive with recipient.	A*02, A*29 B*08, B*44	A*02, A*30 B*08, B*45
C	One antigen of donor is not present in recipient and non-crossreactive with recipient.	A*02, A*29 B*08, B*44	A*02, A*29 B*08, B*78
D	Two antigens of donor are not present in recipient and non-crossreactive with recipient.	A*02, A*29 B*08, B*44	A*02, A*29 B*7, B*78

\* Four antigens refer to both alleles at the HLA-A and HLA-B loci.

\*\* U: unknown/blank and is generally interpreted as homozygosity (designated XX) at one or both loci. However, the classification system was created when serology typing techniques were employed, which were not sensitive enough to detect all antigens, especially those within a CREG. As such, the assay might not identify antigens that were present, and thus the U designation.

NOTE: This classification scheme is included more for historical purposes and because some centers still use this terminology. However, HLA molecular typing has superseded the utility of matching grading.

### Antibody specificity prediction (ASP)

Despite best efforts, HLA-matched platelets are frequently unavailable for many refractory patients either due to rarity of recipient HLA type and/or insufficient pool of HLA-typed donors. In such cases, the use of the patient's HLA antibody specificity for the selection of platelet products has been demonstrated to widen the pool of compatible donors. Antibody specificity prediction (ASP) allows the procurement of platelet products from donors who lack HLA to which the patient has antibody. This approach is akin to supplying appropriate red blood cell (RBC) units to patients with blood group antibodies. One study compared ASP platelets to those selected by standard HLA matching criteria and by platelet crossmatching. All three methods were found to have similar platelet recoveries; however, the number of potential donors for each group was vastly different. For 29 alloimmunized patients, the mean number of potential donors found among 7247 HLA-typed donors was 6 for grade A HLA matches, 39 for BU matches, but 1426 (20% of total) for the ASP method. Hence, careful HLA antibody specificity identification could greatly enhance the number of potential donors by identifying nonmatched products that lack the corresponding HLA antigens.<sup>98</sup>

Current sensitive techniques for detecting and identifying HLA Class I alloantibodies using flow cytometry or Luminex (see Chapter 54 for HLA specificity testing methods) provide a precise way of determining the relative strength of multiple HLA alloantibodies that may be present in patient sera.<sup>99–101</sup> Though these test allow for the identification of HLA alloantibody specificities, their sensitivity can also detect low levels of alloantibodies that may not

induce platelet destruction but could limit the donor pool if they are considered in donor selection. Running antibody specificity assays in parallel with platelet crossmatches can help determine levels of HLA alloantibodies that correspond to positive crossmatches.<sup>102</sup> Such correlation studies may help set thresholds for risk stratifying HLA alloantibody specificities. Also, HLA alloantibody testing should be obtained periodically (e.g., weekly and monthly), given that the ASP method may lead to additional sensitization while a patient is transfused using platelet concentrates from nonmatched HLA donors.

In a small number of patients who fail to respond to HLA-matched or ASP-selected platelets and for whom no other nonimmune explanation can be found for refractoriness, platelet-specific alloantibodies may be responsible. Though there are many platelet-specific antigens (see Table 16.2), only a few, as discussed above, are commonly associated with platelet refractoriness.<sup>103</sup> Approximately 8% of multitransfused patients develop platelet-specific alloantibodies.<sup>20</sup> Many of these patients are also alloimmunized against HLA.<sup>104</sup> Patients with platelet specific alloantibodies can be successfully supported with platelets that are HPA typed and matched or selected by platelet crossmatching.

### Platelet crossmatching

Platelet crossmatching provides yet another modality to supply immune refractory patients with compatible platelet products. Many platelet and leukocyte antibody detection methods have been assessed as platelet crossmatch compatibility tests. One method that has gained wide acceptance is the commercially available solid-phase red cell adherence (SPRCA) assay. In this assay, patient serum is added to microwell plates with wells that have been coated in platelets from donor units.<sup>105</sup> Indicator RBCs coated in antihuman globulin are then added. In the presence of recipient antibodies, the indicator cells will adhere to the side of the wells; whereas in the absence of recipient antibody, indicator cells will aggregate at the bottom of the well. The test is rapid and sensitive, particularly for the detection of HLA alloantibodies, and is therefore suitable for routine platelet crossmatching.<sup>106,107</sup> Good correlation between test results and post-transfusion platelet counts has been achieved with the SPRCA assay.<sup>107,108</sup> Other methods for platelet crossmatching include a modified capture ELISA (MACE) and flow cytometry.

Platelet crossmatching has been shown to be efficacious in increasing the platelet count in immune refractory patients.<sup>109,110</sup> When compared to HLA-matched and ASP-selected platelets, crossmatch platelets also demonstrate comparable clinical efficacy.<sup>109,111,112</sup> However, some studies suggest that HLA selected platelets may be more long lived in vivo than crossmatched platelets.<sup>109</sup> Moreover, platelet crossmatching permits large numbers of platelet concentrates to be routinely tested and identification of compatible pools of platelets for refractory patients. The speed at which crossmatching can be performed can shorten the time to identify compatible platelets for immune refractory patients. One caveat is the delay incurred by institutions that are reliant on blood centers to perform platelet crossmatches. In addition to quick turnaround time, crossmatching offers the benefit of identifying compatibility in the face of platelet specific alloantibodies to platelet glycoproteins. If a patient is not responding to HLA-matched or ASP-selected platelets and the patient is found to have platelet-specific alloantibodies, crossmatching serves as an alternative to HPA-matched platelets. However, there are some drawbacks to platelet crossmatching. The degree to which a patient is

alloimmunized has an impact on the success of platelet transfusions selected by crossmatching assays. For patients who are highly HLA sensitized, numerous platelets may have to be crossmatched before finding a single compatible platelet. Indeed, if a patient has a PRA of 99%, all platelet crossmatches may yield incompatible results. In these cases, HLA-matched or ASP-selected platelets may be better alternatives. Another disadvantage of platelet crossmatching is the need to perform the assay every time transfusion is required.<sup>113</sup>

### Platelet refractory evaluation

The considerable effort and expense entailed in their procurement, HLA- or crossmatch-selected platelet products should be reserved for those refractory patients who (1) have had nonimmune refractory etiologies ruled out and (2) have definite evidence of alloimmunization by either HLA or platelet alloantibody testing. For those unfamiliar HLA alloantibody and antigen testing, selecting the appropriate tests at the right time opens up the possibilities of inappropriate test utilization and test misinterpretation. Complicating the evaluation is the possibility of platelet specific antibodies. Although the incidence of platelet-specific alloantibodies causing patients to be refractory to most or all attempted platelet transfusions is small, this possibility should be investigated when most of the attempted crossmatches are positive or when HLA-matched transfusions fail. If platelet-specific alloantibodies are present, donors of known platelet antigen phenotype or family members, who may be more likely to share the patient's phenotype, should be tested. In the event that testing fails to document alloimmunity (i.e., no HLA- or platelet-specific alloantibodies are identified), then support with randomly selected or ABO compatible platelet products should resume.

Given the complicated clinical workup, selecting the most appropriate method of platelet selection (HLA matching vs. ASP vs. crossmatching) can be difficult. The method selected should be made by weighing the pros and cons of each method (Table 16.6) in the context of the clinical picture and laboratory test results. To assist in both the workup of platelet refractoriness and product selection method, many have proposed diagnostic and management algorithms (Figure 16.2).<sup>102,113,114</sup> Employing diagnostic management team models, in which laboratorians assist in proper test utilization and interpretation, some have developed full platelet refractory consults, which have taken the onus off the clinical providers and can provide the appropriate products in timely fashion.<sup>115</sup> However, it is worth noting that despite the effort and expense involved in procuring compatible platelets, a positive impact on morbidity and mortality related to bleeding has not been demonstrated by using methods such as platelet crossmatching.<sup>116</sup> Furthermore, procuring serologically compatible platelets does not guarantee increased platelet count increments as there may be nonimmune mediated factors contributing to poor response. Indeed, up to 20% of refractory cases have been reported to have immune and nonimmune etiologies contributing to the overall clinical picture.<sup>117</sup>

### Overcoming established alloimmunization

Strategies attempting to reverse established alloimmunity to HLA have been met with limited success to date. Attempted methods include (1) methods to neutralize recipient alloantibody by absorption or removal, (2) temporal blockade of immune mediated

**Table 16.6** Advantages and Disadvantages of the Different Methods for Compatible Platelet Allocation

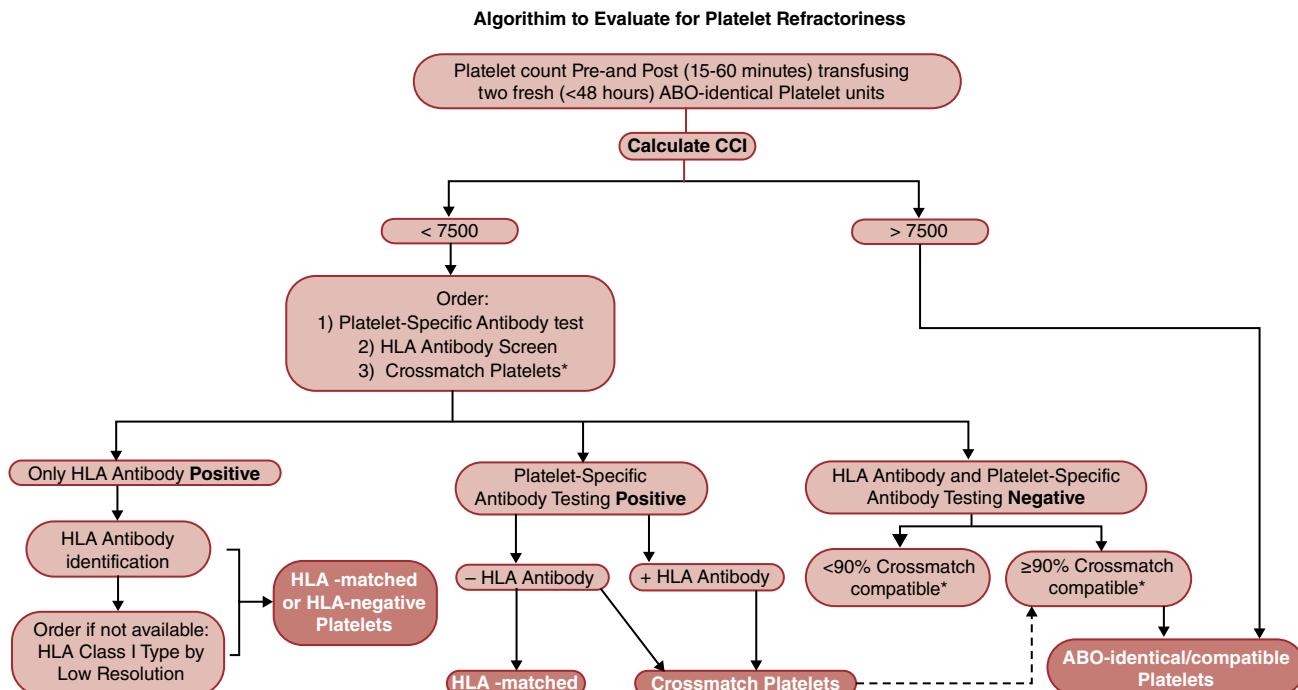
	Advantages	Disadvantages
HLA-matched	<ul style="list-style-type: none"> <li>• Useful for highly sensitized patients</li> <li>• Limits further sensitization</li> </ul>	<ul style="list-style-type: none"> <li>• Requires donor and recipient HLA typing</li> <li>• Restricts donor pool</li> <li>• Requires recruitment of HLA-typed donors</li> <li>• Does not help when platelet-specific alloantibodies are responsible for refractoriness</li> </ul>
ASP	<ul style="list-style-type: none"> <li>• Widens donor pool</li> <li>• Does not require recipient HLA typing</li> </ul>	<ul style="list-style-type: none"> <li>• Does not help when platelet-specific alloantibodies are responsible for refractoriness</li> <li>• Requires donor HLA typing</li> <li>• Requires repeat HLA antibody specificity testing to monitor HLA alloantibody profile and ensure continued provision of HLA compatible platelets</li> <li>• Risk of further sensitization to unmatched antigens</li> </ul>
Crossmatched	<ul style="list-style-type: none"> <li>• Rapid turnaround time</li> <li>• Does not require recipient or donor HLA typing</li> <li>• Detects HLA and platelet-specific alloantibodies</li> <li>• Suitable for patients who are minimally to moderately sensitized</li> </ul>	<ul style="list-style-type: none"> <li>• Not ideal for highly sensitized patients</li> <li>• Requires frequent crossmatching each time patient needs platelet transfusion</li> <li>• Risk of further sensitization to unmatched antigens</li> </ul>

Table modified from Forest and Hod.<sup>118</sup>

destruction of platelets, (3) suppression of the immune response to decrease the production of relevant alloantibodies, and (4) provision of modified platelets or alternative (nonplatelet) hemostatic compounds.

In thrombocytopenic immune refractory patients who are bleeding or who are at risk of bleeding, there is a theory that alloantibodies can be absorbed by large platelet dose transfusions. Some experts explain that they implement such strategies by repeat transfusions of platelets expressing antigen(s) to which the recipient has alloantibodies.<sup>34</sup> After antibody depletion, platelets expressing the same antigen(s) as those in the “in vivo adsorption” will hopefully yield better increment count responses as the corresponding alloantibody should no longer be present. Though similar anecdotal protocols exist, this method has not been heavily studied and/or published.<sup>119</sup>

Several studies investigated the utility of high-dose intravenous immunoglobulin (IVIG) to increase the platelet count in association with transfusion in refractory patients. The results of these studies have been highly variable.<sup>120–122</sup> To date the literature does not appear to support the use of IVIG in lieu of HLA-matched platelet transfusions.<sup>121</sup> Hence, IVIG is not commonly utilized in the treatment of immune refractory patients. Rh immune globulin (RhIG) infusions have also been studied in preventing refractory responses; however, patients treated with weekly intravenous RhIG infusions have similar rates of refractoriness to those treated with placebo, indicating that this form of reticuloendothelial system blockade is not helpful in preventing refractory responses.<sup>123</sup> Other strategies evaluated to overcome the platelet refractory state include the use of vinblastine-loaded platelet transfusions,<sup>124</sup> treatment with cyclosporin A,<sup>125</sup> immunoabsorption using staphylococcal protein



\* Crossmatch platelets may be ordered concurrently to the platelet-specific antibody test and HLA antibody screen if clinical need warrants quick provision of compatible platelets, or could be ordered depending on the results of the platelet-specific antibody test and HLA antibody screen if time is not a limiting factor.

**Figure 16.2** Algorithm to evaluate for platelet refractoriness.

A columns,<sup>126,127</sup> and plasmapheresis. A more recent report describes the coincident improvement in platelet response in a patient treated with bortezomib for multiple myeloma.<sup>128</sup> In addition, eculizumab, a complement inhibitor, has also been evaluated and indicated a potential to overcome platelet refractoriness in some patients.<sup>129</sup> Each strategy has had some limited success, but none can be recommended for routine use in this situation.

One approach that has been developed involves the transfusion of platelets that have undergone acid treatment, which modifies HLA Class I, making them less recognizable to the immune system.<sup>130,131</sup> Several potential platelet substitutes are also being investigated including lyophilized platelets, infusible platelet membranes, thromboerythrocytes, and thrombospheres. Whether any of these methods will have clinical relevancy remains uncertain.<sup>132,133</sup> The role of platelet growth factors such as interleukin-11 (IL-11)<sup>134</sup> and recombinant human thrombopoietin has also been studied, and shown to not only shorten the duration but also blunt the severity of thrombocytopenia after chemotherapy; however, toxicity and the development of thrombopoietin-neutralizing antibodies render these agents less effective.<sup>135</sup> These drugs may play more of a role in preventing rather than managing platelet refractoriness because responsive patients would require fewer platelet transfusions. There have been only limited studies of the newer thrombopoietin mimetic agents romiplostim or eltrombopag for refractory patients.<sup>136</sup> Finally, studies suggest an adjunct role of antifibrinolytics and recombinant coagulation factors in the prevention and management of bleeding in platelet refractory patients.<sup>137,138</sup>

### **Prevention of alloimmunization**

Supporting effective hemostasis in patients with immune-mediated platelet refractoriness is challenging once alloimmunization has occurred. Strategies to overcome immune mediated refractoriness are expensive and primarily consist of additional matched or cross-matched transfusions.<sup>2</sup> Thus, as a more practical modality to ensure continued successful platelet transfusion support, attention has turned to developing approaches to prevent alloimmunization from occurring.

One method to decrease the risk of forming an immune response to alloantigens on transfused platelet concentrates is the use of apheresis platelet units. Apheresis allows for the collection of platelet units that are from a single donor rather than a pool of whole blood derived platelet concentrates that typically consist of 5–8 different donors.<sup>139</sup> Thus, the major advantage of using apheresis platelet concentrates is that it limits antigenic exposure within a given transfusion as well as overtime. Both animal studies and clinical trials have shown that alloimmunization to HLA Class I molecules can be delayed, if not reduced by the provision of apheresis platelet units. Accordingly, the majority of platelet transfusions in the United States are now collected from apheresis.<sup>140</sup>

As an early murine study demonstrated that the removal of leukocytes from platelet concentrates could eliminate alloimmunity,<sup>141</sup> it is hypothesized that residual leukocytes are the immunogen responsible for alloimmunization to HLA following platelet transfusion. Due to this, strategies to reduce the number of residual leukocytes in platelet concentrates have been developed. Indeed, differential centrifugation of blood products reduces leukocyte numbers by separating distinct cellular populations. However, it does not sufficiently reduce alloimmunization to HLA following platelet transfusions. As a result, the removal of leukocytes is most often accomplished by passing blood components through third-generation leukocyte reduction filters that use a combination of adhesion and pore size to remove leukocytes. These filters result

in a 3–4 log reduction of leukocytes in platelet concentrates, meeting the US Food and Drug Administration's requirement of  $<5 \times 10^6$  total leukocytes per platelet concentrate. While these filters can be used at the bedside, the preferred method is to leukocyte reduce platelet concentrates prior to storage. Doing this allows for platelet concentrates to be generated according to good manufacturing practice regulations with well-defined quality control. Moreover, leukocyte reduction of platelet concentrates prior to storage prevents the formation of leukocyte fragments that can pass through filters and consequently sensitize recipients.<sup>142</sup> Leukocyte reduction of platelet concentrates can be accomplished through the use of a leukocyte reduction filter prior to storage.

The majority of clinical trials to date support the use of leukocyte reduction to decrease the risk of forming an alloimmune response to HLA in blood products. In 1998, 18 clinical trials (some randomized controlled and some nonrandomized) using leukocyte-reduced RBCs and platelet concentrates to prevent alloimmunization were reviewed.<sup>143</sup> Fourteen of these trials demonstrated a positive trend, if not a statistically significant reduction in alloimmunization to HLA and platelet refractoriness following the transfusion of leukocyte-reduced platelet concentrates, while three other trials<sup>27,144,145</sup> illustrated no statistically significant benefit from leukocyte reduction. However, a subsequent meta-analysis of eight randomized controlled trials demonstrated a clear protective effect of leukocyte reduction in decreasing the risk of alloimmunization to HLA and platelet refractoriness.<sup>146</sup>

The most definitive and landmark clinical trial demonstrating the importance of leukocyte reduction in decreasing the incidence of alloimmunization to HLA and refractoriness to platelet transfusion was the TRAP study.<sup>20</sup> This multi-institutional, randomized clinical trial compared de novo formation of alloantibodies and refractoriness in AML patients transfused with (1) unmodified, pooled platelet concentrates from random donors (control), (2) leukocyte-reduced pooled platelet concentrates from random donors, (3) ultraviolet B (UVB) irradiated pooled platelet concentrates from random donors, and (4) filtered platelet concentrates obtained by apheresis from single random donors. Of the 530 patients with no detectable alloantibodies at baseline, lymphocytotoxic alloantibodies were observed in approximately 45% of the control group compared with 17–21% of treated groups ( $p \leq 0.0001$  for each treated group compared with control). Moreover, roughly 13% of patients in the control group developed refractoriness in association with alloimmunization to HLA compared to only 3–5% of those in the treated groups ( $p \leq 0.03$  for each treated group compared to control). No statistical difference in alloantibody formation or platelet refractoriness was observed among the treated groups.

A second large, single institute study that found leukocyte reduction of platelet concentrates to be effective in reducing the rate of alloimmunization and refractoriness to subsequent platelet transfusions was a retrospective study from Canada, where universal leukocyte reduction of blood components was instituted between 1997 and 1999.<sup>21</sup> In this study, the rate of alloimmunization and refractoriness before and after the introduction of universal leukocyte reduction was examined in patients with acute leukemia or those undergoing stem cell transplantation. Patients transfused with universal leukocyte-reduced platelet concentrates were found to demonstrate a significantly decreased rate of alloimmunization to HLA, immune-mediated refractoriness, and a need for HLA-matched platelets. Indeed, the trigger for platelet transfusions was changed during the period of this study from 20,000 to 10,000 platelets/ $\mu\text{L}$ , resulting in considerably lower number of platelet transfusions for patients administered universal

leukocyte-reduced platelet transfusions. However, the trial found that leukocyte-reduced transfusions were a significant independent variable driving the lower rates of alloimmunization and refractoriness in these patient populations.

An alternative strategy that has been explored in parallel with leukocyte reduction technology to prevent alloimmunization following platelet transfusion is to treat platelet concentrates with ultraviolet irradiation.<sup>147</sup> Ultraviolet irradiation inactivates HLA Class I and II bearing cells (e.g., antigen presenting cells), thereby preventing the activation of recipient T cells even upon recognition of cognate HLA molecules. With the inability to activate, donor antigen presenting cells cannot upregulate costimulatory molecules and secrete cytokines, both of which are essential for optimal activation of recipient T cells. Instead, studies suggest that inactivated antigen presenting cells in ultraviolet B (UVB) irradiated blood products may possess the ability to induce immune tolerance.<sup>148–153</sup> However, murine studies demonstrate that ultraviolet irradiation of allogeneic platelet concentrates only diminishes the risk of developing alloantibodies to MHC Class I.<sup>150,151,154</sup> Similarly, clinical data from the TRAP study found that the transfusion of UVB-irradiated platelet concentrates associated with a decreased incidence (5%) of alloimmunization to HLA and platelet refractoriness in patients with acute myeloid leukemia compared to those individuals transfused with unmodified platelet concentrates (13%).<sup>20</sup> This rate of alloimmunization was found to be comparable to individuals that were transfused with leukocyte-reduced pooled or apheresis platelet concentrates. Nevertheless, despite being a promising and effective strategy to reduce the risk of forming alloimmune responses to HLA, UVB irradiation of platelet concentrates is not approved for use in the United States.

Pathogen reduction technology, initially developed to improve the safety of blood products by inactivating pathogens, has been explored as a means to reduce alloimmunization to platelet transfusions. Pathogen reduction technology essentially combines the therapeutic benefits of ultraviolet irradiation with a photosensitizer (e.g., riboflavin or psoralen) to kill or inactivate residual leukocytes that may also carry intracellular pathogens. Using this technology, several animal studies have demonstrated that pathogen-reduced platelet concentrates, whether in the presence or absence of leukocyte reduction or ultraviolet irradiation, generated a weak to undetectable alloimmune response to MHC.<sup>155–159</sup> As both ultraviolet irradiation and photosensitizer treatments damage DNA, the inability of pathogen-reduced platelet concentrates to induce an alloantibody response is likely due to reduced immunogenicity of residual leukocytes. Several preclinical studies have demonstrated that pathogen reduction induces tolerance through a variety of mechanisms.<sup>159–161</sup> However, several clinical studies evaluating the efficacy of various pathogen-reduced technologies (e.g., riboflavin + ultraviolet irradiation or psoralen + ultraviolet irradiation) unexpectedly demonstrated that alloimmunization and refractoriness appear to be increased in patients receiving pathogen-reduced platelet concentrates.<sup>162–166</sup> It is worth noting that the interpretation of these studies may be complicated by known confounding factors that could in general increase the risk of alloimmunization, and others have concluded that pathogen reduction is not associated with higher rates of alloimmunization.<sup>167</sup> For instance, trials to date have used stored pathogen-reduced platelet concentrates and pathogen reduction is known to enhance the development of platelet lesions as well as increase phosphatidylserine on platelets and downstream phagocytosis.<sup>168–171</sup> Thus, the disparate observations made in preclinical and clinical studies may be due to differences in the utilization of fresh or stored pathogen-reduced platelet

concentrates. In addition, the trials differed in whether concurrent gamma irradiation was used and exposure to other untreated allogeneic blood products, all of which could increase alloimmunization risk independent of pathogen reduction.

Several future directions are being explored as strategies to prevent alloimmunization. One such method is to strip platelet concentrates of HLA using chloroquine diphosphate or a citrate buffer. The rationale behind this approach is that lower antigen expression may render cells in platelet concentrates less susceptible to recognition; low antigen expression on RBCs has been shown to induce antigen-specific tolerance compared to RBCs expressing the antigen at normal levels.<sup>172</sup> Early studies indicated that HLA could be eluted from the surface of platelets following treatment with chloroquine or a low pH buffer.<sup>130,131,173–175</sup> However, subsequent studies found that citric acid treatment actually dissociates  $\beta_2$ -microglobulin from the heavy chain of HLA Class I molecules, resulting in a denatured HLA molecule on the surface of platelets.<sup>176</sup> Although not decreasing the expression level of HLA, the denatured HLA on citric-acid-treated platelets may possess the ability to modulate the immune response to HLA; a recent study demonstrated that acid treatment prevented the binding of alloantibodies against HLA, activation of the classical complement pathway, and antibody-mediated endocytosis of platelets.<sup>177</sup> Unfortunately, treatment with chloroquine or citric acid impacts the long-term functionality and viability of platelets.<sup>130,177,178</sup> Moreover, the clinical application of transfusing acid-treated platelets remains unclear, with some studies demonstrating a benefit but others not.<sup>179–181</sup> Thus, though there is potential for a therapeutic benefit of acid-treated platelets, additional studies investigating the impact of acid treatment on platelet function and the possibility of reassembling<sup>182</sup> HLA molecules or de novo synthesis<sup>183</sup> of HLA are still needed. Finally, over recent years, significant progress has been in the use of human-induced pluripotent stem cells (iPSCs) as a source for producing autologous, HLA-matched or HLA-universal donor platelets ex vivo.<sup>184–186</sup> iPSCs can be produced without donor dependency and possess the potential to not only eliminate alloimmunization to HLA but supply shortages of platelets. However, the clinical application of iPSC-derived platelets is still extremely premature, as this strategy still necessitates major optimization and characterization of the functionality as well as longevity of these platelets.

## Summary

Platelet transfusion remains an essential therapy for the maintenance of hemostasis in patients with thrombocytopenia or bleeding. Alloantibodies directed at platelet antigens, most notably HLA or HPA, may negatively impact transfusion responses, rendering patients platelet refractory and necessitating specific approaches to mitigate their effects and allow for efficacious transfusion. Ongoing efforts focused on both the prevention of alloimmunization and the development of new treatment strategies for patients with existing alloantibodies will aid in overcoming this significant clinical hurdle.

## Disclaimer

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## CHAPTER 17

# Preparation, preservation, and storage of platelet concentrates

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Platelet concentrates used for transfusions can be obtained from either pooling 4–6 whole-blood-derived units or be selectively collected from a single donor by various apheresis instruments. While the method may differ, the total number of platelets in each transfusable unit must contain at least  $3 \times 10^{11}$  platelets. In both methods, platelets are suspended in autologous plasma or a mixture of plasma and a saline-based additive solution.

### Preparation of platelets from whole blood: platelet-rich plasma method

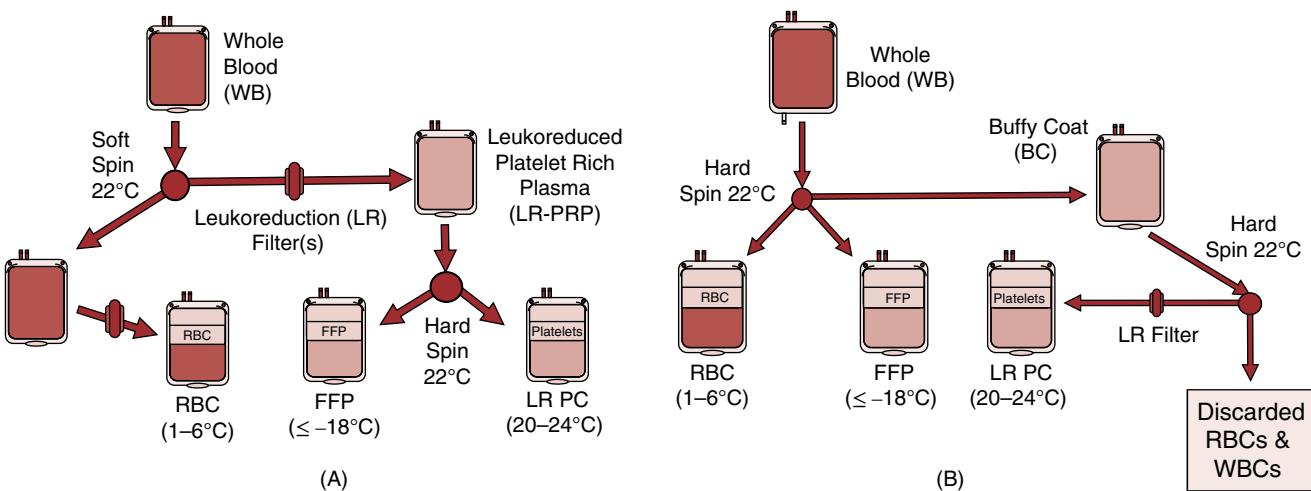
Platelet component therapy was facilitated by the advent of plastic storage containers in the late 1960s, enabling the direct transfusion of platelet-rich plasma to thrombocytopenic patients after a one-step “soft” centrifugation of collected whole blood.<sup>1</sup> At this time, blood was processed under refrigeration to prevent bacterial growth. The effort to increase the concentration of platelets in each unit by an additional “hard” centrifugation step was complicated by irreversible clumping at the bottom of the refrigerated bag. Mourad discovered that by allowing the centrifuged platelets to rest for 30 minutes at room temperature, they can be manually resuspended in residual plasma without major clumps.<sup>2</sup> A landmark finding by Murphy *et al.* demonstrated that platelet viability was best maintained by storage at ambient room temperature (20–24 °C).<sup>3</sup> Building upon this knowledge, Slichter and Harker introduced a preparation method that increased both the platelet yield and viability.<sup>4</sup> They discovered that a stepwise centrifugation process can recover 86% of the platelets from a unit of whole blood at room temperature: A soft spin to procure platelet-rich plasma (PRP) followed by a hard spin to concentrate the platelets (Figure 17.1A).

Following whole blood collection (WB), the product is held between 20 and 24 °C in storage. During transport from the collection center to the processing facility, the temperature is kept as close to 20–24 °C as possible. In the early 1970s, United States Food and Drug Administration (FDA) guidelines stipulated that platelet components be prepared within four hours of WB collection. However, Kahn *et al.* showed that platelet yield and recovery

in vivo were comparable with hold times of four, six, and eight hours. As a consequence, WB holding time was extended to six hours in the late 1970s.<sup>5</sup> In the 1980s, with the introduction of more gas-permeable storage containers, FDA guidelines were modified to allow component separation within eight hours of collection.<sup>6</sup> Many changes have been implemented over the years to optimize the production process. For example, companies have introduced new anticoagulants, improved bag materials, and integrated leukocyte reduction filters into collection sets. Blood centers have improved centrifugation protocols. Nevertheless, PRP remains the only FDA-licensed method of producing whole-blood-derived platelets in the United States at this time.

Whole-blood-derived platelets are formally known as *Platelets* per FDA nomenclature (colloquially, random-donor platelets [RDPs]). AABB and the FDA mandate that a unit of *Platelets* contains at least  $0.55 \times 10^{11}$  platelets suspended in 40–70 mL of plasma.<sup>7,8</sup> Modern concentrates usually contain an average of  $0.9 \times 10^{11}$  platelets with a relatively wide ( $\sim 0.27 \times 10^{11}$ ) standard deviation. This requires a pool of at least five concentrates to ensure 99% of pools contain at least  $3 \times 10^{11}$  platelets, the minimum dose as stipulated by AABB/FDA for apheresis platelets.<sup>9</sup> An integrated filter between the primary blood container and the platelet bag facilitates leukoreduction. PRP is expressed off of the RBC product through the filter after the initial centrifugation.<sup>10</sup> While filtration can cause ~4–5% loss of platelet content, leukocyte reduction results in a  $\geq 3$  log reduction in white blood cell (WBC) content, satisfying the FDA’s requirement for residual WBC count  $<0.83 \times 10^6$  per unpooled unit and  $<5 \times 10^6$  per pooled unit.<sup>11</sup>

Individual units of *Platelets* or *Platelets Leukocytes Reduced* can be pooled either pre- or poststorage. Prestorage pools are more convenient as they can be placed in licensed and validated containers, and thus have a five-day outdate compared to the four-hour outdate for poststorage pools performed in an open system. *Platelets* or *Platelets Leukocytes Reduced* are primarily used for neonatal transfusion, for which one or two units constitute a therapeutic dose.



**Figure 17.1** Platelet production methods. RBC: red blood cell; FFP: fresh frozen plasma; LR: leukocyte reduced; PC: platelet concentrate.

### Preparation of platelets from whole blood: buffy-coat method

In contrast to the United States, the buffy-coat (BC) pooling method is widely used in Europe. Europeans noted the substantial number of residual WBCs  $>10^9$  in RBC products after the initial “soft spin” and the formation of microaggregates between WBCs and platelets.<sup>12</sup> Thus, Prins and colleagues in the Netherlands began using the buffy-coat method to produce blood components in the 1970s.<sup>13</sup> Whole blood donations undergo a “hard spin” initially to separate plasma and RBCs from the buffy-coat layer, containing platelets and WBCs. Four to six buffy coats of the same ABO blood group are pooled, resuspended in plasma from a male donor (in the United Kingdom and Canada) or in a platelet additive solution (PAS; most of continental Europe) and a second centrifugation step “soft spin” to separate the WBCs and residual RBCs from the platelets (Figure 17.1B).<sup>14</sup> The resultant product is passed through a leukocyte reduction filter to produce a prestorage pool with  $<1 \times 10^6$  residual WBCs per unit per European leukocyte reduction standards.<sup>15</sup> Compared to the PRP method, the BC method yields an average platelet product with nearly equivalent platelet content and 10% fewer contaminating white cells before leukoreduction. In addition, the RBC component has about 20 mL fewer red cells and one log fewer white cells. There is also about 30–75 mL more recovered plasma.<sup>16</sup> In 2005, Canadian Blood Services switched from PRP method to BC method to alleviate the chronic strain to platelet inventory and improve logistic hurdles. The BC method used butanediol plates within transport containers to rapidly cool and hold WB at 20–24 °C for up to 24 hours before manufacturing. The overnight hold at room temperature permits exclusive day-shift manufacture and eliminates multiple trips from collection sites to the processing lab. A more theoretical advantage of BC-method production may be less early activation that contributes to platelet loss during storage as platelets are centrifuged against a cushion of RBCs rather than the nonphysiologic plastic container of the PRP method.<sup>17</sup>

Recently, there have been calls by US blood centers to transition from the PRP method to the BC method to optimize the use of all donated products.<sup>18</sup> Automated expression technology contributes to process control, making the BC method potentially even more economical while improving product

standardization.<sup>19</sup> Currently, ~70% of platelet units are prepared from whole blood in Canada, while >85% of platelets are prepared from WB in Denmark, Spain, and the Netherlands.<sup>20</sup> These countries demonstrate that a national platelet supply can be derived predominantly from WB instead of from apheresis. Between 2008 and 2017, the proportion of BC platelet distributions from 11 centers outside the United States increased from approximately 35–60%.<sup>21</sup>

### Preparation of platelets from plateletpheresis

Platelets produced by apheresis are called *Platelets, pheresis* by the FDA (informally, single donor platelets [SDPs]). Recognition by Yankee *et al.* that poor platelet increment in recipients was associated with human leukocyte antigen (HLA) alloimmunization in 1970s led to increased single-donor utilization apheresis platelets. This was implemented not only for platelet-refractory patients but also to minimize donor exposure for the general patient population.<sup>22</sup> It is estimated that apheresis platelets account for ~90–95% of all platelet usage in the United States.<sup>23</sup>

Automated apheresis instruments have evolved over the years to obtain multiple platelet doses from a single collection. In-process leukocyte reduction became possible with better separation controls, so *Platelets, pheresis Leukocytes Reduced* now contain  $<5 \times 10^6$  WBCs. Unlike WBD platelets, red cell content in apheresis platelets is very low and often (but not always) below the dose of red cells associated with RhD alloimmunization.<sup>24,25</sup> There are several licensed apheresis instruments that separate platelets in different ways (see Chapter 22 for more detail). Some devices employ a disposable plastic centrifuge chamber from which a layer of PRP is siphoned into a collection bag. Others use a spinning channel with two-stage plastic inlays to initially separate the RBCs from the PRP and then remove plasma from the platelet product. Yet another device uses an elutriation mechanism. This device moves platelets away from the RBC layer, separates the PRP, and then hyperconcentrates platelets along a collection bag wall before plasma resuspension in a storage container. This heterogeneity of collection technologies leads to apheresis products that are not necessarily similar and may not always produce equal platelet increments in a given recipient.<sup>26</sup>

## Alternative sources of platelets

An entirely different approach to collecting platelets from peripheral blood is the generation of platelets from stem cells ex vivo. Possible advantages are preparing and storing patient-specific precursor cells and an inventory-independent, donor-independent reservoir of platelets. Different groups reported the differentiation of CD34+ hematopoietic precursor cells from peripheral blood, cord blood, bone marrow, stromal cells, and fetal liver cells.<sup>27–36</sup> However, the low proliferation capacity of these cells leads to a dependence on donors and thereby eliminates some of the postulated advantages of in vitro generation. The utilization of embryonic stem cells and induced pluripotent stem cells (iPS) represents a source with unlimited proliferation capacity.<sup>37–42</sup> While utilizing embryonic stem cells raises ethical concerns limiting their application to research questions, this reservation does not apply to iPS cells. Another advantage of iPS-derived platelets is using patient-specific, reprogrammed cells to generate a personalized product for alloimmunized patients. In vitro generated megakaryocytes and platelets have similar characteristic features as cultured, native megakaryocytes.<sup>43</sup> Platelets from iPS and embryonic cells are hypo-reactive compared to adult platelets.<sup>44–47</sup> Whether this is beneficial or detrimental for transfusion outcomes remains to be investigated. Age-mismatched transfusions have recently been problematic when higher transfusion triggers of adult platelets worsened premature infants' outcomes.<sup>48</sup> High costs and low platelet yields per megakaryocyte still represent major bottlenecks for the routine clinical application of these cells. Another more manageable challenge is the application of good manufacturing practices. Transfusion of in vitro generated megakaryocytes could represent an alternative. Using this approach, the lung could represent a natural bioreactor for platelet generation in vivo. At least in mice, the lung appears to be involved in platelet biogenesis at a steady state.<sup>49</sup> Furthermore, human data, including radiolabeling studies with megakaryocytes or platelets in humans, are largely missing.<sup>50</sup>

## Storage conditions

Once the circulatory survival benefit of room temperature processing and storage was appreciated, platelets were kept for up to three days in the first generation of plastic containers. As improvements were made in platelet storage technology, shelf life was first increased to five days and then to seven days. In 1986, the FDA mandated a return to a five-day shelf life because of increasing reports of bacterial sepsis associated with prolonged 20–24 °C storage.<sup>51</sup> Detection methods for bacterial contamination were required in 2004 (discussed in detail in Chapter 53), but the re-extension of storage duration was delayed because of concerns about the poor sensitivity of standard testing (29–40%).<sup>52–54</sup> However, improvements in bacterial testing and pathogen reduction technologies have led to the extension of platelet storage life to seven days in several countries, including Canada, the Netherlands, Switzerland, and the United Kingdom. Draft guidance from the FDA permitted the extension of platelet storage up to seven days with rapid bacterial testing or up to five days following pathogen reduction.<sup>55</sup> A new FDA guidance published in 2019 will bring widespread changes in bacterial testing by blood centers and hospitals in the United States.<sup>56</sup>

When released into circulation from the bone marrow, platelets survive for approximately 9–10 days and are removed by senescence, consumption during maintenance of vascular integrity (normally,  $\sim 7.1 \times 10^9/\text{L/day}$ )<sup>57</sup>, or clot formation to control pathologic bleeding. Room temperature storage appears to slow platelet

metabolic activity by almost 60% compared with in vivo activity at 37 °C.<sup>58</sup> This suggests that seven days of storage may lead to only three days' worth of in vivo senescent loss. Efficacy-wise, it is well known that platelet recovery and survival continuously decline over storage time.<sup>59</sup> Progressive loss of recovery can be observed over the first five days of storage<sup>60</sup> and further decline by seven days.<sup>61,62</sup> Some storage lesion features, such as pH, differ significantly between five-day stored and seven-day stored platelets.<sup>63</sup> Three systematic studies examined the effect of storage time on clinical outcomes.<sup>64–66</sup> The freshest units (<2–3 days of storage) consistently resulted in the highest corrected count increments (CCIs). In stable hematology patients, six- or seven-day stored platelets resulted in similar corrected count increments, bleeding events, and time to next platelet transfusion compared with 2–5-day stored platelets, supporting extended room temperature storage.<sup>67</sup>

Storage conditions that minimize platelet loss by inhibiting platelet activation, senescence (apoptosis), or metabolic exhaustion might, in theory, permit the extension of PC shelf life well beyond seven days. Certain experimental conditions appear to allow for extended storage at room temperature with remarkable in vivo characteristics. The investigators collected platelets with an elutriation apheresis device and replaced most plasma with an additive solution.<sup>68</sup>

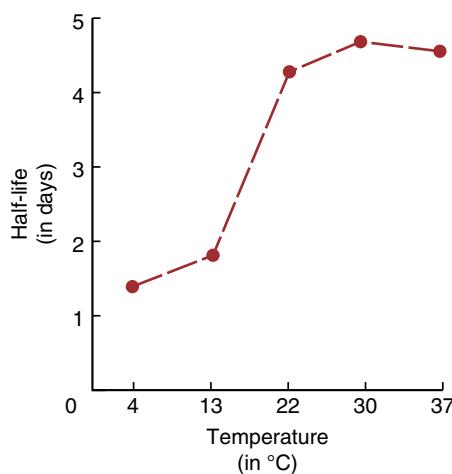
The critical variables affecting stored platelet health are (1) temperature, (2) metabolic fuel availability, and (3) respiratory capacity. The last variable is dependent on platelet content, gas diffusion through the plastic container, and agitation. Activation occurring during collection and processing appears to be somewhat reversible, but excessive activation may also lead to platelet loss.

## Metabolic concerns during storage: temperature

When platelets are exposed to the cold, their plasma membranes undergo phase transitions that result in the coalescence of lipid rafts. This leads to shape change and activation priming, clustering of neopeptope-expressing glycoprotein (GP)-I $\beta$  $\alpha$  domains with enhanced von Willebrand factor (vWF) binding capacity, and acceleration of platelet apoptosis.<sup>69–73</sup> The in vivo platelet cold sensitivity presumably facilitates activation at wound sites on the cooler body surface while removing repetitively primed platelets to prevent core thrombosis.<sup>74,75</sup>

Membrane phase transitions begin at temperatures below 30 °C, bringing phosphoinositide-rich lipid rafts together.<sup>74,76</sup> Resultant phosphoinositide release results in an increase in intracellular calcium, which is associated with activation priming. Indeed, the in vitro function of cold stored platelets exceeds that of room temperature stored platelets, prompting a call for the re-evaluation of the role of cold stored platelets when immediate hemostasis is paramount (e.g., after trauma).<sup>77</sup> Hypothermic membrane changes also result in a clustering of the principal vWF receptor, GP-Ib/IX/V.<sup>74</sup> The function of the receptor appears to be enhanced by clustering. However, lectin-binding domains of hepatic macrophages and hepatocyte receptors recognize sugar changes on clustered GP-I $\beta$  $\alpha$  subunits and rapidly remove these platelets. Hence, the circulation time of these potentially more hemostatically active chilled platelets is shortened.<sup>77</sup>

The reversibility of cold-induced platelet membrane changes is both time- and temperature-dependent. Figure 17.2 shows the effect of 18 hours of storage at various temperatures upon the half-life of radiolabeled autologous platelets after reinfusion into healthy volunteers. Temperatures above 20 °C resulted in normal platelet survival.<sup>3</sup> Holme *et al.*<sup>78</sup> reported that significant loss of platelet circulatory capacity occurred after 24 hours at 18 °C, 16 hours at



**Figure 17.2** *in vivo* survival of radiolabeled autologous platelets stored at various temperatures for 18 hours.<sup>3</sup> Source: Reproduced from Dr. Ralph Vassallo.

16 °C, 10 hours at 12 °C, and 6 hours at 4 °C. Storage above the 20–24 °C temperature described as optimal by Murphy and Gardner did not result in measurable damage but was accompanied by increased metabolism compared with room temperature storage. The salutary metabolic effects of room temperature storage include decreased in vitro aging and slower accumulation of toxic metabolites within the platelet container. There is, however, a reversible loss of function that recovers only after a variable period of time in circulation.<sup>77</sup> The ability of room temperature stored platelets to circulate for longer periods of time, despite transiently decreased activation capacity, is desirable in preventing spontaneous hemorrhage in chronically thrombocytopenic transfusion recipients.

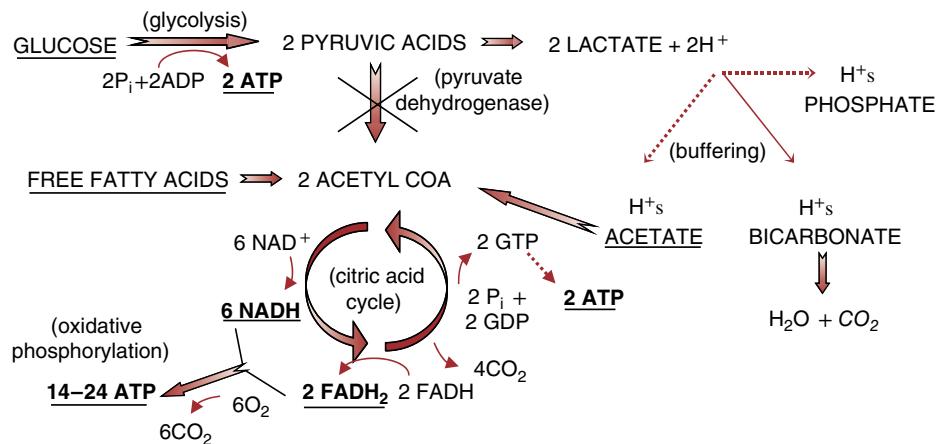
### Metabolic concerns during storage: metabolic fuel availability

Stored platelets must catabolize substances within their suspension medium to regenerate adenosine triphosphate (ATP), the cell's principal "energy currency." Figure 17.3 shows the pathways for ATP regeneration.

Seminal studies of glucose consumption and lactate production in PRP-PCs revealed an approximately 1:2 ratio consistent with predominantly anaerobic glucose metabolism. Pyruvate dehydrogenase, which produces acetic acid for the aerobic citric acid cycle, appears to be downregulated in stored platelets. Glucose metabolism in platelets ends with only 2 moles of ATP per mole of glucose consumed. Lactic acid is buffered primarily by plasma bicarbonate, which results in the production of CO<sub>2</sub>.<sup>79</sup> This CO<sub>2</sub>, a volatile acid, diffuses through the storage container wall. Progressive depletion of bicarbonate thus occurs through lactic acid buffering and slow spontaneous dissociation to CO<sub>2</sub> and water. Once bicarbonate is consumed, continued lactic acid production ultimately results in a deleterious decrease in product pH.

Aerobic metabolism through the citric acid cycle and oxidative phosphorylation is a far more efficient energy source, yielding up to 13 moles of ATP per mole of acetic acid entering the cycle. Comparison of platelet oxygen consumption (approximately 6 moles of ATP produced per mole of O<sub>2</sub> consumed) with lactate production (1 mole of ATP per mole of lactate) suggests that approximately 85% of platelet energy is derived from aerobic metabolism in continuously agitated PCs.<sup>80</sup> Studies using radiolabeled palmitate identify free fatty acids derived from plasma triglycerides as the predominant metabolic fuel for platelets stored in plasma.<sup>73</sup> A small, rather insignificant component results from the metabolism of the amino acid glutamine.<sup>72</sup> Efforts to identify plasma-sparing PAs led to the discovery that acetate could serve as an effective alternative fuel to plasma free fatty acids.<sup>71</sup> As described below, acetate also provides a valuable lactate-buffering function by consuming a hydrogen ion during its metabolism.

Under low oxygen conditions, glycolysis is upregulated six- to sevenfold, the so-called Pasteur effect.<sup>70</sup> Although this satisfies energy requirements in the absence of aerobic metabolism, it results in a considerable increase in lactic acid production, which can exacerbate plasma buffer exhaustion and produce deleterious declines in pH. Mechanical factors also affect platelet oxygenation. Mitochondrial oxygen starvation can occur with interruption of agitation during shipping, accompanied by an expected increase in lactate generation and pH decrements.<sup>69</sup> Studies have shown that platelets begin to suffer deleterious effects below a pH of 6.5–6.8 at room temperature (22 °C) and are irreversibly damaged in the face



**Figure 17.3** Pathways of platelet adenosine triphosphate (ATP) regeneration from inorganic phosphate (P<sub>i</sub>) and adenosine diphosphate (ADP). Metabolic fuels are underlined. Energetic intermediates are underlined. Carbon dioxide (CO<sub>2</sub>) is produced from hydrogen ion (H<sup>+</sup>) buffering and oxidative metabolism of acetyl CoA. AMP: adenosine monophosphate; PPi: pyrophosphate; GDP/GTP: guanosine di/triphosphate; NAD/NADH: nicotinamide adenine dinucleotide/reduced NAD; FADH/FADH<sub>2</sub>: reduced flavin adenine dinucleotide forms. Source: Reproduced from Dr. Ralph Vassallo.

of a sustained pH below 6.2 at 22 °C.<sup>79,81</sup> Efforts to extend platelet storage have been limited by the constitutive glycolytic production of lactate, even with maintenance of adequate oxygenation. Glycolysis can also be transiently upregulated by activation associated with platelet separation.<sup>82</sup> Attempts to develop glucose-free additive solutions to minimize the production of lactate have not been successful. Despite the presence of sufficient amounts of alternate fuels (e.g., acetate), once PCs exhaust their supply of glucose, they deplete their adenine nucleotides and a host of other metabolic intermediates, leading to cell death.<sup>71,83</sup> The reasons for this are poorly understood but may be related to substrate depletion of the antioxidant pentose phosphate shunt and an as-yet-unexplained link between glucose metabolism and mitochondrial function. Adenine nucleotide depletion during storage has been characterized in PRP-PCs.<sup>84</sup> After seven days of storage, adenine nucleotide levels in PCs (in both metabolic and storage granular pools) decline to 70% of those initially present.<sup>85</sup> Although platelet ATP levels are not predictive of platelet quality except at extremely low levels, energy depletion is responsible for a host of deleterious effects that ultimately culminate in platelet senescence or necrosis.

Proteomic and metabolomic studies have shed additional light on platelet storage metabolism.<sup>82,86,87</sup> Regardless of the separation method, there appears to be a transition from active aerobic and glycolytic metabolism to a state of metabolic decay characterized by progressive depletion of intermediates. This is posited to occur due to mounting activation stimuli, both outside-in and inside-out, which reach an irreversible tipping point after three days of storage under current conditions. In vitro suppression of signaling proteins appears to delay the metabolic shift from maintenance to activation, but this laboratory approach has not resulted in an inhibitor suitable for clinical use.<sup>82</sup>

### **Metabolic concerns during storage: respiratory capacity**

Maintenance of aerobic metabolism requires a PO<sub>2</sub> above 5–10 mmHg.<sup>80</sup> Because oxygen consumption is relatively constant per platelet, the presence of a high total platelet bag content stresses the storage container's oxygen permeability. When PC consumption exceeds the ability of the container to transport oxygen, the PO<sub>2</sub> will decline to hypoxic levels over several days, leading to the upregulation of glycolysis, lactic acid accumulation, and a deleterious decrease in pH. Gases diffuse across storage container walls driven by their gradients once the plastic is saturated. Each manufacturer's container has a different gas permeability related to the wall thickness and the plastic's unique gas transport capacity. Oxygen delivery also depends upon the container's ratio of the volume-to-surface area and continuous agitation, which promotes gas diffusion and helps maintain normal platelet oxygen consumption.

Kilkson *et al.*<sup>80</sup> demonstrated that across the CO<sub>2</sub> concentrations seen in PCs stored in the least permeable containers (10–100 mmHg), little pH change is attributable to the carbonic acid formation from aqueous CO<sub>2</sub> and H<sub>2</sub>O. Lactate concentration was shown to be the principal determinant of pH. Thus, high CO<sub>2</sub> permeability is not as critical as adequate oxygen permeability. High CO<sub>2</sub> permeability can lead to an early increase in pH as the PCO<sub>2</sub> falls from physiologic plasma levels, unbalanced by significant lactic acid accumulation. A pH >7.4 at 22 °C may result, which has been associated with cellular damage with earlier forms of agitation. Although European regulators have set 7.4 pH as the upper limit of acceptable end storage at 22 °C, a comprehensive study noted that

with modern forms of agitation, a pH >6.3 at 22 °C, had no predictive value for platelet viability (in PCs compliant with licensed maximum and minimum platelet contents and concentrations).<sup>88</sup>

From the foregoing discussion of metabolic fuel requirements, it is evident that high platelet content will increase constitutively produced levels of lactic acid and that high platelet concentrations also result in fewer plasma buffers per respiring platelet, both of which may lead to deleterious drops in pH. Less intuitive are the harmful effects of low platelet contents and concentrations. Adverse effects in these situations result from low CO<sub>2</sub> and lactate production, which initially increase PC pH. Why these products develop metabolic exhaustion and adenine nucleotide depletion is not entirely clear, but some forms of agitation further increase this effect at higher pH values.

The very first containers, made of polyvinyl chloride (PVC) plasticized with di-(2-ethylhexyl) phthalate (DEHP), were originally designed for freezing plasma. Serendipitously, they allowed enough oxygen and CO<sub>2</sub> diffusion across their walls to permit room temperature platelet storage of relatively lower content PCs for three days before the pH decreased to unacceptable levels. Containers were later specifically designed for improved gas permeability as trial and error revealed that PCs stored in more gas-permeable containers had lower pH failure rates (pH <6.2 at 22 °C).<sup>89</sup> The so-called second-generation containers appear to store PRP-PCs for at least seven days without deleterious pH decrements. Because apheresis PCs and prestorage pooled PRP-PCs have such heterogeneous contents, each unit must be assayed to ensure that total platelet content and concentration limits validated by the container manufacturer are not exceeded. The platelet concentration of donor blood limits individual WBD-PC content to values below those accommodated by newer storage containers. Second-generation containers currently in use are manufactured from polyolefin, ethylene-vinyl acetate (EVA), or PVC that is thinner or plasticized with different compounds such as triethyl hexyl trimellitate (TOTM) and butyryl-tri-hexyl citrate (BTHC).<sup>90</sup> These bags provide nearly twice the oxygen permeability of first-generation DEHP-plasticized PVC containers.<sup>91</sup>

The need for continuous agitation was also recognized as a means of facilitating oxygen utilization.<sup>89,92</sup> Several different types of platelet rotators have been used over the years. Ferris wheel and elliptical rotators have given way to face-over-face tumblers (3–6 rpm) and flatbed platform shakers (50–70 cpm) because of inefficient maintenance of pH or damage to platelets at pH >7.4 at 22 °C.<sup>79</sup> Initially thought to promote oxygen diffusion throughout the PC, agitation appears to exert a more complex effect on platelet metabolism. Several groups have shown that PO<sub>2</sub> was maintained in unagitated PCs and that mixing of products even once a day could prevent injurious pH decrements in relatively lower content platelet products.<sup>93,94</sup> Agitation, therefore, may also facilitate oxygen utilization by platelet mitochondria or prevent settling and a platelet contact-mediated switch from oxidative to glycolytic metabolism.<sup>95</sup> In any case, even with relatively higher content PCs, agitation may be safely interrupted for 24 hours (as during shipping) without undue lactate accumulation and subsequent development of injuriously low pH.<sup>95,96</sup>

### **The platelet storage lesion**

Stored platelets experience a progressive decline in function accompanied by characteristic morphologic changes. Studies document up to 20% loss of platelet recovery through five days of storage.<sup>60</sup> In apheresis containers currently licensed in the United States, Day 7

recovery is approximately 83% of Day 5 recovery.<sup>61,62</sup> This progressive damage has been termed the *platelet storage lesion*.<sup>97,98</sup> Well-characterized changes are seen in common tests assessing platelet morphology, activation, cell metabolism and function, and apoptosis. Some, but not all, of these changes are reversible upon platelet transfusion.<sup>99</sup>

The gold standard for evaluating product efficacy remains post-transfusion bleeding assessments. However, the large patient numbers required and related time and expense usually lead to the substitution of surrogate measurements such as patient-adjusted count increments assuming that a circulating platelet is a functional one. An even less costly alternative involves the reinfusion of healthy volunteers with autologous stored platelets radiolabeled with chromium-51 or indium-111. (Cell kinetics are described in greater detail in Chapters 7, 18, and 22.) Percent recovery and survival may be calculated by serial assay of postinjection blood radioactivity. Platelet recovery is significantly affected by individual differences in splenic sequestration (usually ~33%) and blood volume estimation errors during calculations. Murphy proposed that products be evaluated against concurrently injected fresh autologous platelets to establish a fixed, rather than floating, standard.<sup>100</sup> This facilitates the comparison of interventions designed to ameliorate the storage lesion. Although in vitro tests have attempted to rapidly and economically predict the ability of transfused platelets to circulate, only extreme values have documented any correlation with outcomes.<sup>101,102</sup> Accordingly, batteries of tests are often examined to ensure product equivalency after minor changes or as the first step in the assessment of more substantive changes in storage methods.

Alterations from normal discoid morphology are observed during platelet storage. Kunicki described a scoring system that assigned four points to discs, two to spheres, one to dendritic forms, and none to ballooned platelets.<sup>103</sup> Multiplied by the percentage of each form seen under phase contrast microscopy, scores decline from a maximum of 400 as changes associated with the storage lesion progress. Others have advocated simply reporting the percentage of discoid forms. The extent of shape change assay uses aggregometry to measure the decrease in light transmission after adenosine diphosphate stimulation of stirred PRP in the presence of EDTA to prevent aggregation.<sup>104</sup> Because only discoid platelets can change shape and block more light, the assay provides a more objective measure of the percentage of discoid platelets. All three approaches appear to correlate somewhat with *in vivo* recovery and survival at high or low assay values but predict less well for in-between values. The mean platelet volume decreases throughout storage when membrane loss from dendritic forms occurs or when activation or apoptosis results in microvesiculation.<sup>105</sup>

Platelet activation during processing and throughout storage is accompanied by surface expression of sequestered granular membrane proteins (P-selectin and CD63) and conformational changes of the fibrinogen receptor,  $\alpha$ IIb $\beta$ 3 (GP-IIb/IIIa). Release of specific granular contents into the supernatant ( $\beta$ -thromboglobulin, platelet factor 4, etc.) also occurs throughout platelet storage as a manifestation of activation. However, this may not be specific for activation because granular content release also occurs with platelet lysis. Similarly, platelet procoagulant activity (surface expression of membrane phosphatidylserine) appears not only with activation but also during cellular apoptosis. None of these indicators of platelet activation appear to correlate reliably with platelet recovery and survival. However, studies of storage medium supplementation with activation inhibitors suggest that activation contributes in part

to the platelet storage lesion.<sup>106</sup> As previously noted, the use of activation inhibitors in storage media has not gained widespread acceptance because of inconsistent results, toxicity, and the expense of licensing novel storage systems.

The ability of stored platelets to function normally has been assessed in several ways, and only one functional assay—hypotonic shock response—has proven to be of value in assessing platelet viability. Measurements of platelet ATP levels do not consistently provide reliable information.<sup>85</sup> Aggregation studies similarly fail to predict platelet viability. Responsiveness to single agonists rapidly declines during storage but can recover with plasma rescue or *in vivo* reinfusion.<sup>82</sup> Responses to agonist mixtures, although better preserved, are still not helpful in assessing viability.<sup>107</sup> The hypotonic shock response or osmotic reversal reaction exposes platelets to a hypotonic environment. Metabolically active platelets rapidly extrude water after initial swelling. Recovery of normal intracellular water content can be quantified using light transmittance. Significant changes in values for this assay have been shown to correlate with *in vivo* recovery and survival.

Markers associated with cellular apoptosis accumulate throughout platelet storage with evidence of caspase activation, structural protein degradation, and mitochondrial membrane potential changes.<sup>108,109</sup> Although apoptotic inhibitors can ameliorate some of these changes, they have not improved platelet viability, casting doubt upon cellular senescence as a significant contributor to the platelet storage lesion.

### **Platelet additive solutions (synthetic storage solutions)**

In Europe, synthetic storage solutions, also called PASs, have been used routinely for two decades.<sup>110</sup> In the United States, PASs were approved for use in 2010. The use of PASs allows<sup>1</sup> greater plasma recovery from whole blood donations for transfusion or fractionation;<sup>2</sup> minimization of the adverse effects mediated by plasma (e.g., allergic and febrile nonhemolytic transfusion reactions [FNHTRs] and possibly the reduction of ABO isoagglutinin-mediated hemolysis or antibody-mediated transfusion-related acute lung injury [TRALI]); and potential improvements in platelet storage through engineered manipulation of the storage medium.<sup>111,112</sup> Platelet collection with the Amicus collection system allows for the addition of PAS and thereby facilitates the use of photochemical pathogen reduction technology. In contrast, pathogen-reduced platelets from the Trima system are all in plasma as per FDA.

Early studies demonstrated the feasibility of suspending PRPPCs in a commercially available, neutral intravenous crystalloid solution, Plasmalyte A.<sup>113</sup> The 10–20% plasma carried over in the storage medium provided the sole source of glucose and bicarbonate. *In vitro* results were quite encouraging, and versions of this PAS containing additional glucose and citrate were shown to be promising *in vivo*. However, the solution was not successfully commercialized.<sup>114</sup> When Murphy tested another balanced salt solution, the *in vivo* results were disappointing.<sup>115</sup> In retrospect, the deletion of acetate from Murphy's so-called PSM-2 solution was likely responsible for the pH failures seen *in vitro*. Acetate can efficiently substitute in the citric acid cycle for free fatty acids, the primary *in vivo* substrate for oxidative metabolism (Figure 17.3), decreasing both the glycolytic rate and lactic acid generation.<sup>116</sup> Acetate must also be transformed to acetic acid to enter the cycle, removing hydrogen ions produced by the anaerobic metabolism of glucose. This bicarbonate-sparing buffering effect also helps preserve pH. From Murphy's experience and other studies, it appears likely that

some glucose must be available throughout storage. Holme and Gulliksson noted the association of glucose exhaustion (even in the presence of alternate fuels) with adenine nucleotide depletion and loss of platelet viability.<sup>71</sup> The addition of glucose to PAS is technically difficult because glucose caramelizes upon heat sterilization at the neutral or slightly basic PAS pHs best suited to maintain PC pH. Accordingly, most commercially available PASs do not contain additional glucose and require 20–40% plasma carryover. Two as-yet FDA-unapproved solutions have gotten around this problem by adding bicarbonate to a separately sterilized glucose-containing low-pH base solution.<sup>117,118</sup> Table 17.1 lists the components of various solutions, including those currently FDA-approved and under investigation in the United States.

In addition to sparing bicarbonate, PAS contents have been designed to modulate the glycolytic rate and downregulate platelet activation. Citrate, important in maintaining anticoagulation, upregulates glycolysis and renders platelets more susceptible to activating stimuli. Consequently, the use of the lowest possible concentrations of citrate in the medium appears desirable.<sup>71</sup> Phosphate similarly has both salutary and detrimental effects in PAS.<sup>119</sup> Phosphate serves as a buffer, and in apheresis platelets it is important in maintaining adenine nucleotide levels. The difference in anticoagulants can explain this: apheresis platelets are suspended in acid–citrate–dextrose (ACD), while PRP-PCs and BC-PCs use CPD/CP2D. Phosphate also upregulates glycolysis and consequently mandates more plasma carryover or addition of glucose to PAS. Magnesium and potassium both appear to decrease platelet activation and may downregulate glycolysis as well.<sup>119</sup> Studies with several platelet activation inhibitors (e.g., prostaglandin E1 and theophylline) suggest that these substances may enhance PAS efficacy, but safety concerns related to routine transfusion in various patient populations significantly hinder commercial applicability.<sup>120</sup>

Clinical studies comparing platelets stored in plasma with FDA-approved additive solutions have demonstrated that allergic reactions and FNHTRs were reduced by approximately two-thirds.<sup>121</sup> Dutch investigators reported a nonsignificant reduction from 11% to 9% in mostly mild reactions when PAS-C was used compared to 100% plasma.<sup>122</sup> PAS-C also proved efficacious in reducing the relative risk of all transfusion-related adverse rates (incidences of 1.37% vs. 0.55%).<sup>121</sup> A large retrospective study found that storage time was associated with allergic transfusion reactions in platelets stored in PAS-B and PAS-C. For PAS-B, storage time was also associated with FNHTRs. However, due to the lower overall incidence of reactions in the PAS-C group, the authors considered PAS-C the best choice to prevent transfusion reactions.<sup>123,124</sup> The effect of PAS on TRALI is

less clear, likely because of the lower incidence of TRALI. A large retrospective study from France reported a significant reduction of TRALI cases with PAS platelets compared to plasma platelets. However, this was only when buffy-coat-derived platelet (pooled) concentrates were used and not with apheresis platelets.<sup>125</sup> A possible explanation is that the amount of plasma from one individual donor is much smaller in pooled units, and further dilution may reduce concentrations below a disease-causing threshold.<sup>126</sup> Replacing plasma with PAS could also have negative effects. PAS may enhance bacterial growth by reducing the bactericidal properties of plasma.<sup>127</sup> Faster growth could allow for quicker detection and reduce false positives, but this remains to be investigated. Retrospective hemovigilance data from the Netherlands show that the relative risk is four-fold higher for transfusion-transmitted bacterial infections with PAS-B and PAS-C compared to 100% plasma.<sup>128</sup> An additional negative side effect is that the corrected count increments after transfusion are approximately 25% lower with PAS platelets. This effect may be less severe with PAS-E.<sup>129,130</sup> Nevertheless, when the conditions are optimized, platelets in PAS can be superior to platelets in 100% plasma, as shown by a recent radiolabeling study using PAS-F and the Haemonetics MCS+ elutriation collection system.<sup>68</sup> Additive solutions containing glucose and bicarbonate require 5% or less plasma carryover. Once commercialized, these “low plasma” PAS may further reduce transfusion reaction rates, including antibody-mediated reactions such as TRALI and ABO-mediated hemolysis. However, even with currently licensed PAS/plasma ratios, desirable ABO antibody titer thresholds can be achieved.<sup>131</sup>

With the re-emergence of cold stored platelets, platelet additive solutions have gained further attention since additive solutions prevent cold-induced micro- and macroaggregates associated with wastage.<sup>132,133</sup> Hemostatic in vitro properties of cold stored platelets appear to be preserved in additive solution, but in vivo recoveries were reduced compared to 100% plasma in a radiolabeling study.<sup>134</sup> Additive solutions appear to have beneficial effects beyond the prevention of aggregates and the preservation of hemostatic function. When treated with pathogen reduction techniques, cold stored platelets in PAS-C/plasma showed less plasma membrane damage than platelets stored in 100% plasma.<sup>135</sup> In other studies, PAS led to better preservation of mitochondria and reduced apoptosis during cold storage.<sup>136</sup> Whether PAS or 100% plasma is the better storage solution for cold stored platelets remains to be investigated.

### Component modification

Several PC modifications are occasionally required for various subsets of patients. Cellular products containing viable lympho-

**Table 17.1** International Council for Commonality in Blood Banking Automation (ICCBBA) Designations for Platelet Additive Solution Products (with Annotation of FDA-Approved Products and Those Under Investigation in the United States).

	Citrate	Phosphate	Acetate	Magnesium	Potassium	Gluconate	Glucose
PAS-A	A	A					
PAS-B	B						
PAS-C*	C	C	C				
PAS-D	D		D	D	D	D	
PAS-E	E	E	E	E	E		
PAS-F		F	F	F	F	F	
PAS-G	G	G	G	G	G		G*

\* Haemonetics’ “PAS-G” includes HCO<sub>3</sub><sup>-</sup>, and Fenwal’s “PAS-5” includes HCO<sub>3</sub><sup>-</sup> and Ca<sup>++</sup>.

<sup>†</sup> B. Braun’s Isoplate®, a PAS-F product, includes PO<sub>4</sub><sup>3-</sup>.

<sup>\*</sup> Fenwal’s Intersol® is a PAS-C product.

Source: Reproduced from Dr. Ralph Vassallo.

cytes must be irradiated before transfusion to patients who are susceptible to graft-versus-host disease. An irradiation dose of 25 Gy (15–50 Gy) has no significant effect on common in vitro parameters even after seven days of storage following irradiation as early as Day 1.<sup>137</sup> Similarly, little or no effect of irradiation was seen on in vitro storage parameters or in vivo recovery and survival with five-day stored platelets irradiated with up to 30 Gy on Day 1 or 3.<sup>138,139</sup>

Patients with moderate to severe allergic reactions to plasma components or newborns with neonatal alloimmune thrombocytopenia receiving maternal platelets benefit from plasma removal by platelet washing. Some institutions also wash platelets to remove ABO-incompatible plasma.<sup>140</sup> However, washing leads to a significant loss of platelets and a decrement in platelet function while also reducing the product shelf life to four hours. A standard saline wash procedure using the COBE 2991 instrument (TerumoBCT, Lakewood, CO) has been described. Platelet losses range from 8% to 29%.<sup>141,142</sup> Functional impairment of saline-resuspended washed platelets resulted in a two-thirds loss of recovery versus an unwashed control in one study.<sup>143</sup> Other studies have reported better in vitro properties of washed platelets resuspended in additive solutions instead of the saline commonly used in the United States.<sup>144</sup> For IgA-deficient patients, saline alone may not remove as much IgA as citrate-buffered saline because of IgA's low isoelectric point and its coprecipitation with platelets in an acidic saline wash fluid.<sup>145</sup>

For transfusions in utero or for volume-sensitive neonates, the additional concentration of PCs can be achieved through volume reduction by centrifugation and plasma removal. This technique has also been used to minimize infusion of maternal antibodies or ABO-incompatible plasma, although washing is preferred when antibody removal is critical. Some have questioned the need for volume reduction for most neonates and have pointed out that nonmanipulated PCs are well tolerated in neonates not *in extremis*.<sup>146</sup> Procedures have been described for volume reduction, resulting in <15% loss of platelets and relatively preserved in vivo viability.<sup>147</sup> British standards call for a concentration over  $2000 \times 10^9/L$  for in utero transfusion.<sup>148</sup> Volume-reduced PCs maintain acceptable in vitro properties in plastic syringes at room temperature or in neonatal incubators (at 37 °C).<sup>149</sup> PCs should be transfused as soon as possible after volume reduction in a closed system, employing sterile connection devices, and must be used within four hours if prepared in an open system, such as via spike entry.

Most apheresis platelets are leukocyte reduced during the separation process. Leukocyte reduction of WBD-PCs is usually accomplished by prestorage leukofiltration during separation or at prestorage pooling. Prestorage leukocyte reduction has resulted in a 75–90% reduction in FNHTRs after platelet transfusion.<sup>150,151</sup> Leukocyte reduction also reduces cytomegalovirus transmission and platelet alloimmunization.<sup>152,153</sup>

### Novel storage techniques

Room temperature platelet storage addressed the problem of rapid clearance that occurs with cooler storage temperatures but is not without limitations. Bacterial proliferation occurs more easily, and platelet metabolism is increased compared with cold storage. Prolonged storage at cold temperature reduces platelet recovery and survival, but membrane integrity may be more effectively preserved.<sup>154</sup> Additionally, in vitro suggest cold stored platelets may have better hemostatic function.<sup>77</sup> Sustained survival following transfusion is important for prophylactic transfusions, but the

possibility of improved function with cold storage is appealing for transfusion in bleeding patients.

The in vivo significance of cold-related platelet activation is uncertain. Cold stored platelets are more effective in correcting prolonged bleeding time (BT) in healthy volunteers taking acetylsalicylic acid (ASA) but not both ASA and clopidogrel.<sup>155–159</sup> Both cold- and room-temperature stored platelets reversed ASA effects measured using the VerifyNOW assay. However, the duration of reversal was shorter with cold stored platelets.<sup>160</sup> In a pilot study, patients undergoing cardiac surgery were randomized to receive platelets stored at room temperature for up to seven days or at 2–6 °C for up to 14 days without agitation. The primary outcome of chest tube output was not significantly different between groups, supporting the feasibility of prolonged cold storage but not suggesting a hemostatic advantage to cold storage.<sup>161</sup> Attempts to mask clustered GP-Ib/IX/V receptors with sugar moieties, a successful manipulation in mice transfused with briefly cold stored platelets, have not reversed the rapid clearance of longer stored chilled platelets observed in humans.<sup>162</sup>

Cryopreserved platelets can be prepared and stored for up to 10 years, but cost, inconvenience, procedure-related platelet loss, and functional decrements remain barriers to wider implementation. There is, however, considerable military interest in frozen platelets because of their low volume, rapid availability, and less demanding transportation requirements. The most common freezing method employs 5–6% dimethylsulfoxide (DMSO) as a cryoprotectant and requires <65 °C storage, with DMSO removal possible before freezing.<sup>163,164</sup> The use of cryopreserved platelets results in corrected count increments that are approximately half of the increments seen with liquid apheresis platelets.<sup>163,165</sup> Contemporary autologous reinfusion studies with modern cryopreserved platelets have observed a recovery of 52% and survival of 89% of fresh platelets. Controlled rate freezing appears superior to uncontrolled temperature reduction.<sup>166,167</sup> Nonmilitary use is generally limited to broadly alloimmunized patients refractory to allogeneic transfusion. Collected between successive rounds of high-dose chemotherapy, these units can support patients through short periods of severe thrombocytopenia. Functionally, one unit of frozen platelets has been shown to correct the aspirin-prolonged bleeding time in healthy humans.<sup>168</sup> Results from several retrospective studies suggest safety and efficacy, but the usual limitations, including possible biases and confounders apply.<sup>169–171</sup> Only very few controlled trials with limited patient numbers are available. A multicenter trial included 28 thrombocytopenic hematology-oncology patients with WHO grade II–IV bleeding. Up to three cryopreserved platelet doses were administered, and no major adverse events were observed.<sup>172</sup> The majority of recipients showed improved bleeding scores after receiving cryopreserved platelets.<sup>172</sup> In a randomized, controlled trial in trauma patients, cryopreserved platelets resulted in comparable clinical and laboratory outcomes, but the trial was underpowered to allow for any conclusive statements about efficacy.<sup>173</sup> Similarly, the CLIP-I trial showed comparable outcomes and safety in a cardiac surgery population comprising 23 patients receiving cryopreserved platelets and 18 receiving liquid stored platelets.<sup>174</sup> The group receiving cryopreserved platelets received more FFP than the control group making a definitive interpretation difficult.<sup>174</sup>

Lyophilized platelets have been studied without great success in a resulting product. Animal studies in the 1950s showed no hemostatic effect of early preparations.<sup>175,176</sup> Bode and Read described a

new technique using 1.8% paraformaldehyde to fix washed platelets, followed by freezing in 5% bovine albumin and lyophilization.<sup>177</sup> This preparation has been stored at -80 °C but may be stable at room temperature for shorter periods. Paraformaldehyde treatment appears to confer an additional benefit by killing bacteria and many viral pathogens. Although morphologically normal and able to support adhesion and thrombin generative functions, these platelets do not activate or aggregate. Observation of thrombosis, splenic accumulation, and short circulatory time in animal models has led to limited enthusiasm for the product.<sup>178</sup> A trehalose-based formulation of freeze-dried platelets (thrombosomes) underwent a Phase I dose-escalation study in 10 healthy humans. While no serious adverse events were observed, three out of four subjects in the group with the highest dose had treatment-related adverse events, including EKG abnormalities, elevated D-Dimers, and a platelet autoantibody.<sup>179</sup> In vitro, thrombosomes were able to adhere to collagen, expressed phosphatidyl-serine and promoted thrombin generation. Similar to previous products, minimal aggregation was observed.<sup>180</sup> The role of platelet substitutes, including platelet microvesicles, liposome-based hemostatic agents, fibrinogen-coated albumin microspheres, and red cells coated with fibrinogen or RGD peptides, is beyond the scope of this chapter, and readers are referred to published reviews for additional information.<sup>181-185</sup>

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## CHAPTER 18

# Neutrophil production and kinetics: neutropenia and neutrophilia

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## Introduction

Changes in neutrophil count are frequently encountered in clinical medicine in that increased or decreased neutrophil counts are often seen with infections and other disorders. Neutrophils are the body's primary defense against invading pathogens, particularly bacteria and fungi. Thus, neutrophil changes most often represent a response to an underlying disorder, and they may be the first clue to its presence. At other times, high or low neutrophil counts can be a crucial indicator of a primary hematologic disorder. Increased neutrophils are characteristic of chronic myeloid leukemia and other myeloproliferative disorders, and decreased neutrophils can signal aplastic anemia, myelodysplastic syndrome, acute leukemia, or other disorders of bone marrow function. This chapter does not focus on primary hematologic disorders, but rather on normal neutrophil kinetics and common reasons for aberration, and briefly deals with esoteric disorders that primarily affect myeloid cells.<sup>1</sup>

## Normal neutrophil kinetics

### Production in marrow

Neutrophils are produced in the bone marrow, an extremely proliferative organ of the body. Neutrophils are among the most numerous cells being produced there with approximately  $10^{11}$  generated daily under baseline circumstances. There is a capacity to increase this several fold when stressed.<sup>2</sup> The production of neutrophils and other granulocytes (eosinophils; basophils) is termed *granulopoiesis* or *myelopoiesis*. This occurs in the extravascular space of the bone marrow. Mature cells are stored there until mobilized and released.

Neutrophils originate from multipotent myeloid progenitor cells. Once committed to the granulocyte line, precursor cells differentiate along morphologically distinct myeloblast, promyelocyte, myelocyte, metamyelocyte, band, and then segmented neutrophil stages. The stages of granulopoiesis can be defined not only morphologically, but also by cell surface markers (detected on flow cytometry) and by the mitotic capacity. The mitotic compartment includes myeloblast, promyelocyte, and myelocyte stages, with promyelocytes and myelocytes

comprising the neutrophil-committed mitotic bone marrow pool. The postmitotic pool includes metamyelocytes and band neutrophils. Mature neutrophils then participate in a state of exchange between the bone marrow, blood circulation, and other tissues.<sup>3</sup>

Granulopoiesis is regulated by hemopoietins, cytokines that can stimulate colony formation from progenitor cells in the bone marrow. Hematopoietins include stem cell factor, interleukin-3 (IL3), granulocyte macrophage colony-stimulating factor (GM-CSF), and granulocyte colony-stimulating factor (G-CSF). In addition to stimulating progenitor cells, GM-CSF and G-CSF directly enhance neutrophil function. G-CSF is the principal cytokine regulating granulopoiesis, providing a stimulus at several stages of differentiation.<sup>4</sup> G-CSF induces commitment of multipotential progenitor cells down the myeloid lineage and impacts the proliferation of granulocytic precursors. It also reduces the transit time through the differentiating granulocytic compartment and facilitates neutrophil release from the bone marrow. Thus, G-CSF both markedly expands the marrow neutrophil mitotic pool and it shortens the transit time of differentiating neutrophils through the postmitotic pool. The postmitotic transit time in healthy volunteers shortens from 6.4 days to 2.9 days after G-CSF administration.<sup>5</sup> Genetic expression related to granulocyte differentiation is regulated mainly by myeloid transcription factors PU.1, members of the CCAT enhancer binding protein family, and GFI-1.

The total transit time from myeloblast to mature neutrophil is 10–12 days *in vitro*.<sup>6</sup> Primary granules appear in the promyelocyte stage, containing myeloperoxidase and serine proteases, including neutrophil elastase. In myelocytes, secondary and tertiary granules appear. Secondary granules contain lactoferrin and collagenase; tertiary granules contain gelatinase. Secretory vesicles also appear near neutrophil maturity. Other changes during the maturation process include increases in adherence capabilities, deformability, responsiveness to chemoattractants, and the development of the characteristic segmented nucleus.

### Mobilization of neutrophils

Mature neutrophils remain in the bone marrow for 4–6 days as a storage pool, poised to respond to microbial invaders. These cells are estimated to number  $4.4 \times 10^9/L$  under basal conditions, with  $0.87 \times 10^9$  cells/kg

released daily into the circulation. Only about 2% of neutrophils circulate in the bloodstream at any given time, with about 90% retained in the bone marrow. The half-life of neutrophils in the circulation is 6–8 hours, and they then survive longer in tissues, up to one or two days.<sup>7</sup> The number of neutrophils produced and released from the bone marrow greatly increases in response to stimuli such as corticosteroids, exercise, cytokines such as G-CSF, chemokines, or bacterial products.<sup>5</sup>

The retention, mobilization, and homing of neutrophils to and from the bone marrow are regulated by molecular mechanisms involving CXCR4 (C-X-C motif receptor 4), CXCR2 (C-X-C motif receptor 2), and CXCL12 (C-X-C motif ligand 12). CXCR4 is a cytokine receptor essential for the homing of stem cells and more mature neutrophils to the bone marrow. When deleted in mice, it causes a shift in the pool of mature neutrophils from bone marrow to the circulation with no change in the lifespan of circulating neutrophils.<sup>8</sup> A mutation in CXCR4 causing enhanced responsiveness to CXCL12 underlies the WHIM syndrome (warts, hypogammaglobulinemia, infections, and myelokathexis) in which there is deficiency of neutrophils in the circulation with accumulation of mature neutrophils in the bone marrow. The CXCR4 antagonist plerixafor, FDA-approved to mobilize stem cells to the peripheral blood for collection and transplantation, can treat leukopenia in patients with WHIM syndrome. CXCR2 is a chemokine receptor expressed on myeloid cells that, when deleted, causes the retention of mature neutrophils in the bone marrow. Thus, CXCR4 seems to be necessary to maintain neutrophils in the bone marrow, whereas CXCR2 is necessary for their release. CXCL12, also known as stromal-cell-derived factor 1 $\alpha$ , is a ligand of CXCR4. Bone marrow stromal cells, including vascular endothelial cells and osteoblasts, express CXCL12.<sup>9</sup> CXCL12 expression is high in the bone marrow during normal steady state, acting to retain neutrophils in the bone marrow by binding the CXCR4 on the neutrophils.

Inflammation causes an increase in cytokine levels, including G-CSF, in the bloodstream. This decreases CXCL12 expression in the bone marrow, allowing egress of neutrophils toward circulation where there is a higher level of cytokine. G-CSF treatment decreases CXCL12 mRNA in the bone marrow, decreasing CXCL12 protein expression. G-CSF also decreases surface expression of CXCR4 on neutrophils over 5–18 hours. With less CXCR4 receptors on neutrophils and less CXCL12 expression in the bone marrow, more neutrophils are mobilized into circulation.

After release from the bone marrow, neutrophils become distributed between the circulating and marginal pools, freely exchangeable pools of approximately equal size.<sup>10</sup> Cells in the circulating pool are readily sampled by drawing blood. Cells in the marginal pool are loosely adherent to the endothelium of blood vessels, often slowly rolling along endothelial surfaces.<sup>11,12</sup> They are prepared for rapid egress into the tissues at times of need. Some studies suggest that the marginal pool is not diffusely distributed throughout the vasculature, but is largely localized to the liver, spleen, and lungs.<sup>7</sup> Stressors can influence allocation of neutrophils in each pool. Administration of prednisone increases the size of both the circulating and marginating pools, whereas exercise and administration of adrenaline cause a shift of cells from the marginal pool to the circulating pool.<sup>13</sup>

### Migration into tissues

Neutrophils must cross the vascular wall to get to sites of inflammation, a process referred to as diapedesis. P-selectin and E-selectin on endothelial cells capture neutrophils and mediate rolling by binding platelet sialoglycoprotein ligand-1 (PSGL1),

L-selectin, and CD44. This activates neutrophil integrins that interact with intracellular adhesion molecules (ICAMs) on endothelial cells. Migration through the endothelium is mostly paracellular, but 20% is thought to be transcellular.<sup>8</sup> In paracellular migration, neutrophils loosen the tight junctions by presenting alternative binding partners, platelet-endothelial cell adhesion molecule-1 (PCAM1), JAM-A, and  $\beta_2$  integrins (LFA1 and Mac1), facilitating the ability to squeeze through the tight junctions. Integrin  $\alpha 3\beta 1$  is upregulated and appears crucial to neutrophil diapedesis.<sup>14</sup> In transcellular migration, transmigratory cups expressing high ICAM-1 and VCAM-1 capture the crawling neutrophils, which allows neutrophils to go through the endothelial cells. Leukocyte adhesion deficiency (LAD) syndromes can result from the lack of adhesion molecules such as  $\beta_2$  integrins on the neutrophil surface, producing an inability to deliver neutrophils to sites of infection. Patients with LAD have neutrophilia and recurrent bacterial infections.

Once in the tissue, neutrophils initiate the generation of cytokines, including IL8 and Gro $\alpha$ , allowing recruitment of more inflammatory cells. Neutrophils then can phagocytose microorganisms and undergo apoptosis. Neutrophils in tissues are more actively phagocytic than neutrophils in the circulation.

### Neutrophil clearance

To prevent neutrophil-mediated tissue damage, a negative feedback mechanism controls the influx of neutrophils. Apoptotic neutrophils are phagocytosed by macrophages, which reduces proinflammatory chemokine production and stimulates the release of anti-inflammatory mediators such as prostaglandin E<sub>2</sub> and transforming growth factor- $\beta$ .<sup>1</sup>

The spleen, liver, and the bone marrow clear circulating neutrophils that have not migrated into the tissues. The CXCR4–CXCL12 chemokine pathway mediates the homing of neutrophils back to the bone marrow. Circulating neutrophils upregulate expression of CXCR4 and become “senescent” within a few hours of their release in blood. A chemotactic gradient of CXCL12 created by bone marrow stromal cells allows the neutrophils with upregulated CXCR4 to home back to the bone marrow. In the bone marrow, senescent neutrophils become apoptotic and are phagocytosed by macrophages.<sup>9</sup>

### NETosis

Neutrophils may extend their antimicrobial activity, beyond phagocytosis and degranulation, and beyond their lifespan, by forming neutrophil extracellular traps (NETs). During this process called NETosis, the nuclear and granule membranes dissolve, decondense chromatin, and extrude into the extracellular space along with proteins that were contained in the cytosol and granules.<sup>15</sup> NETs, therefore, are large, extracellular web-like structures consisting of neutrophil cytosolic and granule proteins on a scaffold of decondensed chromatin. NETs can trap pathogens, including bacteria, fungi, viruses, and parasites within the sticky meshwork of chromatin, and expose them to highly concentrated antimicrobial enzymes trapped within the chromatin. Numerous NETosis triggers have been identified, ranging from pathogens themselves to activated platelets and immune complexes. Microorganism size appears to influence whether phagocytosis or NETosis occurs.<sup>16</sup> Although NETs can help clear infections, NETs may also damage tissues. Excessive formation of NETs has been linked to various neutrophil-mediated pathologies, including vasculitis, vascular injury, and thrombosis. Excessive NETosis can

damage the epithelium in pulmonary fungal infections and the endothelium in transfusion-related acute lung injury. It is thought that NETs forming in the circulation can provide a scaffold for deep vein thrombosis formation. Thus, NETs may promote vaso-occlusion. Some have hypothesized that intravascular NETosis plays an important role in the vascular complications related to COVID-19.<sup>17</sup>

## Neutrophilia

Neutrophilia, defined as a neutrophil count greater than  $7.5 \times 10^9/\mu\text{L}$ , may be acute or chronic. (Much higher neutrophil counts are normal in infants less than one-month old (up to  $26 \times 10^9/\mu\text{L}$ ).) Table 18.1 summarizes causes and mechanisms of acute and chronic neutrophilia. Neutrophilias are acquired disorders, most commonly reactive phenomena with only a small minority representing a primary hematologic disorder.<sup>18</sup>

Faced with a symptomatic patient, primary care and emergency room physicians often equate neutrophilic leukocytosis with the presence of infection, and they may even base a decision on the need for hospital admission on its presence. While neutrophilic leukocytosis is the rule with most bacterial infections, it should be recognized that this is a very nonspecific finding that may occur with many causes of inflammation or stress. Mild chronic neutrophilia can even be due to smoking or obesity, where it has been associated with elevated leptin levels. Further, neutrophilic leukocytosis is certainly not sensitive for bacterial infections, and in fact many infections may occasionally or characteristically produce neutropenia.

The evaluation of neutrophilic leukocytosis begins with a history focused on potential causative factors, particularly infections. The history would include systemic symptoms such as fevers, night sweats, and weight loss. Questions are further directed to possible foci of infection such as cough, shortness of breath, dysuria, and abdominal symptoms. Medication history particularly assesses glucocorticoid use. Special attention on physical examination should be devoted to oropharyngeal and lung exams, lymph nodes, and spleen. As with any abnormality of blood cell counts, the review of the peripheral blood smear may be crucial to expeditiously pinpoint the problem. Eosinophils are generally decreased with bacte-

rial infection, so an increase in eosinophils and basophils may point to a primary hematologic disorder (or possibly a parasitic, allergic, or vasculitic disorder).

Pseudoneutrophilia may occur during exercise or time of stress because of epinephrine effects, shifting marginal pool cells to the circulating pool. The shift is rapid but short-lived, and should no more than double or triple the neutrophil count. Immature neutrophils should not be seen.

*Neutrophil left shift* refers to the presence in the circulation of relatively immature cells such as band neutrophils. With a marked left shift, myelocytes and metamyelocytes circulate. Left shift is nonspecific, occurring with infection or with any cause of neutrophilia.

Glucocorticoids cause neutrophilia by decreasing the uptake of neutrophils from the intravascular space. This is similar to the mechanism of neutrophilia in patients with leukocyte adhesion deficiency (CD11/CD18 deficiency). Other drugs known to cause neutrophilia include catecholamines, which affect demargination, and lithium, which may cause G-CSF release.

Leukemoid reaction is a nonclonal neutrophilic leukocytosis with white blood cells (WBCs)  $>50,000/\text{mm}^3$ . It is generally seen with an uncontrolled inflammatory state, cancer, asplenia, infection, recovery from neutropenia with or without the administration of colony stimulation factor, or some combination of these causes. In chronic infection and/or inflammation, the neutrophil production rate can increase threefold. G-CSF or GM-CSF administration can further increase neutrophil production, with a maximum response seen in 7–10 days. Overshoot in patients recovering from neutropenia is common, such as after myelosuppressive chemotherapy.

*Leukoerythroblastosis* is defined as the presence of immature myeloid cells (myelocytes) and nucleated RBCs in the peripheral blood, often with giant platelets as well. Leukocytosis is usually present. Two-thirds of patients with this finding have an underlying infiltrative disorder of the bone marrow, such as fibrosis, granuloma, necrosis, or metastatic tumor. Leukoerythroblastosis may also commonly accompany brisk hemolytic anemia. In about one-tenth of cases, it may result from hypoperfusion of the bone marrow (shock from sepsis, hemorrhage, anaphylaxis, or cardiac failure) causing disruption of the microenvironment. If an underlying cause of leukoerythroblastosis is not readily apparent, bone marrow biopsy is usually warranted. In the past, a major differential diagnosis was to distinguish reactive leukoerythroblastosis from chronic myelogenous leukemia (CML) or another myeloproliferative neoplasm. Helpful to differentiate these were the clinical situation, splenomegaly, eosinophilia, and basophilia. Leukocyte alkaline phosphatase, high with reactive neutrophilia but low with CML, was sometimes a final arbiter but is becoming obsolete with the availability of molecular tests for the Philadelphia chromosome t(9;22) (and for Jak2 mutations).

Chronic neutrophilic leukemia is a very rare myeloproliferative neoplasm characterized by sustained neutrophilia and splenomegaly. It mainly occurs in the elderly and carries a poor prognosis. Bone marrow biopsy shows neutrophilic hyperplasia without excess of myeloblasts.<sup>19</sup>

## Neutropenia

An absolute neutrophil count (ANC) of less than  $1500/\text{m}^3$  is considered to be neutropenia. ANC is the percentage of neutrophils multiplied by the WBC count. An ANC of  $1000\text{--}1500/\text{m}^3$  is considered mild neutropenia,  $500\text{--}1000/\text{m}^3$  moderate, and below

**Table 18.1** Causes and Mechanisms of Neutrophilia

	Cause	Mechanism
Acute	Stress	Decreased margination
	Epinephrine	Decreased margination
	Exercise	Mobilization from marrow
	Infection	Mobilization from marrow
	Inflammation	
	Stress	
	Corticosteroids	Mobilization from marrow
	Granulocyte colony-stimulating factor	Prolonged survival
Chronic	Rebound from neutropenia	Increased marrow proliferation
	Corticosteroids	Prolonged survival
	Leukocyte adhesion deficiency	Prolonged survival
	Myeloproliferative disorders	Increased marrow proliferation
	Infection	Increased marrow proliferation
	Inflammation	Increased marrow proliferation

500/m<sup>2</sup> severe neutropenia. The risk of infection is greatly increased with severe neutropenia, more so with ANC below 100/m<sup>2</sup>. Agranulocytosis is a condition of acute severe neutropenia with markedly diminished or absent white cell precursors in the bone marrow.

Neutropenia is commonly encountered in clinical practice. Like neutrophilic leukocytosis, the clinical significance of neutropenia varies greatly, starting with conditions that have no impact on overall health and longevity and that may require no evaluation beyond history, physical exam, and review of the blood smear. On the opposite end of the spectrum there are neutropenic disorders that can severely impact health and survival. Acute neutropenia with fever can be a life-threatening medical emergency. Other than the ANC, critical factors in determining the importance of neutropenia and the urgency of evaluation include whether it is an affected child or adult, if it can be determined to be congenital or acquired, if it is encountered in an inpatient or in the clinic, whether there have been infectious sequelae, what are the concordant symptoms and signs (e.g., arthritis, skin rash, and/or splenomegaly), and whether the patient is acutely ill. On physical exam, careful attention should be directed to the oropharynx, lymph nodes, and spleen.

Review of the peripheral blood smear is invaluable in the initial approach to neutropenia. Helpful findings may include neutrophil inclusions, such as Döhle bodies, and hypogranularity (pale cells due to a paucity of secondary granules). Most neutrophils have 3–4 lobes in their nucleus; 5% with five or more lobes indicates hypersegmentation. This is constantly found in megaloblastic processes and may also occur with antimetabolite drugs and with uremia. Changes in other cell lines may bear on the cause of neutropenia, such as large granular lymphocytes (LGLs) or other atypical lymphoid cells. Reactive lymphocytes can be a clue to viral infection. Blast cells may indicate leukemia.

Beyond the blood smear, tests commonly helpful in the evaluation of neutropenia include rheumatoid factor and antinuclear antibodies when there is reason to suspect an autoimmune problem. A direct Coombs test may also help to confirm an autoimmune diathesis. Flow cytometry may clarify the etiology of neutropenia in a number of disorders. This is particularly true when blood smear findings or splenomegaly creates suspicions for LGL syndrome/T-NK leukemia, hairy cell leukemia, or other clonal proliferations. Bone marrow exam is often valuable, particularly in more severe neutropenic disorders, although it should be realized that morphologic findings may be nonspecific. One example is that a hypercellular marrow with “maturation arrest” picture could be compatible with a stem cell maturation defect, antibodies to late precursors, early release in times of stress, or recovery from a toxic insult.

Conditions commonly resulting in neutropenia are listed in Table 18.2, and they are briefly discussed below.

### Congenital neutropenias

The molecular mechanisms underlying severe congenital neutropenias (SCNs) have been greatly clarified in recent years, and the prognosis for these patients has improved due to advances in supportive care, the use of granulocyte growth factors, and allogeneic bone marrow transplantation.<sup>20</sup> These patients have suffered from recurrent severe infections starting in infancy, and oral ulcerations and painful gingivitis by two years of age. As enumerated in Table 18.2, there are many defined conditions causing such neutropenia. Notable congenital neutropenias include the following:

**Table 18.2** Some Conditions Causing Neutropenia

#### Hereditary Neutropenias

- Severe congenital neutropenia (SCN)
  - SCN1: ELANE gene mutation
  - SCN2: GF11 gene mutation
  - SCN3 (Kostmann disease): HAX1 mutation
  - SCN 4: G6PC3 gene mutation
  - SCN 5: VPS45 gene mutation
  - X-linked SCN: WASP gene mutation
- Shwachman–Bodin–Diamond syndrome
- Cyclic neutropenia
- Duffy null red cell phenotype (previously “Benign Ethnic Neutropenia”)
- Myelokathexis (WHIM syndrome)
- Chediak–Higashi syndrome
- P14 deficiency syndrome
- Glycogen storage disease type 1 (von Gierke)
- Barth syndrome
- Cohen syndrome
- Charcot–Marie–Tooth disease
- Hermanski–Pudlak syndrome
- Gricelli syndrome
- GATA2 deficiency (MonoMAC syndrome)

#### Infections

- Overwhelming bacterial sepsis
- Particular pathogens (such as typhoid, ehrlichiosis, brucellosis, and rickettsia)
- Measles, rubella, and varicella
- HIV, EBV, CMV, hepatitis A, and hepatitis B
- Other viruses
- Granulomatous marrow infection (such as histoplasmosis and TB)

#### Drugs

(see Table 18.3)

- #### Autoimmune Conditions
- Systemic lupus erythematosus
  - Evan’s syndrome (ITP and AIHA)
  - Felty syndrome

#### Malignancies

- Acute leukemias
- LGL leukemia
- Hairy cell leukemia
- Myelodysplastic syndromes

#### Dietary

- Global caloric malnutrition
- Copper deficiency
- Alcoholism
- Vitamin B<sub>12</sub> and folate deficiency

#### Other Conditions

- Hypersplenism
- Hyperthyroidism

- SCN1 is an autosomal dominant disorder caused by mutation in the neutrophil elastase gene on the ELANE gene on chromosome 19p13.3.
- The same ELANE gene is mutated in cyclic neutropenia.<sup>21</sup>
- Kostmann syndrome is an autosomal recessive disorder linked to mutations in HAX1. It is associated with mental retardation.
- Myelokathexis is caused by a mutation in the CXCR4 chemokine receptor, part of the WHIM syndrome mentioned in this chapter. It consists of severe neutropenia in the setting of hypercellular marrow containing hypersegmented neutrophils.
- Shwachman–Bodin–Diamond syndrome (SBDS) is an autosomal recessive disorder usually with mutations in the SBDS gene, consisting of exocrine pancreatic insufficiency, bone marrow dysfunction, skeletal abnormalities, and short stature. There is a variable degree of neutropenia.
- Chediak–Higashi syndrome patients have cutaneous and ocular hypopigmentation, and mild neutropenia with large abnormal granules in the neutrophils. Neutrophil dysfunction leads to fre-

- quent pyogenic infections. Platelet granules are also affected resulting in a bleeding diathesis.
- Cyclic neutropenia is characterized by recurrent fevers, mouth ulcers, with infections due to regularly recurring severe neutropenia. G-CSF can be used to treat cyclic neutropenia.
  - MonoMAC syndrome is an autosomal dominant disorder due to mutations in the GATA2 transcription factor. It may be associated with mild chronic neutropenia and persistent severe monocytopenia, leading to opportunistic infections and a high risk of eventual leukemic progression.
  - The p14 deficiency syndrome, lacking an adaptor protein involved in mitogen-activated protein kinase signaling, is characterized by severe neutropenia, partial albinism, short stature, and recurrent infections.
  - Neonatal alloimmune neutropenia occurs when there is transplacental passage of IgG antibodies to neutrophil-specific antigens. The neutropenia can be moderate to severe.<sup>22</sup>

### Neutropenias in adults

In the hospital, the most common causes of neutropenia are medication reactions or sepsis, and these become especially suspect if the neutropenia has its initial onset during hospitalization. While almost any drug can rarely cause neutropenia, it is most closely associated with certain medications and classes. It is important to consider whether there is agranulocytosis (sudden- and severe-onset neutropenia) vs. a gradual decline in ANC, as the former is particularly characteristic of a few drugs. Table 18.3 reviews more common drug associations.<sup>23</sup>

Sepsis characteristically leads to increased neutrophil count, but neutropenia can also be seen relatively often, particularly in those with compromised marrow reserve such as an underlying hematologic disorder, chemotherapy, or malnutrition. Neutropenia in this context augurs a high mortality rate. Other infections that may be characterized by neutropenia include bacteria, such as typhoid fever and brucellosis, and nonbacterial infections, such as anaplasmosis, ehrlichiosis, and rickettsia. Neutropenia or pancytopenia is seen with granulomatous marrow involvement by histoplasmosis or mycobacteria. Transient neutropenia is very common with viral infection, so intensive evaluation of a low neutrophil count should ordinarily be delayed in those where an acute viral etiology seems likely. Viruses with a particular predilection to cause this include HIV, hepatitis A, hepatitis B, Epstein–Barr virus (EBV), cytomegalovirus (CMV), measles, rubella, and varicella.

Neutropenia is an expected and dose-limiting side effect of many cytotoxic and/or immunosuppressive drugs, such as those used for cancer chemotherapy or to prevent transplanted organ rejection.

Prophylaxis with growth factors and/or antibiotics is recommended if the risk of developing febrile neutropenia is greater than 20%. This risk can be calculated from age, extent of primary cancer, comorbidities, and the known myelotoxicity of the chemotherapy regimen (according to the National Comprehensive Cancer Network [NCCN], Multinational Association of Supportive Care of Cancer [MASCC] score, and Infectious Diseases Society of America [IDSA] risk assessment). With high risk, G-CSF or a long-acting analog should begin 24–72 hours after the completion of myelotoxic chemotherapy.

Other medications may cause neutropenia with variable frequency, severity, and mechanisms.<sup>24</sup> Neutropenia is sometimes sudden and severe. Dose- and duration-related myelosuppression may be seen with chlorpromazine, ticlopidine, and valganciclovir. Chloramphenicol can cause neutropenia by both dose-related and idiosyncratic mechanisms. Clozapine has been shown to induce apoptosis of myeloid precursors. Immune mechanisms have been implicated in neutropenia from antithyroid drugs and aminopyrine. An epidemic of severe neutropenia was first appreciated in 2007 in intravenous cocaine abusers and was linked to levamisole adulteration. Also, increasingly recognized in recent years is neutropenia related to rituximab. This is unusual for its late onset, typically three months after the last dose, and may be related to imbalanced recovery of B-lymphocyte subsets with resultant deficiency of stromal-derived factor 1.

Immune mechanisms may be implicated relatively commonly in neutropenia, sometimes in the context of autoimmune disorders such as systemic lupus erythematosus (SLE) or rheumatoid arthritis (RA). Immune neutropenia can vary from mild to severe. Antineutrophil antibodies are classically imputed, but autoreactive cytotoxic lymphocytes can also play a role. Bone marrow examination most often shows normal to increased cellularity with maturation arrest, but this picture is nonspecific (as discussed above). Available antineutrophil antibody tests are not clinically reliable, creating substantial challenges to validate the diagnosis. Sometimes, empiric immunosuppressive therapy (e.g., methotrexate) may enlighten in retrospect as to whether immune factors were at play. Felty syndrome is a complication of RA with splenomegaly and neutropenia that may be severe, and it is traditionally attributed to antineutrophil antibodies. This occurs in 1% of patients with RA and may be complicated by severe infections. Felty syndrome may be on a continuum with large granular lymphocyte proliferation (see below). The mainstay treatments for Felty syndrome are methotrexate, cyclosporine, or cyclophosphamide. G-CSF engenders a risk of exacerbating underlying inflammatory problems, so it should be used cautiously.

Splenomegaly can be a cause of leukopenia, anemia, thrombocytopenia, or any combination of these. The degree of cytopenia is

**Table 18.3** Drugs Commonly Causing Neutropenia and/or Agranulocytosis. Source: Based on Gibson and Berliner (2014).<sup>23</sup>

Antimicrobials	Vancomycin TMP-SMX Dapsone	Cephalosporins Semisynthetic Penicillins Ganciclovir Captopril Phenytoin	Chloramphenicol Flucytosine Linezolid Procainamide Ethosuximide
Cardiovascular Anticonvulsants	Ticlopidine Carbamazepine Valproate		
Psychotropic Agents Antithyroid Anti-inflammatory	Clozapine Methimazole Sulfasalazine Phenylbutazone	Phenothiazines Propylthiouracil	
Immunosuppressives Others	Mycophenolate mofetil Levamisole	Diclofenac Azathioprine Deferiprone	Indomethacin Methotrexate Rituximab

generally proportional to the degree of splenic enlargement. Additional factors such as drugs or autoimmune diseases should be considered if neutropenia is out of proportion to the splenomegaly and to the reduction in other cell lines.

T-cell LGL leukemia and other natural killer (NK) cell proliferations should be considered in adults with neutropenia. NK cell proliferations can vary from nonclonal reactive processes, to indolent clonal proliferations, to very aggressive neoplasms. Reactive LGLs can be idiopathic, or they can be seen in autoimmune disorders, after organ transplant or during therapy with dasatinib, a tyrosine kinase inhibitor.<sup>25</sup> Similarly, clonal T-NK-cell/LGL leukemia can be associated with RA or can be idiopathic. Felty syndrome can be associated with increases in T-NK cells. Peripheral blood smear shows large lymphocytes with mature chromatin, excess cytoplasm, and usually prominent cytoplasmic granules. Flow cytometry shows CD3 and CD57 positive cells, and molecular studies show T-cell receptor gene rearrangement and, in about half of cases, stat3 gain-of-function mutations. T-NK clonal disease most often responds to methotrexate, cyclosporine, or cyclophosphamide therapy. Pure NK cell proliferations are generally CD3 negative and CD56 positive.

A large majority of people of sub-Saharan African descent display a Duffy null [Fy(a-b-)] red blood cell phenotype, protective from malaria, and associated with a lower number of circulating neutrophils. Thus, African Americans have a lower mean ANC compared to Caucasians, with 5% falling below 1500, but there is not an increased risk of infection. This can also be seen in other ethnic groups, including those of Middle Eastern heritage. There is a current effort to no longer designate this as “Benign Ethnic Neutropenia” so as not to stigmatize people who have no clinical disease.<sup>26</sup>

Chronic idiopathic neutropenia is the default diagnosis in adults who have acquired the problem but do not meet diagnostic criteria

for established etiologies.<sup>21</sup> This usually runs a benign course, but some may require growth factor support at times of illness.

When treatment for neutropenia is indicated, one addresses the specific cause (e.g., withdraw suspicious medications), but additionally G-CSF may facilitate recovery. This may be required episodically or on a regular basis with chronic afflictions. This does not come without a price. After 10 years of G-CSF therapy, the risk of death from MDS and AML was reported as 21% in patients with SCN,<sup>27</sup> higher than 13% after eight years of G-CSF therapy reported by the Severe Chronic Neutropenia International Registry in 2003.<sup>28</sup> The later report did find a statistically significant association between the dose of G-CSF and the risk of MDS and AML in SCN. Prompt empiric initiation of appropriate antibiotics is an additional measure that may be life-saving in the situation of fever with severe neutropenia. As stressed in this chapter, changes in neutrophil counts are most often reactive phenomena, but occasionally they are manifestations of a primary hematologic process. Accelerated progress continues in our ability to understand and address these disorders.

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## CHAPTER 19

# Granulocyte collection and transfusion

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Life-threatening infections with bacteria, yeast, and fungus continue to be a consequence of severe neutropenia, with an absolute neutrophil count less than 500/ $\mu$ L, and disorders of granulocyte (PMN) dysfunction. The most frequent situation is neutropenic fever and infection with yeast or fungus during either hematopoietic progenitor cell (HPC) transplantation or intense chemotherapy for hematologic malignant disease. Neutropenic infections cause considerable morbidity, occasionally are fatal, and add considerable cost to the treatment of these patients. One approach has been the use of granulocyte transfusions (GTXs).

Granulocytes can be collected by apheresis but because of the relatively low concentration of granulocytes in the circulation it is necessary to increase the level of circulating cells to collect an adequate dose for transfusion. Historically, the granulocyte count was elevated by the use of corticosteroids but even this did not yield a very meaningful dose of granulocytes. More recently, the hormone granulocyte-colony stimulating factor (G-CSF) has been used to mobilize the donors to produce high-dose granulocyte concentrates. This has led to renewed interest in granulocyte transfusions.

Therapeutic GTX to treat known or suspected infection has not gained broad acceptance despite many reports documenting benefits.<sup>1,2</sup> This lack of enthusiasm for GTX can be explained by the continuing development of effective antimicrobial drugs to prevent and manage infection, by the availability of recombinant hematopoietic growth factors to quicken granulocyte recovery, by preference for peripheral blood HPC over marrow-derived HPC transplantation to hasten patient recovery from myelotoxic therapy and to shorten the period of severe neutropenia, and by the lack of familiarity/experience by most physicians with the markedly improved PMN concentrates.

This chapter discusses the current technology of granulocyte collection and provides a critical assessment of the potential for the use of therapeutic and prophylactic GTX.

## Collection of granulocytes for transfusion

### Donor selection

Several factors must be taken into consideration beyond the standard allogeneic blood donor's eligibility screening criteria. These elements include the following: (1) ABO and Rh type, (2) CMV status,

(3) recent blood or apheresis donation, and (4) medical conditions that would contraindicate the use of corticosteroids and/or the hormone granulocyte-colony stimulating factor (G-CSF) as well as exposure to the erythrocyte sedimenting agent hydroxyethyl starch.

Donor granulocyte concentrates must be ABO and Rh compatible with the patient because of the red cell content found within the final GTX product. An in vivo study of indium labeled granulocytes shows that normal migration occurs in a chemotactic chamber containing ABO-incompatible granulocytes that are free of red cells.<sup>3</sup> This result confirms the lack of ABO antigens on the granulocytes cell.<sup>4,5</sup>

Pediatric and transplant patients should receive products from donors with CMV negative status as the virus is known to reside within leukocytes.<sup>6</sup> For the typical whole blood donation, this viral transmission risk would be significantly reduced by leukoreduction, but as this manufacturing step is not performed with granulocyte concentrates, it is best to consider providing a CMV negative donor if the patient's need requires it.

As the collected product will more than likely be distributed prior to having the infectious disease test results made available, consider scheduling donors who have undergone a blood donation in the past month. Another benefit for using platelet donors is that they are likely to have undergone anti-human leukocyte antigen (HLA) antibody screening to reduce the risk of a type of transfusion reaction called transfusion-related acute lung injury (TRALI).

Medical assessment must be performed to determine if the donor could tolerate the administration of drugs received for granulocyte stimulation and the exposure to the erythrocyte sedimenting agent, hydroxyethyl starch (HES). For example, contraindications for the use of corticosteroid include the following: diabetes, glaucoma, gastrointestinal ulcers, hypertension, and history of allergy to corticosteroid use. For G-CSF, it is required to determine if there was a prior exposure to the drug that resulted in an allergic reaction. HES requires a healthy donor who does not have a recent history for renal dysfunction, active heart disease, as well an allergic history to the reagent.<sup>7</sup>

### Histocompatibility matching

Granulocyte surfaces are coated with HLA and granulocyte-specific antigens<sup>8</sup> but probably not ABO.<sup>4,5</sup> Older studies of CML cells show that leukoagglutinating or lymphocytotoxic antibodies interfere

with the in vivo localization of granulocytes.<sup>9,10</sup> More specific studies of Indium labeled cells demonstrate the failure of granulocytes to localize at sites of inflammation<sup>9</sup> and also excess sequestration of granulocytes in the lungs,<sup>10</sup> indicating that incompatibility interferes with the clinical effect of the cells. One difficulty in developing a practical approach to this is that most of these patients' serum contains both HLA and granulocyte-specific antibodies, making it difficult to distinguish whether HLA is important. Clearly, granulocyte agglutinating antibodies interfere.<sup>9</sup> However, the practical application of this is difficult because granulocytes must be transfused within eight hours of collection. With such a short time frame, it is not feasible to collect and have available several granulocyte units to crossmatch and select a compatible unit for transfusion.

If the specificity of the granulocyte antibodies is identified, then consideration should be made to have family members tested to find compatible donors. It is unlikely that the general apheresis donor population is phenotyped on a routine basis for granulocyte-specific antigens; however, as a phenotype is more likely among familial relations due to genetic inheritance, a closer look at family members is of interest. Such testing is not only time consuming but is not always accessible as the technology for granulocyte antibody antigen workup is available only in a small number of research laboratories. Because of these factors, performing phenotype testing of donors would not be an option for acute needs.

### **Donor granulocyte stimulation**

A limitation of GTx has been the insufficient number of granulocytes collected to meet clinical need. Granulocyte concentrates collected from healthy donors who are not stimulated with corticosteroids or G-CSF will contain between 0.2 and  $1.0 \times 10^{10}$  PMNs—about 1% of a healthy marrow's output. To ensure adequate numbers and quality of PMNs for transfusion, granulocytes must be collected from optimally stimulated donors by automated leukapheresis using an erythrocyte-sedimenting agent, such as hydroxyethyl starch.

Donor stimulation with properly timed corticosteroids ( $\leq 12$  hours before leukapheresis) increases the number of circulating granulocytes by releasing those that were adhering to the vascular walls. In this manner, the granulocyte yield can expand two- to threefold higher from baseline for a healthy individual blood donor.<sup>11,12</sup>

The use of granulocyte colony-stimulating factor mobilizes PMNs directly from the marrow, will produce, especially in combination with corticosteroids, as much as 14-fold higher granulocyte yields when compared to unstimulated blood donors.<sup>13–17</sup> An example of a typical G-CSF stimulation protocol includes administering 300–480 µg of the drug subcutaneously and 8 mg dexamethasone orally, approximately 12 hours before beginning leukapheresis. Yields of  $4\text{--}8 \times 10^{10}$  PMNs are achieved regularly.

Adverse effects following G-CSF and dexamethasone are frequent but generally mild in nature.<sup>17</sup> Most donors experience minor discomfort including myalgia, headache, and generalized bone pain that is readily relieved with acetaminophen or ibuprofen. Donors given G-CSF for PMN collection have no reportable significant long-term adverse effects. One reassuring study found no cytogenetic abnormalities in individuals who had received G-CSF years earlier.<sup>18</sup> However, as a few reports suggest that the use of corticosteroids might cause posterior subcapsular cataracts in PMN donors<sup>19,20</sup> it seems logical to include cautionary information in the donor consent forms regarding the use of G-CSF and corticosteroids.

### **Apheresis collections and granulocyte concentrate manufacture**

Donation of granulocytes consists of utilizing an automated apheresis collection device such as the Spectra Optia (Terumo BCT) and the Trima Accel (Terumo BCT). The continuous flow separators typically processes 8–10 L of donor blood with the use of a sedimenting agent, such as hydroxyethyl starch (HES), for a minimum target volume of 250 mL ( $\pm 50$  mL). The final product consists of a minimum  $1 \times 10^{10}$  granulocytes per unit and approximately 20–50 mL red blood cells.<sup>21</sup>

Use of an erythrocyte-sedimenting agent, such as HES or pentastarch, during centrifugation leukapheresis is necessary to improve efficiency of cellular collection.<sup>11</sup> Although pentastarch was once advocated due to decreased impact on coagulopathy and increased removal from circulation,<sup>11</sup> HES is more widely used.<sup>22–24</sup> Hydroxyethyl starch is known to have a higher collection efficiency resulting in a larger PMN collection yield and has a sufficient safety profile that allows for its continued use with donors who undergo recurring collections.<sup>22,25,26</sup> Typical side effects of HES are mild in nature and include bone pain, headaches, generalized rash, and fatigue.<sup>17</sup> A decrease in the hematocrit is not surprising, due to the volume of red cells collected, which is a secondary effect of the sedimenting activity of HES.<sup>27</sup>

### **Function**

These granulocytes have normal bacterial killing, chemotaxis, and chemiluminescence superoxide production.<sup>28–31</sup> Thus, they should function normally in vitro. In vivo there is normal intravascular recovery and survival and particularly important normal migration to sites of inflammation.<sup>32–34</sup> The use of a G-CSF for donor stimulation does not alter these normal functions in vitro and in vivo.<sup>32</sup>

### **Granulocyte storage and distribution conditions**

As granulocytes have a short shelf life, it is best to transfuse the product as soon as possible after collection, preferably within 6–8 hours to ensure donor granulocyte function is retained.<sup>33</sup> The product is generally stored at room temperature, 20–24 °C, with little or no agitation for no more than 24 hours.<sup>21</sup> This was arrived at by in vivo studies of indium labeled granulocytes, where intravascular recovery, survival, and migration to sites of inflammation or in a skin window were used to assess in vivo function. Storage at 1–6 °C for 24 hours resulted in decreased intravascular circulation and reduction in migration to skin window reference,<sup>33</sup> but storage at room temperature for eight hours did not reduce the intravascular recovery or migration into the skin chamber. Storage at 1–6 °C for even eight hours reduced in vivo granulocyte function; thus, storage for up to eight hours at room temperature is recommended.

### **Granulocyte transfusion in clinical medicine**

#### **Historical overview of therapeutic granulocyte transfusion**

Granulocyte transfusions may be considered the oldest form of cell therapy with "leukocyte cream" reported to treat neutropenia in 1934. Subsequent studies in aplastic dogs showed that transfused granulocytes migrated to sites of infection. Initial studies were challenging due to difficulties obtaining enough granulocytes from healthy volunteers until the development of blood-flow separator in 1969. Case series suggested favorable outcomes and were followed

**Table 19.1** Historical Controlled Trials of Therapeutic Granulocyte Transfusions

Investigators	Success	Survival		Characteristics of Neutrophil Concentrates				
		Study Group N	(%)	Control Group N	(%)	Dose ( $\times 10^{10}$ )	Schedule	HLA/WBC*
Higby et al. <sup>36</sup>	Yes	17	76	19	26	2.2	Daily	Yes
Vogler and Winton <sup>37</sup>	Yes	17	59	13	15	2.7	Daily	Yes
Herzig et al. <sup>35</sup>	Yes	13	75	14	36	1.7	Daily	Yes
Alavi et al. <sup>38</sup>	Partial	12	82	19	62	0.4	Daily	
Graw et al. <sup>39</sup>	Partial	39	46	37	30	5.9	Daily	No
Winston et al. <sup>41</sup>	No	48	63	47	72	2	Daily	Yes
Fortuny et al. <sup>40</sup>	No	17	78	22	80	0.6	Daily	Yes

by randomized controlled trials with conflicting results (Table 19.1). Three of the seven studies<sup>35–37</sup> reported a significant overall benefit for GTx while in two other studies<sup>38,39</sup> only certain groups of patients were found to benefit significantly. An analysis of these studies suggested that patients in the successful studies received relatively high doses of PMNs ( $>1.7 \times 10^{10}$  per day), and the donors were both erythrocytes and leukocytes compatible. Furthermore, two of the four studies with negative findings used PMNs collected by filtration leukapheresis for some patients<sup>38,39</sup> while it is now established that such PMNs have functional deficiencies.<sup>28</sup> Finally, control subjects responded particularly well to antibiotics alone in three of the four negative studies,<sup>38,40,41</sup> suggesting that these patients fared relatively well with conventional treatment alone, and it was not possible to document additional benefit by adding GTx. The results of the seven controlled GTx trials have been analyzed by formal meta-analysis,<sup>42</sup> and conclusions were that the low doses of PMNs transfused and the relatively high survival rate of the nontransfused control subjects were primarily responsible for the differing success rates of these studies.

### Contemporary experience with granulocyte transfusions

No satisfactorily designed and conducted randomized clinical trials of therapeutic GTx collected after G-CSF and dexamethasone donor stimulation have been reported to establish either the efficacy or the potential toxicity of modern GTx. Two randomized clinical trials<sup>43,44</sup> have been reported, but both have shortcomings and flaws that preclude firm conclusions or guidelines for clinical practice. Accordingly, practices must be based on an assessment of the overall literature.

Many case reports, which must be viewed with caution, suggest success for GTx. Most of them are reports of single patients responding favorably to GTx<sup>45,46</sup> reported patients with aplastic anemia, undergoing progenitor cell transplantation. Ozsahin et al.<sup>47</sup> and Bielorai et al.<sup>48</sup> reported single patients with chronic granulomatous disease and fungal infections. Bielorai et al.<sup>49</sup> reported a single patient with acute leukemia and sepsis with vancomycin-resistant *Enterococcus*. Lin et al.<sup>50</sup> or a single patient with multidrug-resistant *Pseudomonas* sepsis who underwent a successful HPC-A transplantation supported by GTx.

There are six studies of larger numbers of infected, neutropenic patients given GTx from G-CSF-stimulated donors (Table 19.2). However, even with investigation of larger numbers of patients, the benefits of GTx remain unclear because some lack concurrent control patients treated with antibiotics alone. In addition, in some studies<sup>51,52</sup> granulocytes were collected from donors stimulated only

with G-CSF and selected without regard for leukocyte compatibility. Although GTxs were successful in most patients, it was not possible to distinguish responses of fungus versus yeast infections. In three studies,<sup>52–54</sup> success was excellent for bacterial and stable fungus infections but was quite poor for progressive fungus infections with organ dysfunction.<sup>54</sup> Historical control patients ( $n = 74$ ) who did not receive GTx were included. Comparative success was similar between GTx recipients and nontransfused patients, and non-transfused controls had better success than PMN recipients for bacterial infections. The authors suggested that by including only patients with microbiologically documented infection they may have selected a group that was too advanced for the intervention to be of benefit. The study also indicated that allogeneic transplant recipients given GTx from unrelated donors experienced grade IV graft-versus-host disease more (GVHD) than controls who were not given GTx; however, relatively few patients were assessed for this end point. A recent retrospective review<sup>55</sup> showed that GTx did not affect the incidence of (GVHD).

Two additional studies reported results in ways that did not lend themselves to tabulation. Illerhaus et al.<sup>58</sup> transfused 42 neutropenic patients with GTx collected from donors stimulated with 5 µg/kg G-CSF. Eighteen of the patients had severe infections with a variety of organisms, and each received a median of three GTx each containing a median of  $2.6 \times 10^{10}$  leukocytes. Of these 18 patients, 8 (44%) improved, 4 (22%) stabilized, and 6 (33%) deteriorated—with 4 of these last 6 dying of pulmonary aspergillosis. Rutella et al.<sup>59</sup> transfused 20 patients with hematologic malignancies and neutropenic infections. Donors were HLA-identical siblings, and each received 5 µg/kg G-CSF. Favorable responses were seen in 54% of patients with bacterial and 57% of fungal infections. Underlying cancer status (i.e., complete or partial remission achieved) and recovery of blood PMN counts to 500/µL or more were significantly correlated with a favorable response to GTx.

No firm conclusions could be drawn from these larger reports of GTx for several reasons: (1) no concurrent control subjects were included (i.e., randomly assigned to receive antimicrobial drugs, but no GTx); (2) the number of patients reported, generally, was quite small; and (3) granulocyte collection methods were variable with a broad range of granulocyte doses transfused. Based on these preliminary findings, bacterial infections appeared to respond quite well to GTx, and relatively mild fungus and yeast infections responded modestly well. However, serious fungus infections with tissue invasion often resisted even the large doses of granulocytes<sup>51,53,57,58</sup>

There are two randomized, multicenter phase III trials, one from Europe<sup>43</sup> and the other from the United States.<sup>44</sup> The European

**Table 19.2** Neutropenic Patients Treated with GTx Collected from G-CSF-Stimulated Donors

Investigators	PMNs $\times 10^{10}$ per GTx	Stimulation	Leukapheresis	Outcomes
Hester et al. <sup>56</sup>	4.1	G-CSF 5 µg/kg	Pentastarch 7 L processed	60% (9 of 15) success with fungus (11 patients) and yeast (4 patients)
Grigg et al. <sup>53</sup>	5.9*	G-CSF 10 µg/kg	Dextran 10 L processed	100% (3 of 3) success with bacterial infection 0% (0 of 5) success with progressive fungus
Peters et al. <sup>51</sup>	3.5*	G-CSF 5 µg/kg or prednisolone	Hetastarch 6.4 L processed	67% (2 of 3) success with stable fungus 82% (14 of 17) success with bacterial infection
Price et al. <sup>52</sup>	8.2	G-CSF 600 µg/kg plus dexamethasone 8 mg	Hetastarch 10 L processed	54% (7 of 13) success with fungal infection 100% (4 of 4) success with bacterial infection 0% (0 of 8) success with invasive fungus
Hubel et al. <sup>54</sup>	4.6–8.1	G-CSF 600 µg/kg with or without dexamethasone 8 mg	Hetastarch or pentastarch 10 L processed	57% (4 of 7) success with yeast infection 55% (unrelated donor) success with bacterial infection 75% (family donor) success with bacterial infection 70% (unrelated donor) success with yeast infection 40% (family donor) success with yeast infection 15% (unrelated donor) success with fungal infection 25% (family donor) success with fungal infection
Lee et al. <sup>57</sup>	5.1–10.6	G-CSF 5 µg/kg and/or dexamethasone 3 mg/m <sup>2</sup>	Pentastarch 6–10 L processed	40% (10 of 25) success with multiple-organism infections

\* Assumptions made as PMN dose expressed  $\times 10^{10}$  unclear in these reports. PMN dose calculated using values for the total number and differential count of leukocytes collected, and the volume of units collected (Grigg et al.<sup>42</sup>). Dose calculated that would be given to a 70-kg recipient for Peters et al.<sup>44</sup> GTx, granulocyte transfusion; G-CSF, granulocyte colony-stimulating factor; PMNs, granulocytes.

multicenter randomized trial included 74 patients in five centers in Austria and Germany randomized between 1999 and 2005. Due to declining recruitment, the published study included less than 50% of the expected sample size. The patients did not have to have a microbiologically documented infection. Other limitations of the study included low number of transfusions (17 of 39 patients in the GTx arm received only one or two GTx before neutrophil recovery) and significant crossover between arms. Donors were stimulated only with G-CSF, and the doses of granulocytes per infusion were also lower than desired: 16% of the transfusions had less than the recommended by the study design neutrophil dose of  $3 \times 10^8$  per kg (which is still significantly less than the currently ideal  $4 \times 10^{10}$  dose). There was no subgroup identified that seemed to benefit more than the others. The RING (Resolving Infection in Neutropenia with Granulocytes) study was able to only partially overcome the limitations of prior trials. As in the previous trial, accrual was too slow and only 114 patients were randomized out of a target sample of 236. Originally, only patients with proven infection were recruited; however, as a result of the slow accrual, the inclusion criteria were changed to allow patients with presumed infection, and the time between meeting eligibility and randomization was extended from the original 24 hours after diagnosis to one week. The primary end point of clinical success was defined as survival to Day 42 plus clinical response of the infection. The adjudicating panel was blinded to the subject's study arm. Donors were stimulated with 480 mg of G-CSF and 8 mg of dexamethasone orally. Continuous flow apheresis with hydroxyethyl starch as a sedimenting agent was used, processing 7–10 L of blood. The goal was to collect  $4 \times 10^{10}$  granulocytes per transfusion ( $0.6 \times 10^9$ /kg); however, this was not always possible and approximately one-quarter of the subjects received a lower dose. The dose differences were site-specific and not due to dose or timing of G-CSF or dexamethasone, nor to amount of blood processed during the collection. There was no difference in the success rate

between the GTx and control groups, whether analyzed per protocol (PP: 49% vs. 41%) or by modified intention to treat (MITT: 42% vs. 43%). There was no discernible difference in the response rate based on the type of infection (bacterial vs. fungal), location of infection, or risk category. A model that adjusted for baseline prognostic factors like Zubrod score or mechanical ventilation did not find any difference either. In their discussion, the investigators pointed out the low power that did not allow to exclude with certainty a beneficial effect of GTx. In a post hoc analysis, they compared subjects who received high dose as intended with those who received low dose and found a significant difference in the success rate (high dose vs. low dose: 59% vs. 15%,  $P < 0.01$ ). Subjects in the control group did better than subjects in the low-dose group, and worse than subjects in the high-dose group, but these differences were not statistically significant. A retrospective review of patients with invasive Fusarium infections<sup>50</sup> also concluded that high-dose GTx may effectively control life-threatening infections to “bridge” periods of severe neutropenia until marrow function is restored, though another single-center, retrospective study from Italy found that recipients of both low-dose ( $<1.5 \times 10^8$  cells/kg) and high-dose ( $>3 \times 10^8$  cells/kg) GTx had higher infection-related mortality than recipients of “standard doses” ( $1.5\text{--}3 \times 10^8$  cells/kg).<sup>60</sup>

Thus, despite attempts<sup>43,44</sup> to perform a properly designed and conducted randomized clinical trial to test the efficacy of high-dose GTx collected from donors stimulated with G-CSF + dexamethasone, the definitive/proven role of GTx in the management of infected, neutropenic, oncology/transplant patients awaits definition by a successfully completed randomized clinical trial—something that seems elusive to date. A Cochrane review of therapeutic granulocyte transfusion<sup>61</sup> included 10 trials with a total of 587 participants conducted between 1975 and 2015. Overall, the quality of the evidence was very low to low across different outcomes. There may be no difference in all-cause mortality over

30 days between participants receiving therapeutic granulocyte transfusions and those that did not (six studies; 321 participants; RR 0.75, 95% CI 0.54–1.04). There were no differences between the granulocyte dose subgroups ( $<1 \times 10^{10}$  per day vs.  $\geq 1 \times 10^{10}$  per day) (test for subgroup differences  $P = 0.39$ ). There was a difference (reduction) in all-cause mortality between the studies based on the age of the study (published before 2000 vs. published 2000 or later) (test for subgroup differences  $P = 0.03$ ). There was no difference in clinical reversal of concurrent infection between participants receiving therapeutic granulocyte transfusions and those that did not (five studies; 286 participants; RR 0.98, 95% CI 0.81–1.19). There was insufficient evidence to determine whether there is a difference in pulmonary serious adverse events (one study; 24 participants; RR 0.85, 95% CI 0.38–1.88). None of the studies reported number of days on therapeutic antibiotics, number of adverse events requiring discontinuation of treatment, or quality of life. The authors concluded that in people who are neutropenic due to myelosuppressive chemotherapy or a hematopoietic stem cell transplant, there is insufficient evidence to determine whether granulocyte transfusions affect all-cause mortality. To be able to detect a decrease in all-cause mortality from 35% to 30% would require a study containing at least 2748 participants (80% power, 5% significance).

### Prophylactic granulocyte transfusions

Another approach is the use of granulocyte transfusions as prophylaxis to prevent infection. There are 12 such studies. Based on historical reports, prophylactic GTxs were of marginal value. Some measure of success was found in 7 of 12 studies.<sup>62–68</sup> Five studies failed to show a benefit for prophylactic GTxs.<sup>69–73</sup> In none of these five negative studies, there were large numbers of granulocytes obtained from matched donors and transfused daily. In a situation analogous to that for the negative therapeutic GTX trials, the failure of prophylactic GTX might be explained, as least in part, by inadequate dose of granulocytes.

In 1997, data from 8 of the 12 controlled trials of prophylactic GTX were analyzed quantitatively by means of formal meta-analysis.<sup>74</sup> The findings of the meta-analysis confirmed that variability in the dose of PMNs transfused, inconsistent attempts to provide leukocyte-compatible GTX, and the varying durations of severe neutropenia in different patient groups were primarily responsible for the differing success rates in the reported controlled trials. It was recommended that high doses of compatible PMNs should be transfused, if future trials are conducted.<sup>74</sup>

The role of high-dose prophylactic GTX (i.e., from G-CSF-stimulated donors) has not been established by definitive clinical

trials. Table 19.3 summarizes the few published studies. In a Cochrane database systematic review,<sup>61</sup> the authors concluded that in patients who are neutropenic due to chemotherapy or HSCT, there is low-grade evidence that prophylactic granulocyte transfusions decrease the risk of bacteraemia or fungemia. Prophylactic GTxs appear promising, but their efficacy, potential adverse effects, and economic analysis await definition by randomized clinical trials of sufficient numbers of patients.

### Pediatric granulocyte transfusion

There are few publications on the use of GTX in pediatric patients, and no randomized controlled trials have been performed. Table 19.4 offers a summary of some the published data. GTX was determined to be beneficial in retrospective analyses<sup>78–81</sup> and prospective uncontrolled studies.<sup>82</sup> In an open, single-center, prospective phase II clinical trial of early-onset G-CSF-mobilized GTX in neutropenic children with severe refractory infections,<sup>83</sup> GTX was well tolerated, with 93% clearance of initial infection and no other series.<sup>84</sup> There is a possible role for GTX in preventing infections or progression of infections in children with anticipated prolonged neutropenia after HSCT or chemotherapy<sup>85</sup> or in children with chronic granulomatous disease.<sup>86–88</sup> There is also a possible role for GTX in preventing infections or progression of infections in children with anticipated prolonged neutropenia after HSCT or chemotherapy.<sup>85</sup>

Most patients with congenital disorders of PMN dysfunction have adequate numbers of blood PMNs, but they are susceptible to serious infections because their PMNs fail to kill pathogenic microorganisms.<sup>92</sup> Patients with severe forms of PMN dysfunction are relatively rare, and no randomized clinical trials have been reported to establish the efficacy of therapeutic GTX in their management. Several studies have suggested that children with chronic granulomatous disease could benefit for GTX,<sup>93–97</sup> however, because of lifetime problems with infections, prophylactic GTxs are impractical.

Neonates (infants within the first month of life) are another group of patients who may suffer life-threatening bacterial infections caused, at least in part, by PMN dysfunction and neutropenia. A Cochrane review<sup>98</sup> included four trials and showed that there was no significant difference in all-cause mortality (RR 0.89, 95% CI 0.43–1.86; typical RD -0.05, 95% CI -0.31 to 0.21) when GTX was compared with placebo or no transfusion. The study concluded that there is inconclusive evidence to support or refute the routine use of granulocyte transfusions in neutropenic, septic neonates.

**Table 19.3** Prophylactic GTX Using PMNs from G-CSF-Stimulated Donors in Hematopoietic Progenitor Cell Recipients

Investigator	PMNs $\times 10^{10}$ per GTX	Stimulation	Leukapheresis	Outcomes
Bensinger et al. <sup>16</sup>	4.2	G-CSF 3.5–6 µg/kg	Variable	Not reported
Adkins et al. <sup>75</sup>	4.1 (Day 1)	G-CSF 5 µg/kg $\times$ 5 days after transplantation	Hetastarch	60% (6 of 10) Afebrile
Adkins et al. <sup>76</sup>	5.1 (Day 3) 6.1 (Day 5) 5.6 (Day 2)	G-CSF 10 µg/kg	7 L processed Days 1, 3, and 5 Hetastarch	40% (4 of 10) Febrile Three culture positive Reduction of fever and antibiotics if no leukocyte antibodies
Oza et al. <sup>77</sup>	7.0 (Day 4) 8.5 (Day 6) 9.9 (Day 8) 5.9 (Days 3 and 5) 5.2 (Days 6 and 7)	GCSF 10 µg/kg	7 L processed Days 2, 4, 6, and 8 Hetastarch 7 L processed Days 3 and 6 or Days 5 and 7	Less fever and antibiotics when GTX given; no effect on hospital Stay or survival

GTX: granulocyte transfusion; PMNs: granulocytes; F-CSF: granulocyte colony-stimulating factor.

**Table 19.4** Pediatric Patients Treated with Granulocyte Transfusions

Investigator	No. of Patients	Study Design	Stimulation	Outcomes
Grigull et al. <sup>89</sup>	3	Case series	G-CSF	All children survived
Kikuta et al. <sup>82</sup>	13	Phase I/II	G-CSF	Infection resolved in nine patients
Sachs et al. <sup>83</sup>	27	Phase II	G-CSF	92.6% cleared the infection, and 81.5% were alive and without signs or symptoms of their infection one month later.
Drewniak et al. <sup>78</sup>	16	Prospective	G-CSF + dexamethasone	All six children with aspergillosis cleared their infection.
Seidel et al. <sup>90</sup>	49	Prospective	G-CSF or prednisolone	70% showed clinical recovery 28-day survival probability of the total patient cohort was $0.72 \pm 0.06$ 100-day survival was $0.52 \pm 0.07$
Graham et al. <sup>84</sup>	13	Retrospective	prednisolone	50% of children with fungal infection and 100% with bacterial survived to discharge
Atay et al. <sup>80</sup>	35	Retrospective	G-CSF + dexamethasone	Infection-related survival rate was 82.4% and the overall survival rate was 77.1% at Day 30
Ozturkmen et al. <sup>79</sup>	10	Retrospective	G-CSF + dexamethasone	clinical response rate of 69.2% infection-related mortality 30.8%
Diaz et al. <sup>81</sup>	18	Retrospective	G-CSF + dexamethasone	92% of the episodes of acute infection had complete or partial resolution
Nikolajeva et al. <sup>91</sup>	28	Retrospective	G-CSF + dexamethasone	64% of patients survived

## Summary and recommendations for clinical practice

The use of G-CSF + dexamethasone to stimulate granulocyte donors has brought GTx therapy into a new era. It is now possible to collect relatively large numbers of granulocytes ( $>4 \times 10^{10}$ ). To determine whether a local need exists for therapeutic GTx, physicians should survey the outcome of neutropenic infections at their own institutions. If these infections respond promptly to antibiotics alone and the survival rate approaches 100%, therapeutic GTxs are unnecessary and should not be used because possible benefits are too small to outweigh the risks and expense. In contrast, if patients with infection and neutropenia (<500 PMN/ $\mu$ L) do not respond quickly and completely to antibiotics alone, the addition of therapeutic GTx should be considered along with other modifications of therapy, such as selection of different antibiotics, closer monitoring of antibiotic blood levels, intravenous  $\gamma$ -globulin therapy, and treatment with recombinant myeloid growth factors.

Once the decision has been made to provide either therapeutic or prophylactic GTx, granulocytes should be collected and transfused optimally as follows.

Collect granulocytes from allogeneic donors with a goal to transfuse  $6-8 \times 10^{10}$  granulocytes per GTx with an absolute lower limit of  $4 \times 10^{10}$ . Develop and refine methods to consistently ensure desired yields. The need to select donors who are leukocyte compatible with recipients has not been established for high-dose granulocyte transfusions despite the historical importance of doing so when lower doses of PMNs were transfused although this has been established for lower dose transfusions.<sup>9</sup> In studies of high-dose GTx, Price et al.<sup>52</sup> found no apparent adverse effects of several leukocyte antibodies (granulocyte agglutinating, granulocyte immunofluorescent, lymphocytotoxic, and lymphocyte immunofluorescent) that became detectable in the recipient's blood during GTx therapy (i.e., not preexisting antibodies). Adkins et al.,<sup>76</sup> however, found the presence of lymphocytotoxic antibodies to be a poor prognostic factor, in general, although actual donor-recipient incompatibility for individual GTx was rare. Until more data are available, it seems prudent to select donors who are leukocyte compatible (e.g., as done to select platelet donors by HLA matching or leukocyte crossmatching) whenever easily feasible, but never to delay or deny GTx therapy if selecting donors is not readily accomplished.

Stimulate donor neutrophilia by giving 300–600  $\mu$ g G-CSF subcutaneously plus dexamethasone orally 12 hours before beginning leukapheresis. A regimen reported to produce high granulocyte yields with reasonable adverse effects on the donor is one 480  $\mu$ g

vial G-CSF given subcutaneously plus 8 mg dexamethasone given orally 12 hours before leukapheresis.

Process 8–10 L of donor blood using a continuous-flow, centrifugation blood separator with citrated HES (hetastarch preferred) solution infused throughout the entire collection at a starch–donor blood ratio of 1:13.

Transfuse the granulocyte unit as soon as possible after collection to minimize storage damage. Granulocyte function begins to deteriorate within a few hours of storage. Until the efficacy of stored granulocytes from G-CSF-stimulated leukapheresis donors is documented, it seems wise to transfuse preferably within eight hours or so of collection. This timing may require agreement by the patient's physician to perform infectious disease testing on the donor before actual granulocyte collection (e.g., at the time G-CSF is administered before leukapheresis) or accept a donor who has been tested recently for a previous donation (e.g., within the preceding 10–30 days), but has not been tested for the PMN collection in question.

Give GTx daily until resolution of infection—as evidenced by clearing of tissue lesions, negative cultures, or resolution of fever—or until marrow function recovers to produce adequate numbers of endogenous PMNs. Determining marrow recovery may be difficult because PMNs collected from G-CSF-stimulated and dexamethasone-stimulated donors and transfused at doses of  $6-8 \times 10^{10}$  PMNs may elevate the recipient's blood PMN count to more than 1000/ $\mu$ L for more than 24 hours. Accurate differentiation of transfused PMNs from those produced endogenously is challenging, and marrow recovery must be based on a sustained increase in blood PMN count after GTx is discontinued.

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## CHAPTER 20

# Composition of plasma

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The first part of this chapter describes the composition of plasma, focusing on those protein components that are important for treatment with plasma or its derivatives. The second part addresses individual factors influencing plasma levels of therapeutic plasma proteins. The regulation of blood coagulation and fibrinolysis and clinically useful blood coagulation and fibrinolysis screening tests are then discussed.

### Plasma composition

Plasma is the cell-free part of blood composed of water, proteins, electrolytes, lipids, and carbohydrates. It is the transportation medium through which nutrients, hormones, waste products, and drugs are transported through the body. *In vivo*, the fluidity of plasma is maintained through complex interactions between its procoagulant and anticoagulant proteins and between these proteins, circulating blood cells, and the endothelium. Coagulation activation in plasma is prevented and its fluidity maintained *in vitro* by the addition of anticoagulants. Citrate is presently the only anticoagulant used for collecting therapeutic plasma and plasma for fractionation, the starting material for the manufacture of plasma protein concentrates. Although proteomics based on two-dimensional gel electrophoresis and mass spectrometry has revealed about 10,000 different human plasma proteins,<sup>1,2</sup> the number of constituents important for transfusion medicine is small, comprising albumin, immunoglobulins G (IgG) and M (IgM), alpha-1-antitrypsin, C1 inhibitor, von Willebrand factor (vWF), vWF-cleaving protease, blood coagulation factors, and the blood coagulation inhibitors antithrombin (AT) and protein C. The plasma levels and biological half-lives of these plasma proteins are subject to large interindividual variation. Plasma is either prepared from citrate-phosphate-dextrose-anticoagulated whole blood units or collected by automated plasmapheresis. Because of lower final citrate concentrations and lower dilution due to lower anticoagulant-to-blood ratios, apheresis plasma contains higher concentrations of clotting factors V, VIII, IX, and XI than plasma from whole blood.<sup>3,4</sup> A dilution of 1:7 to 1:9 for plasma from whole blood and 1:16 for apheresis plasma has to be considered when plasma protein concentrations are given which are related to circu-

lating undiluted plasma. IgG levels in plasma from whole blood are significantly greater than in apheresis plasma because of a decrease in IgG when individuals donate apheresis plasma repeatedly at short intervals.<sup>4,5</sup>

### Albumin

Albumin is a 66.4-kD protein synthesized in the liver. Considering a total plasma volume of 3 L, about 120–140 g or 40% of the total body content of albumin circulates in the plasma at concentrations between 35 and 50 g/L (Table 20.1), which is more than 50% of the total plasma protein concentration, ranging from 64 to 88 g/L. The albumin concentration in the interstitial space of 10–12 L is about 14 g/L. The intravascular albumin compartment is responsible for 80% of plasma oncotic pressure, thus mainly contributing to maintaining blood volume. An intact liver synthesizes 12 g of albumin per day. In a steady state, the same amount of albumin is eliminated per day. The biological half-life of albumin in plasma is 17–20 days. Compensatory reduction of albumin metabolism occurs when plasma levels decrease because of reduced synthesis in the liver or capillary leakage.<sup>6</sup> A marked acquired hypoalbuminemia is a non-specific marker of severe illness and strongly indicates poor prognosis.<sup>7,8</sup>

The functions of albumin are as follows:

- Maintenance of the intravascular oncotic pressure.
- Binding and transportation of drugs, vitamins, minerals, bilirubin, uric acid, hormones, amino acids, and fatty acids.
- Binding of toxic metabolites, among them free fatty acids, and radical scavenging.

Despite all these important functions of albumin, it is remarkable that congenital analbuminemia causes only mild clinical symptoms (e.g., hypotension or discrete pretibial edema at all ages) or no symptoms at all.<sup>9</sup>

Commercially available albumin preparations are obtained from plasma fractionation, according to Cohn and Oncley<sup>10</sup> or Kistler and Nitschmann,<sup>11</sup> as fraction V and are subsequently pasteurized for virus inactivation.<sup>12</sup> These solutions contain either 3.5–5% or 20–25% (w/v) albumin at a purity of 95–98%. Albumin is also added to many drugs and plasma protein concentrates to stabilize the respective active ingredients.

**Table 20.1** Therapeutic Plasma Proteins: Albumin, Immunoglobulins, Alpha-1-Antitrypsin, and C1 Inhibitor

Plasma Protein	Plasma Concentration (g/L)	Biological Half-Life	Main Functions
Albumin	35–50	17–20 days	Maintenance of intravascular oncotic pressure, transportation protein, radical scavenger
Immunoglobulin G	6–17	23 days	Humoral immunity
Immunoglobulin M	0.5–2.5	5 days	Humoral immunity
Alpha-1-antitrypsin	1.5–3.0	4–7 days	Inhibits neutrophil elastase
C1 inhibitor	0.2–0.3	30–60 hours	Inhibits complement C1 activation

Plasma concentrations represent the 5th and 95th percentiles; biological half-lives represent the means or the 5th and 95th percentiles of elimination half-lives.

### Immunoglobulins G and M

IgG and IgM are glycoproteins synthesized in plasma cells and circulating in plasma at concentrations between 6 and 17 g/L and between 0.5 and 2.5 g/L, respectively. The molecular weights are 150 kD for IgG and 971 kD for IgM, and the half-lives are about 23 and 5 days, respectively.

Polyvalent intravenous immunoglobulin (IVIG) preparations can also be administered subcutaneously and are prepared from fraction II during plasma fractionation.<sup>12</sup> Current virus inactivation and elimination procedures are low pH incubation, pasteurization, solvent–detergent treatment, caprylic acid treatment, and nanofiltration. Although most IVIG preparations are primarily IgG with only trace amounts of other immunoglobulin isotypes, some IgM-enriched IVIGs contain up to 15% IgM and have been used for the treatment of severe sepsis and septic shock.<sup>13</sup>

Hyperimmune immunoglobulin preparations are obtained from the plasma of actively immunized donors using ethanol fractionation or chromatography.<sup>12</sup> They can be administered intravenously or intramuscularly and are used to prevent postexposure infections, intoxication by tetanus toxoid, and immunization to Rh-positive red cells. For more details, see Chapter 23. Convalescent plasma passively transfers antibodies capable of neutralizing viruses including COVID-19 with the goal of reducing the severity of illness, especially respiratory diseases.<sup>14,15</sup>

### Alpha-1-antitrypsin

Alpha-1-antitrypsin synthesized in the liver is the most abundant serine protease inhibitor (serpin) in plasma. It inhibits a variety of serine proteases of which neutrophil elastase is the most important target. The molecular weight is 52 kD, the plasma concentration is 1.5–3 g/L, and the half-life is 4–7 days.<sup>16,17</sup> Hereditary alpha-1-antitrypsin deficiency resulting in plasma levels lower than 0.8 g/L occurs at a prevalence between 1 in 1600 and 1 in 5000.<sup>18</sup> It is associated with lung emphysema and liver disease in childhood. Plasma-derived alpha-1-antitrypsin concentrates are indicated for the treatment of patients with emphysema secondary to congenital deficiency.<sup>19</sup>

### C1 inhibitor

C1 inhibitor is a 105-kD serpin synthesized in the liver. The plasma concentration is 0.24 g/L, and the half-life ranges from 30 to 60 hours.<sup>20,21</sup> C1 inhibitor affects early activation of the classical complement pathway by blocking activated C1 and subsequent C2,4 complex formations.<sup>22</sup> It also inhibits activated factors XI and XII and plasmin, and the formation of factor XIa, kallikrein, and bradykinin.

An inherited or acquired deficiency of C1 inhibitor may cause angioedema, which is characterized by subcutaneous or mucosal swelling in any part of the skin or the respiratory and gastrointesti-

nal tracts.<sup>22</sup> The prevalence of hereditary angioedema is from 1 in 10,000 to 1 in 50,000. C1 inhibitor concentrates purified by chromatography from the cryoprecipitate-reduced plasma produced during plasma fractionation are used successfully for the treatment of acute attacks of angioedema or for prophylaxis including before surgery.<sup>20,23</sup>

### von Willebrand factor–cleaving protease

The vWF-cleaving protease (a disintegrin and metalloprotease with a thrombospondin type 1 motif, member 13, ADAMTS13) is a 70-kD protein<sup>24</sup> expressed in the liver, platelets, and other tissues,<sup>25,26</sup> and circulates in plasma at concentrations between 0.7 and 1.3 mg/L.<sup>27</sup> The elimination half-life is 2–3 days.<sup>28</sup> The plasma enzyme cleaves the A2-domain of vWF. A defective cleaving protease results in unusually large vWF multimers, which are associated with thrombotic thrombocytopenic purpura (TTP). Therapeutic plasma contains normal vWF-cleaving protease activities and is effective for the treatment of TTP through therapeutic plasma exchange.<sup>29,30</sup>

### Clotting factors, coagulation factor inhibitors, and von Willebrand factor

The coagulation proteins discussed here are responsible for normal hemostasis (Table 20.2). Except for factor V, virus-inactivated plasma-derived concentrates have been developed for preventing or treating bleeding caused by hereditary or acquired vWF or any clotting factor deficiency states.<sup>12</sup> Antithrombin and protein C concentrates are also available and are used mainly for prophylaxis or treatment of thromboembolic complications in patients with hereditary deficiency of the respective blood coagulation inhibitor.<sup>12</sup> Because not all clotting factor and inhibitor concentrates are available in all developed countries, factor V deficiency, for which a concentrate has not yet been produced, and other clotting factor deficiency states (e.g., factor XI deficiency) are alternatively treated with plasma.<sup>31</sup>

Fibrinogen is the precursor of fibrin. Thrombin converts fibrinogen into fibrin monomers by cleaving fibrinopeptides A and B. Fibrin monomers polymerize to form an unstable fibrin clot, which is stabilized by factor-XIIIa-induced crosslinkage. Fibrinogen is also necessary for supporting platelet function. Prothrombin (factor II); clotting factors VII, IX, X, and XI; and protein C are proenzymes of respective serine proteases. After activation by thrombin, factor V accelerates the activation of prothrombin, factor VIII, and factor X. Factor XIII is activated by thrombin to form a transglutaminase, which stabilizes fibrin clots by crosslinkage. vWF is essential for platelet–subendothelium adhesion and platelet aggregation, and for the transportation of factor VIII in plasma. vWF protects factor VIII from early proteolysis. Antithrombin is a stoichiometric serpin that strongly inhibits thrombin and factors IXa,

**Table 20.2** Clotting Factors, Von Willebrand Factor, Antithrombin, and Protein C

Plasma Protein	MW (kD)	Plasma Concentration (mg/L)	Plasma Activity (U/dL)	Half-Life (Hours)	Main Functions	Replacement by Plasma-Derived Concentrates <sup>12</sup> or Plasma
Fibrinogen	340	2.5 g/L	1.5–4 g/L <sup>31</sup>	96 <sup>31</sup>	Clot formation, precursor of fibrin; supports platelet function	Fibrinogen concentrate, cryoprecipitate, plasma; locally, fibrin sealant <sup>32</sup>
Factor II	72	150 <sup>33</sup>	70–130	60 <sup>34,35</sup>	Proenzyme of thrombin	Prothrombin complex concentrates (PCCs) and plasma <sup>31,34</sup>
Factor V	330	7 <sup>36,37</sup>	70–125	12 <sup>38</sup>	Cofactor of prothrombin activation by factor Xa	Plasma <sup>31</sup>
Factor VII	50	1 <sup>39</sup>	70–150	5 <sup>34,30</sup>	Proenzyme of factor VIIa, which activates factor IX and factor X	Factor VII concentrates <sup>40</sup> and factor VII containing PCC
Factor VIII	330	0.15 <sup>41</sup>	60–200	14 <sup>42</sup>	Cofactor of factor X activation by factor IXa	Factor VIII concentrates and factor VIII/vWF concentrates
vWF	600–2 × 10 <sup>4</sup>	8 <sup>43</sup>	50–180	12 <sup>44,45</sup>	Platelet–subendothelium adhesion; platelet aggregation; transports factor VIII and protects it from proteolysis	Factor VIII/vWF concentrates and vWF concentrates
Factor IX	57	5 <sup>43</sup>	60–140	30 <sup>42</sup>	Proenzyme of factor IXa, which activates factor X	Factor IX concentrates
Factor X	59	10 <sup>43</sup>	70–130	30 <sup>34</sup>	Proenzyme of factor Xa, which activates prothrombin	PCC, factor X concentrates, <sup>46</sup> and plasma <sup>31</sup>
Factor XI	143	5 <sup>47</sup>	65–130	50 <sup>48,49</sup>	Proenzyme of factor XI, which activates factor IX	Plasma, factor XI concentrates <sup>31,47</sup>
Factor XIII	320	20 <sup>50</sup>	60–220	170 <sup>31</sup>	Proenzyme of factor XIIIa, which stabilizes fibrin by crosslinkage	Factor XIII concentrates <sup>51</sup>
Antithrombin	58	150 <sup>52</sup>	75–120	72 <sup>52</sup>	Stoichiometric serpin, inactivates thrombin, factor IXa, factor Xa, and factor Xla	Antithrombin concentrates <sup>53</sup>
Protein C	62	44 <sup>43</sup>	70–170	9 <sup>54</sup>	Proenzyme of activated protein C, which inactivates factor Va and factor VIIIa	Protein C concentrates <sup>55</sup>

Plasma concentrations represent the means or medians; plasma activities represent the 5th and 95th percentiles; half-lives represent the mean or median elimination half-lives; PCC: prothrombin complex concentrate; vWF: von Willebrand factor.

Xa, and Xla. Once activated by thrombin–thrombomodulin, activated protein C inhibits factors Va and VIIIa.

Most clotting factors, AT, and protein C are synthesized in the liver. Other sites of protein expression are megakaryocytes and endothelial cells for factor V and the  $\alpha$ -chain of factor XIII. vWF is synthesized in megakaryocytes and endothelial cells.

The synthesis of functionally active prothrombin complex factors II, VII, IX, and X and protein C in the liver requires vitamin-K-mediated gamma-glutamyl carboxylation. Impairment of carboxylation occurs in vitamin K deficiency, in the presence of vitamin K antagonists, and in some cases of liver dysfunction. This results in the synthesis of functionally impaired or inactive prothrombin complex factors, called *proteins induced in vitamin K absence or antagonism* (PIVKA).

## Factors influencing individual plasma composition

### Age and gender

The physiologic aging process is accompanied by an increase in plasma levels of fibrinogen; factors V, VII, VIII, IX, XI, and XIII; protein C; and protein S in both genders.<sup>56</sup> A significant decrease of prothrombin activity levels with increasing age has been found in men but not in women.<sup>57</sup> Young women have significantly lower AT plasma levels than males of similar age. Because of a marked increase after menopause, AT levels in older women exceed levels in male contemporaries.<sup>58</sup> Gradually declining AT plasma levels have been observed in males older than 45 years.<sup>58</sup> Pregnancy is associ-

ated with marked increases in levels of fibrinogen, prothrombin, factors VII and VIII, vWF, and factor X, whereas factor V, factor IX, AT, and protein C levels are largely unchanged.<sup>59</sup>

### ABO blood group

Individuals with blood groups O, A<sub>1</sub>O, and A<sub>2</sub>A<sub>2</sub> have on average about 20–25% lower factor VIII and vWF plasma levels when compared with other ABO blood group constellations.<sup>60–62</sup>

### Acute phase reaction

An acute phase response caused by any trigger of inflammation results in an increase in plasma levels of alpha-1-antitrypsin, C1 inhibitor, fibrinogen, prothrombin, factor VIII, and vWF and a decrease in albumin levels.<sup>63</sup> Smoking causes a low-grade systemic inflammatory response and concomitant increases in plasma fibrinogen.<sup>64–66</sup>

### Physical exercise and mental stress

Both acute severe physical exercise and acute mental stress cause increases in fibrinogen, factor VII, factor VIII, and vWF levels.<sup>67–71</sup> In contrast, prolonged severe physical exercise results in a decrease in factor VII levels, whereas fibrinogen levels continue to be increased during observation.<sup>72</sup>

### Hormones

Danazol, a weak androgen, improves the synthesis of C1 inhibitor, factor VIII, AT, and protein C.<sup>73</sup> Combined oral contraceptives induce increases in plasma fibrinogen; prothrombin; factors VII, VIII, IX, X, and XI; alpha-1-antitrypsin; and protein C, whereas AT

and protein S levels decrease.<sup>74–76</sup> Progestogen-only preparations lead to a decrease in factor VIII and an increase in protein S.<sup>77</sup>

### **Desmopressin (DDAVD)**

DDAVP has been used successfully to increase factor VIII and vWF levels in plasma donors.<sup>78,79</sup> The yields of these plasma proteins were markedly improved when cryoprecipitate or factor VIII concentrates were produced from plasma collected after pretreatment of donors with DDAVP.

### **The influence of storage and freezing on plasma composition**

Prolonged storage reduces factor VIII levels regardless of hold at 4 °C or at room temperature.<sup>80,81</sup> Storage at room temperature does, however, affect protein S activity, which is substantially less when compared to hold at 4 °C. Slow freezing at 20 °C reduces factor VIII and protein S activities markedly in comparison to rapid freezing to a core temperature of below 30 °C within one hour.<sup>80</sup> Factor VIII levels decline by 24% even when fresh plasma samples are frozen in liquid nitrogen, stored at 70 °C, and thawed at 37 °C for 10 min.<sup>82</sup>

### **Regulation of blood coagulation and fibrinolysis**

Hemostasis encompasses all processes of clot formation, clot lysis, and wound healing with the aim of maintaining vessel wall integrity. The mechanisms of hemostasis incorporate the vascular endothelium and subendothelium, platelets, leukocytes, red cells, pro- and anticoagulant, and pro- and antifibrinolytic proteins. Primary hemostasis is characterized by the formation of a platelet plug at the site of vessel wall injury. vWF forms a bridge between platelets and vascular subendothelial structures and promotes platelet aggregation. Secondary hemostasis is achieved by the formation of a stable fibrin clot, followed by fibrinolysis and tissue repair. Abnormal bleeding can result from impaired thrombin generation, clot formation, and clot stabilization, or hyperfibrinolysis. Conversely, increased coagulation and diminished fibrinolysis may provoke thrombosis.

Previous models have suggested two independent pathways of coagulation activation via the intrinsic and extrinsic cascades. However, it is now recognized that the generation or exposure of tissue factor (TF) at the site of vessel injury is the first physiologic step in initiating blood coagulation.<sup>83</sup> There are three closely linked phases of coagulation, comprising the initiation, amplification, and propagation processes (Figure 20.1). Clotting is mainly controlled by several circulating inhibitors. Activation of fibrinolysis results in fibrin cleavage by plasmin and subsequent restoration of vessel patency. The central role of thrombin in hemostasis by exerting both procoagulant and anticoagulant effects is illustrated in Figure 20.2.<sup>84</sup>

### **Initiation and amplification of coagulation**

Clotting is initiated by TF-factor VIIa complexes after TF is exposed to the circulating blood by smooth muscle cells, fibroblasts, disrupted or activated endothelial cells, or activated monocytes (Figure 20.1). Circulating factor VIIa represents approximately 1% of total factor VII and can be generated from the inactive zymogen by an autocatalytic mechanism. Factor VIIa does not express its full procoagulant activity until it is bound to TF.<sup>85</sup> TF-factor VIIa activates factors IX and X, and factor Xa generates thrombin by activating prothrombin (*initiation phase*). These small amounts of thrombin ensure that coagulation activa-

tion and further thrombin formation are maintained (*amplification phase*). Thrombin activates factors V, VIII, and XI, and platelets that provide the surface on which the *propagation phase* of coagulation occurs.

### **Propagation of thrombin generation, fibrin clot formation, and fibrin stabilization**

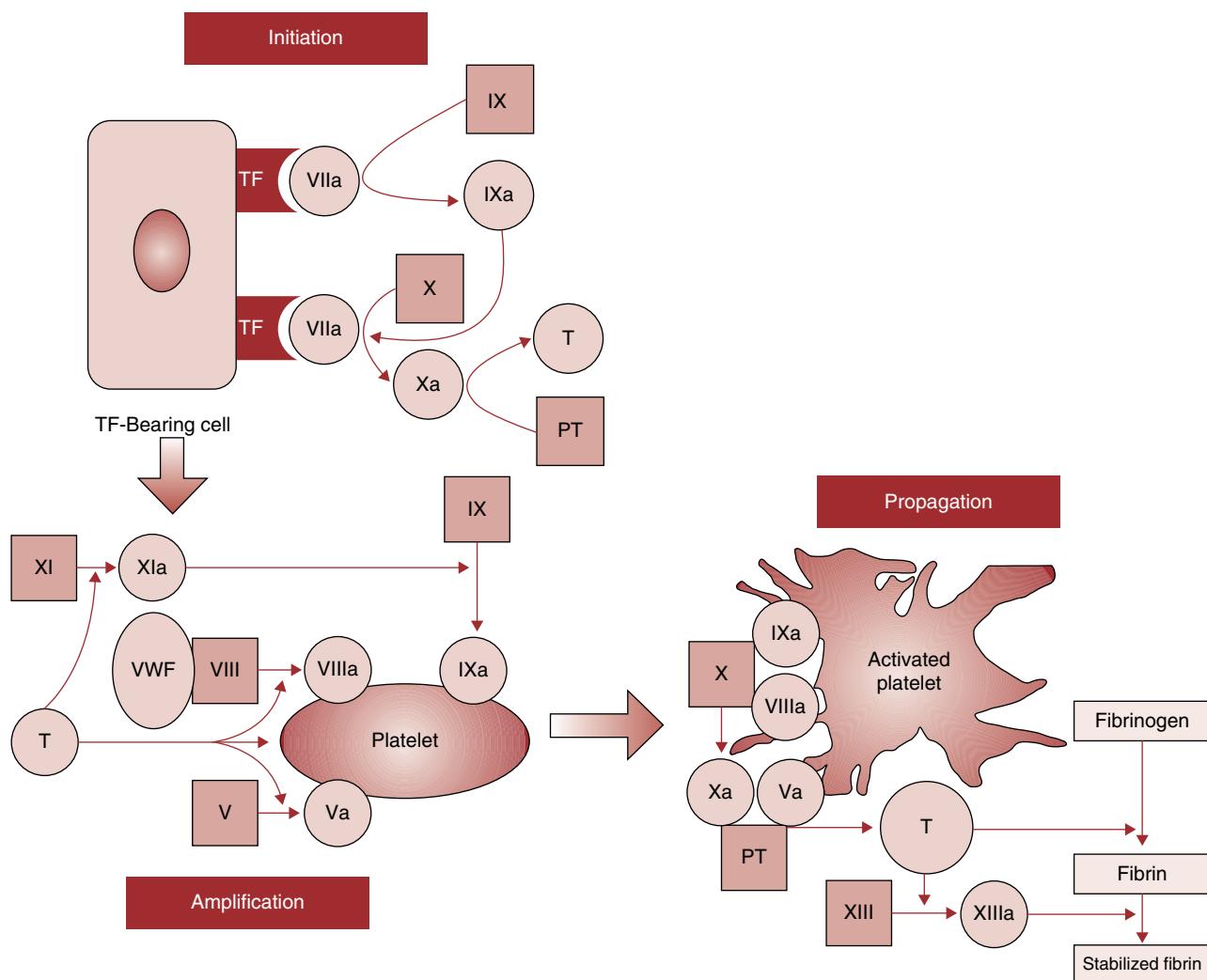
The *tenase complex* consisting of platelet membrane-bound factor IXa, factor VIIIa, and ionized calcium cleaves factor X much more effectively than TF-factor VIIa or factor IXa alone. Factor Xa binds to platelet surfaces and complexes with factor Va and calcium to form the *prothrombinase complex*, which rapidly generates large amounts of thrombin. Fibrin monomers resulting from thrombin-induced splitting of fibrinopeptides A and B from fibrinogen polymerize spontaneously to an unstable fibrin clot. Thrombin also activates factor XIII to the transglutaminase factor XIIIa, which stabilizes fibrin polymers by crosslinkage.<sup>86</sup>

### **Inhibition of blood coagulation and termination of clotting**

Several circulating inhibitors terminate the coagulation process (Table 20.3). The stoichiometric-heparin-binding serpin AT mainly inhibits thrombin and factor Xa and, less strongly, factors IXa and XIa.<sup>52</sup> Thrombin binds to endothelial thrombomodulin, and this complex activates protein C to the serine protease, activated protein C (APC).<sup>87</sup> Endothelial cell protein C receptor augments protein C activation by the thrombin-thrombomodulin complex. Together with its vitamin-K-dependent cofactor protein S, APC inactivates factors Va and VIIIa. Tissue factor pathway inhibitor (TFPI) downregulates blood coagulation by forming a quaternary complex with TF-factor VIIa/factor Xa. Heparin cofactor II is a specific inhibitor of thrombin in the presence of dermatan sulfate or heparin.<sup>52</sup> The protein-Z-dependent protease inhibitor is a serpin inhibiting factor Xa in the presence of the vitamin-K-dependent protein Z, phospholipids, and calcium.<sup>52</sup> Protein C inhibitor, a heparin-binding serpin, predominantly inhibits APC and thrombin.<sup>52</sup>

### **Fibrinolysis and its inhibition**

The role of the fibrinolytic system is to restore vessel patency once wound healing has maintained vessel wall integrity (Figure 20.3). The serine protease plasmin cleaves fibrin (and fibrinogen) at multiple sites, resulting in fibrin and fibrinogen degradation products (FDP), among them D-dimers (DD). Tissue plasminogen activator (tPA) released by endothelial cells is able to activate plasminogen to plasmin. Urokinase plasminogen activator (urokinase, UK) synthesized in endothelial and kidney cells also activates plasminogen to plasmin.<sup>88</sup> Plasmin cleaves not only fibrin but also fibrinogen and other plasma proteins. Fibrin specificity of plasmin is achieved by binding of plasminogen and tPA to the lysine binding sites of fibrin. Both plasminogen activators are mainly inhibited by plasminogen activator inhibitor type 1 (PAI-1) released from endothelial cells and platelets. Plasmin is predominantly inhibited by the very fast-acting plasmin inhibitor (alpha<sub>2</sub>-antiplasmin) and to a lesser extent by alpha<sub>2</sub>-macroglobulin. Plasmin inhibitor crosslinked to fibrin by factor XIIIa inhibits plasmin binding to fibrin. The thrombin-activatable fibrinolysis inhibitor (TAFI) is activated by thrombin-thrombomodulin complexes and protects clots from plasmin-induced degradation by removing lysine residues from fibrin.<sup>89,90</sup>



**Figure 20.1** Cell-based model of coagulation activation. Inactive clotting factors are in squares and active factors are in circles. The coagulation cascade is initiated by tissue factor (TF) release at the sites of cell injury. During the initiation phase, TF complexes with factor VIIa (FVIIa) at the site of cell injury. TF-factor VIIa activates factor IX and factor X. Factor Xa generates small amounts of thrombin, which activates platelets, factor XI, and cofactors V and VIII, allowing multiple positive feedback loops during the amplification phase. The tenase complex (factor VIIIa–factor IXa) generates large amounts of factor Xa on the surface of activated platelets. The prothrombinase complex (factor Va–factor Xa) converts prothrombin to thrombin, which forms fibrin from fibrinogen and activates factor XIII. PT: prothrombin; T: thrombin; TF: tissue factor.

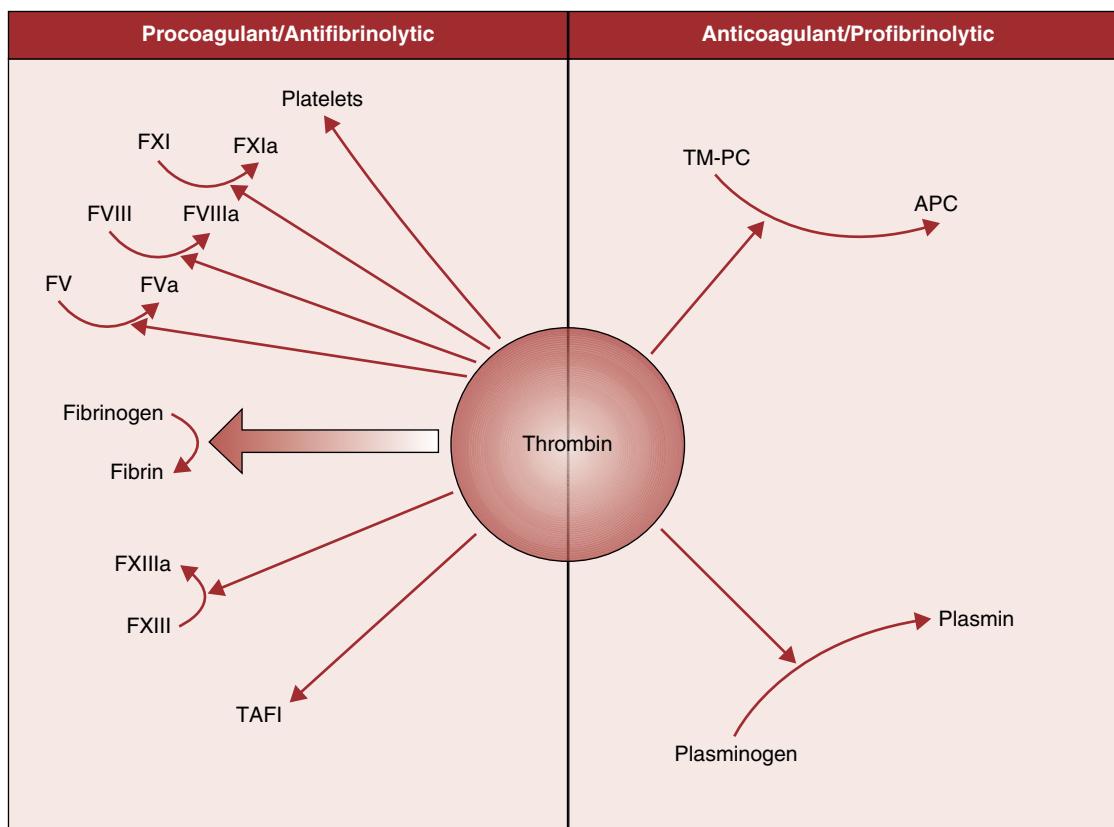
### Clinically useful coagulation and fibrinolysis screening tests

Coagulation and fibrinolysis screening tests are used for detecting disorders of secondary hemostasis caused by coagulation factor deficiencies, dysfunctional coagulation factors, inhibitors against coagulation factors, anticoagulants, or hyperfibrinolysis. In addition to a detailed clinical history and examination and laboratory investigation of disorders of primary hemostasis, coagulation and fibrinolysis screening tests are included in approaches to bleeding patients and to exclusion of bleeding disorders before operations or other invasive procedures.<sup>91</sup> Further applications of these tests are as follows:

- Monitoring of anticoagulant therapy.
- Monitoring of treatment with fresh frozen plasma or clotting factor concentrates.,
- Detection of lupus anticoagulants.
- Detection of hypercoagulable states.

The historical division of coagulation activation and subsequent fibrin formation into an *intrinsic pathway* comprising surface-contact factors and an *extrinsic pathway* (TF-factor VIIa) can be used for a better understanding of blood coagulation screening tests (Figure 20.4). The intrinsic pathway begins with the activation of factor XII and prekallikrein at negatively charged artificial surfaces as found on activated platelets and endothelial cells and at subendothelial collagen. Factor XIIa activates factor XIa and further prekallikrein to kallikrein. Factor XIa and kallikrein accelerate factor XII activation through a positive feedback mechanism. Factor XII and factor XI activation is amplified by high-molecular-weight (HMW) kininogen. Factor XIa activates factor IX, and factor IXa activates factor X. The extrinsic pathway starts with the formation of the TF-factor VIIa complex, also resulting in production of factor Xa.

The common pathway begins with factor X activation, where the intrinsic and extrinsic pathways converge. Factor Xa converts pro-



**Figure 20.2** The central role of thrombin in hemostasis. Procoagulant effects include platelet activation; the activation of clotting factors V, VIII, XI, and XIII; and subsequent formation of a stable fibrin clot. Thrombin acts as an anticoagulant by activating protein C after complexing with thrombomodulin. It promotes fibrinolysis by activating plasminogen and inhibits fibrinolysis by activating TAFI. APC: activated protein C; F: factor; PC: protein C; TAFI: thrombin activatable fibrinolysis inhibitor; TM: thrombomodulin. Source: Modified from Davie and Kulman.<sup>34</sup>

**Table 20.3** Inhibitors of Blood Coagulation and Their Main Functions

Plasma Protein	Main Functions
Antithrombin	Heparin-binding serpin; inhibits thrombin, factor Xa, factor IXa, and factor Xla
Protein C	Serine protease; inhibits factor Va and factor VIIIa
Protein S	Vitamin-K-dependent glycoprotein; cofactor of activated protein C
Thrombomodulin	Endothelial cell-surface glycoprotein; complexes with thrombin and activates protein C
Endothelial cell protein C receptor	Endothelial cell-specific transmembrane protein; augments protein C activation
Tissue factor pathway inhibitor (TFPI)	Trivalent protease inhibitor; inhibits TF–factor VIIa by forming a quaternary complex of TFPI, TF, factor VIIa, and factor Xa
Heparin cofactor II	Dermatan sulfate-binding serpin; inhibits thrombin
Protein-Z-dependent protease inhibitor	Serpin; inhibits factor Xa in the presence of protein Z

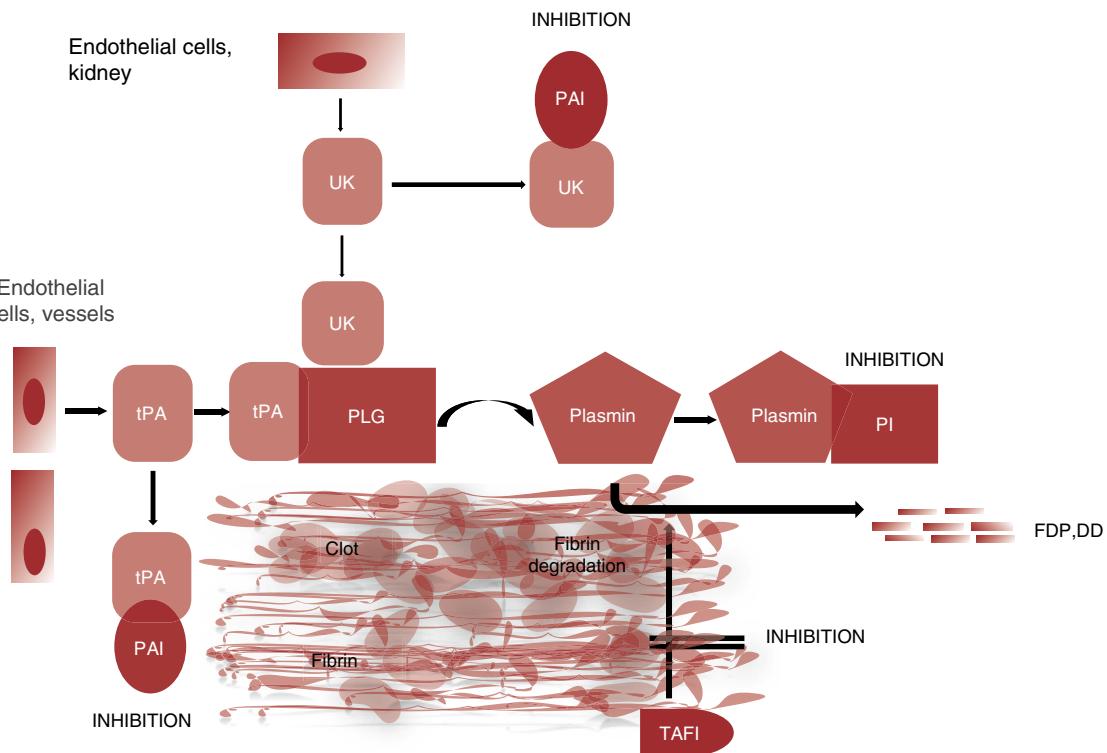
thrombin (factor II) to thrombin (factor IIa) in the presence of factor Va. Thrombin cleaves fibrin to form fibrin monomers, which polymerize spontaneously to an unstable fibrin clot.

Coagulation screening tests use fibrin polymerization as a measuring signal and have limited sensitivity to detect mild coagulation factor deficiencies or weak inhibitors against coagulation factors. Their specificity is also limited by the fact that several causes of abnormal clotting times are not associated with bleeding or thrombosis. Therefore, coagulation screening tests must be combined with the clinical history and symptoms when assessing the

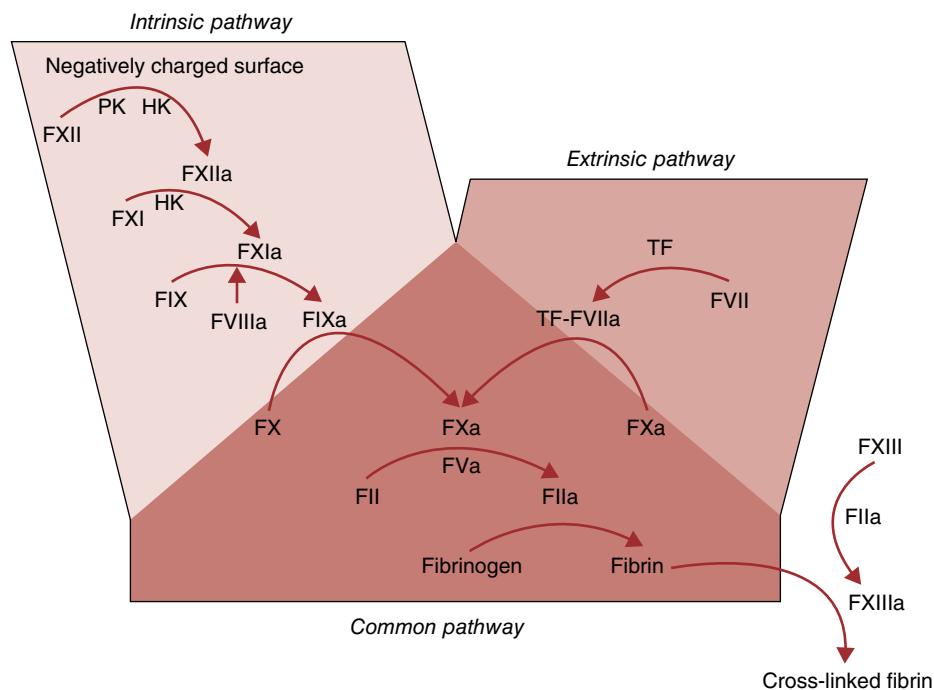
individual risk of bleeding or thrombosis.<sup>91–94</sup> Laboratories performing coagulation screening tests must consider the different sensitivities of reagents to clotting factor deficiencies, therapeutic anticoagulants, or lupus anticoagulants. Each laboratory should establish its own reference ranges, which depend not only on the reagents but also on the instruments used for analyses. It is also important to define a gray area of borderline assay results that should prompt specific coagulation assays despite a lack of clearly abnormal screening test results.

The *activated partial thromboplastin time* (aPTT) measures the intrinsic and common pathways of coagulation activation. The recalcification clotting time of citrated plasma is determined after addition of kaolin, celite, micronized silica, or ellagic acid, providing a large artificial surface area, and phospholipids functioning as a platelet substitute. The *prothrombin time* (PT) is performed by adding brain tissue or recombinant thromboplastin and calcium to citrated plasma and recording the clotting time. This screening test assesses coagulation activation via the extrinsic and common pathways. The *thrombin time* (TT) measures the time for clot formation to occur in citrated plasma after addition of thrombin. It reflects the amount and quality of fibrinogen and the rate of fibrin formation. The reasons for abnormal aPTT, PT, and TT values and their clinical relevance are shown in Table 20.4.

The main use of aPTT is for screening coagulation defects and inhibitors. The test is relatively sensitive to factor VIII, IX, XI, and XII deficiencies and to prekallikrein and HMW kininogen deficiencies. It may also be prolonged by more severe defects of



**Figure 20.3** Activation of fibrinolysis. Endothelial cells release tPA or UK. Both activators can generate plasmin from plasminogen. Plasmin cleaves fibrin and fibrinogen to degradation products. tPA and urokinase are inhibited by PAI-1 and plasmin by PI. TAFI inhibits plasmin-induced cleavage of fibrin by removing lysine residues from fibrin. DD: D-dimers; FDP: fibrin degradation products; PAI: plasminogen activator 1; PI: plasmin inhibitor; PLG: plasminogen; tPA: tissue plasminogen activator; TAFI: thrombin activatable fibrinolysis inhibitor; UK: urokinase.



**Figure 20.4** Classical model of the coagulation cascade, with intrinsic, extrinsic, and common pathways. F: factor; HK: high-molecular-weight kininogen; PK: prekallikrein; TF: tissue factor.

**Table 20.4** Reasons for an Abnormal Activated Partial Thromboplastin Time, Prothrombin Time, and Thrombin Time

Abnormal aPTT	Prolonged PT	Prolonged TT
Associated with Hemorrhage, Prolonged <ul style="list-style-type: none"> <li>Fibrinogen, prothrombin, factor V, X, VIII, IX, or XI deficiency</li> <li>Vitamin K deficiency or antagonism by oral anticoagulants or beta-lactam antibiotics</li> <li>Specific clotting factor inhibitors</li> <li>Unfractionated heparin and low-molecular-weight heparins</li> <li>Factor Xa inhibitors (e.g., apixaban, edoxaban, fondaparinux, and rivaroxaban)</li> <li>Thrombin inhibitors (e.g., hirudins, argatroban, and dabigatran)</li> </ul>	Associated with Hemorrhage <ul style="list-style-type: none"> <li>Fibrinogen, prothrombin, factor V, X, or VII deficiency</li> <li>Specific clotting factor inhibitors</li> <li>Vitamin K deficiency or antagonism by oral anticoagulants or beta-lactam antibiotics</li> <li>Factor Xa inhibitors (e.g., apixaban, edoxaban, fondaparinux, and rivaroxaban)</li> <li>Thrombin inhibitors (e.g., hirudins, argatroban, and dabigatran)</li> <li>High doses of unfractionated heparin</li> <li>Inhibition of fibrin polymerization: fibrin(ogen) degradation products, monoclonal gammopathies, and dysfibrinogenemias</li> </ul>	Associated with Hemorrhage <ul style="list-style-type: none"> <li>Unfractionated heparin and low-molecular-weight heparins</li> <li>Thrombin inhibitors (e.g., hirudins, argatroban, and dabigatran)</li> <li>Inhibition of fibrin polymerization: fibrin(ogen) degradation products, monoclonal gammopathies, and 30% of hereditary dysfibrinogenemias</li> <li>Hypofibrinogenemia</li> </ul> No Association with Hemorrhage or Associated with Thrombosis <ul style="list-style-type: none"> <li>Inhibition of fibrin</li> <li>Polymerization: 50% of hereditary dysfibrinogenemias</li> </ul>
No Association with Hemorrhage or Thrombosis, Prolonged <ul style="list-style-type: none"> <li>Prekallikrein or high-molecular-weight kininogen deficiency, severe factor XII deficiency</li> <li>Transient phospholipid antibodies, predominantly in children with infections</li> <li>Increased hematocrit</li> <li>Heparin contamination</li> <li>Partially activated sample</li> </ul>	No Association with Hemorrhage <ul style="list-style-type: none"> <li>Increased hematocrit</li> <li>Inhibition of fibrin polymerization: dysfibrinogenemia</li> </ul>	Associated with Thrombosis <ul style="list-style-type: none"> <li>Inhibition of fibrin polymerization: 20% of hereditary dysfibrinogenemias</li> </ul>
Associated with Thrombosis, Prolonged <ul style="list-style-type: none"> <li>Lupus anticoagulant</li> <li>Moderate factor XII deficiency?</li> </ul>	Associated with Thrombosis <ul style="list-style-type: none"> <li>Lupus anticoagulant</li> <li>Inhibition of fibrin polymerization: dysfibrinogenemia</li> </ul>	
Associated with Thrombosis, Shortened <ul style="list-style-type: none"> <li>Increased plasma levels of fibrinogen, prothrombin, factors V, VIII, IX, and X; coagulation activation</li> </ul>		

Hereditary dysfibrinogenemia may be associated with bleeding or thrombosis or both, or not associated with any symptoms. aPTT: activated partial thromboplastin time; PT: prothrombin time; TT: thrombin time.

fibrinogen, factors V and X, and fibrin polymerization. Factors affecting the clotting response of the aPTT test include the composition and concentration of phospholipids, the type of contact activator and buffer, and the length of incubation with the plasma. An aPTT should be sufficiently sensitive to record single clotting factor deficiencies of 30% of normal or less.<sup>95</sup> Some aPTT reagents fail to detect individual coagulation factor deficiencies as low as 20%. When used as a screening test for lupus anticoagulant, the detection sensitivity is predominantly determined by the type and concentration of the phospholipid reagent. The aPTT is still the most widely used method for monitoring treatment with unfractionated heparin. Therapeutic thrombin and factor Xa inhibitors also prolong the aPTT. Conditions associated with a prolonged aPTT but not with any increased risk of bleeding include factor XII, prekallikrein, and HMW kininogen deficiencies; lupus anticoagulants; transient phospholipid antibodies; increased hematocrit resulting in citrate anticoagulant excess; and heparin contamination of samples. Recent findings suggest that moderate factor XII deficiency might be associated with vascular mortality.<sup>96</sup> Transient phospholipid antibodies and concomitant prolonged aPTT without any clinical symptoms are frequently observed in children suffering from repeated infections.<sup>97</sup> Increased plasma levels of fibrinogen, factor VIII, and factor IX sometimes cause an abnormally short aPTT, which has been associated with overall poor prognosis and venous thromboembolism.<sup>98,99</sup>

The PT reflects changes in the three vitamin-K-dependent clotting factors (prothrombin and factors VII and X) and the non-vitamin-K-dependent factor V. Its sensitivity to clotting factor deficiencies or to vitamin K deficiency or antagonism by oral anti-coagulants strongly depends on the source and type of TF thromboplastin reagent. PT prolongations not associated with clotting factor deficiencies may be caused by lupus anticoagulants, increased hematocrit, or impaired fibrin polymerization. Because the heparin sensitivity of PT reagents is reduced by adding heparin-neutralizing substances, only very high unfractionated heparin concentrations may cause prolonged PT. The PT is still the most widely used test for monitoring treatment with warfarin or other oral anticoagulants. For this purpose, the PT is calibrated against the international reference thromboplastin and given as an international normalized ratio (INR).<sup>100</sup> Therapeutic thrombin and factor Xa inhibitors also prolong the PT.

The TT detects an impaired fibrin polymerization resulting from fibrin(ogen) degradation products, monoclonal gammopathies, or dysfibrinogenemias more sensitively than the aPTT and PT. Hereditary dysfibrinogenemia is heterogeneous and may be associated with bleeding and/or thrombosis, or it may be asymptomatic.<sup>31</sup> Unfractionated heparin and therapeutic thrombin inhibitors such as hirudins, argatroban, and dabigatran cause a marked prolongation of the TT. The *reptilase time* performed by adding the snake venom reptilase to citrated plasma is unaffected by heparin and even more sensitive to impaired fibrin polymerization than is the

TT. Because none of the coagulation screening tests are usually prolonged until the fibrinogen level falls below 0.5 g/L, a specific assay for *clottable fibrinogen* should be included in the basic screening tests for detecting coagulation factor deficiencies. Functional fibrinogen is either measured by the Clauss method based on thrombin-induced clotting or calculated from the change of turbidity during measurement of the PT (PT-derived fibrinogen).

Coagulation screening tests do not detect factor XIII deficiency, which must be confirmed by appropriate assays.

There is presently no simple and adequately sensitive screening test for detecting mild *hyperfibrinolysis*. D-dimer latex agglutination, and turbidimetric and enzyme-linked immunosorbent assays quantify the plasmin-degraded products of crosslinked fibrin in plasma using monoclonal antibodies. However, elevated D-dimer levels are found in numerous clinical conditions that are not inevitably associated with hyperfibrinolysis-induced bleeding. Severe hyperfibrinolysis can be diagnosed easily by markedly elevated D-dimers, prolonged TT and reptilase time, thrombelastography/thrombelastometry, and low fibrinogen, plasminogen, and plasmin inhibitor levels. However, the detection of mild hyperfibrinolytic states associated with bleeding continues to be a diagnostic challenge.<sup>101</sup>

The need for “global screening tests” allowing on-site assessment of the overall hemostatic potential has resulted in further developments of old viscoelastic tests using new technologies. Thromboelastography originally described by Hartert<sup>102</sup> and rotational thrombelastometry (ROTEM<sup>TM</sup>) are able to detect thrombocytopenia, platelet dysfunction, coagulopathies, impaired fibrin stabilization, and hyperfibrinolysis when performed in whole blood.<sup>103</sup> Although these tests have been used successfully in guiding blood component administration in hepatic and cardiac surgery and in trauma,<sup>104–106</sup> validation studies are lacking on their sensitivity and specificity to detect mild disorders of hemostasis in all clinical settings. Studies demonstrated a low sensitivity of thromboelastography to detect platelet dysfunction and vitamin-K-dependent coagulation factor deficiency.<sup>107,108</sup> The endogenous

thrombin potential as measured by a thrombin generation curve invented by Hemker<sup>109</sup> may be a useful tool for estimating the risks of both bleeding and thrombosis. However, data justifying its use in clinical routine are still sparse<sup>110</sup>.

## Summary

Plasma is used therapeutically or as starting material for manufacturing albumin, immunoglobulins, protease inhibitor, clotting factor, and coagulation inhibitor concentrates. Plasma protein levels are subject to wide intraindividual and interindividual variation. Age, gender, ABO blood group, acute phase reactions, physical exercise, mental stress, and hormones substantially influence the plasma levels of therapeutically used plasma proteins. Hemostasis is now understood to involve complex interactions between vessel walls, platelets, blood cells, proteins circulating in blood or being fixed to cells, and other humoral factors such as ionized calcium. Clinically useful coagulation screening tests include activated partial thromboplastin time, prothrombin time, and thrombin time. The detection of mild hyperfibrinolysis and mild coagulation disorders requires additional specific assays, a clinical history, and an examination.

## Disclaimer

The author has disclosed no conflicts of interest.

## Key references

- A full reference list for this chapter is available at: [www.wiley.com/go/simon/Rossi6](http://www.wiley.com/go/simon/Rossi6)
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## CHAPTER 21

# Plasma and cryoprecipitate for transfusion

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### Plasma for transfusion

Plasma for transfusion can be collected through centrifugation of whole blood or by plasma apheresis. It can then be stored frozen as fresh-frozen plasma (FFP) or used to produce more purified constituents, including cryoprecipitate, concentrates of coagulation factors and fibrin sealant, immunoglobulins (e.g., Rh immune globulin), anticoagulants (e.g., antithrombin and protein C), complement related proteins (C1-esterase inhibitor), and albumin. FFP is frozen within a short-specified time period after collection and then stored at a defined temperature, typically at -25 °C.<sup>1</sup>

Internationally, the methods of manufacturing FFP for transfusion varies between countries<sup>2</sup> and some of these differences are summarized in Table 21.1. For example, in the United States, FFP must be frozen within 6–8 hours of collection, while plasma that is produced from whole blood donation that has been stored at 4 °C for up to 24 hours prior to separation is labeled as a different component (i.e., FP24).<sup>3</sup> Clinically, both FFP and FP24 are used interchangeably. In Europe, FFP can be prepared from whole blood donation and stored at 20–24 °C for 24 hours prior to it being separated and is labeled the same as frozen plasma that is produced <8 hours from donation (i.e., FFP),<sup>1,4</sup> and both components are used for the same clinical indications. Once thawed, FFP and FP24 must be used within 24 hours of thawing. In some countries, thawed plasma can be used beyond 24 hours and for up to five days from initial thawing, if stored at 4 ± 2 °C. It is then relabeled as “Thawed Plasma” or “Extended Thawed Plasma.”<sup>2</sup> In this chapter, we refer to *frozen plasma* as FFP, unless otherwise stated.

A typical unit of plasma derived from a collection of whole blood has a volume of 250–300 mL, and local and national guidelines for usage generally specify a dose of around 10–20 mL/kg. The composition of FFP can be influenced by many factors. These would include gender, ABO-type, age, genetic, and other environmental factors, all potentially modifying levels of individual proteins. The composition of collected plasma would also be affected by the processing procedure, including anticoagulants used, processing (for instance pathogen reduction and pooling), how quickly the plasma is frozen after collection, storage time, and preparation before administration.<sup>5</sup>

Although clinicians tend to assume approximate equivalence in clinical effectiveness between individual units of FFP, it is likely that

there is heterogeneity, reflecting the biological variation in constituents between donors (e.g., vWF and FVIII levels being lower in Group O donors). Such variation might be expected to be less marked for pooled plasma components such as pooled solvent-detergent treated plasma. Figure 21.1 illustrate the variation in content for selected coagulation factors between 66 units of (white-cell-depleted) FFP.

Immediately following thawing, plasma contains near normal levels of most plasma proteins, including procoagulant and anticoagulant components of the coagulation system. Over time, there is a loss of clotting factors in thawed plasma, with FVIII being the worst affected factor with losses ranging from 30% to 40% after storage over five days, and most of the decline occurring in the first 24 hours after thawing.<sup>2</sup> As Factor VIII is a labile clotting factor, its levels are used for quality control for the specification of the product. Other coagulation factors are less affected by extended thawed storage, with the decrease in most clotting factors being typically <10% by Day 5, with negligible change in levels of fibrinogen, antithrombin, and protein C.<sup>2</sup>

An alternative to FFP or extended thawed plasma in some countries (USA, Sweden, UK) is plasma that has never been frozen following manufacturing process, known as “liquid plasma” (LP) with its shelf life ranging from 7 to 21 days, depending on countries.<sup>2</sup> The primary use of LP is for immediate transfusion to treat acutely bleeding patients. In vitro studies have demonstrated increase in contact activation with the duration of LP storage, and this is more pronounced in women than men.<sup>6</sup> It is for this reason that some countries have chosen a shorter shelf life for LP, due to clinical concerns, although a retrospective study of 84,986 patients that had received LP in Sweden showed no evidence that the storage time of LP was associated with an increased risk of mortality.<sup>7</sup>

### Alternative preparations of plasma

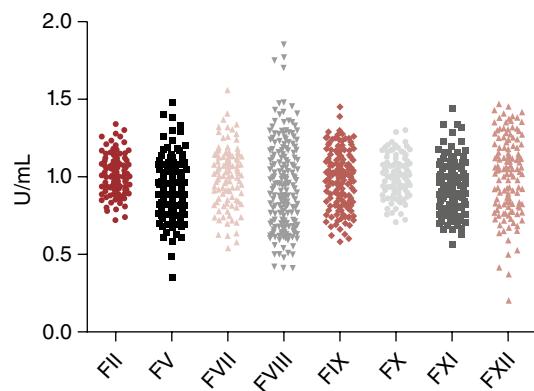
#### Dried plasma

Historically, the use of plasma started as early as the 1930s when methods to produce dried plasma were established.<sup>9</sup> The methods used were lyophilization (freeze drying) or spray drying. Drying plasma allowed for its use in remote areas where freezing facilities were not available, and it was therefore used extensively during the

**Table 21.1** Current Guidelines for the Preparation and Storage of Fresh-Frozen Plasma.

	AABB <sup>3</sup>	Australia <sup>8</sup>	Fresh-frozen plasma (FFP)	Fresh-Frozen plasma (FFP)	Council of Europe <sup>1</sup>
	Fresh-frozen plasma (FFP)	Plasma frozen within 24 hours (FP24)			Pathogen-reduced Fresh-frozen plasma (FFP)
Time from donation to freezing	<8 hours for CPD <6 hours for ACD	<24 hours	Within 18 hours of collection for whole blood derived and within 6 hours for apheresis	Preferably <6 hours <24 hours if whole blood rapidly cooled and stored 20–24 °C	
Freezing process	Not stated		Not stated	<25 °C within 1 hour	
Storage once frozen	≤18 °C: 12 months ≤-65 °C: 7 years	≤-18 °C: 12 months	<-25 °C: 12 months	18 to -25 °C: 3 months ≤-25 °C: 36 months	
Storage once thawed	<24 hours 1–6 °C ("thawed FFP")  <5 days at 1–6 °C labeled as "thawed plasma"	<24 hours 1–6 °C ("thawed FP24")  <5 days at 1–6 °C labeled as "thawed plasma"	Transfuse immediately or stored at 2–6 °C for up to five days	Use immediately or stored for 4 hours at 20–24 °C, 24 hours at 2–6 °C. For patients with major bleeding can be stored at 2–6 °C for up to five days	Use immediately
Clotting factor requirement for routine quality monitoring	None		>0.70 IU/mL of FVIII	>0.70 IU/mL FVIII	>0.50 IU/mL FVIII
Limits for cellular content	Not stated		Not stated	Red cells <6 x 10 <sup>6</sup> /L Leucocytes <1 x 10 <sup>6</sup> /unit if LD Platelets <50 x 10 <sup>9</sup> /L	

CPD: citrate phosphate dextrose; ACD: anticoagulant citrate dextrose; LD: leucocyte-depleted.

Source: Adapted from Cardigan and Green.<sup>2</sup>**Figure 21.1** Coagulation factor concentration in individual units of fresh-frozen plasma tested in the Component Development Laboratory of NHS Blood and Transplant. Source: Rebecca Cardigan, NHSBT. Reproduced with permission.

World War II (WWII). Currently, dried plasma is commercially available from three different companies producing dried plasma in centralized production units: French Lyophilized Plasma (FLYP), produced by the French Military Blood Institute (Centre de Transfusion Sanguine des Armees [CTSA]); LyoPlas N-w, produced by the German Red Cross; and Bioplasma FDP, produced by National Bioproducts Institute, Pinetown, South Africa. All three alternatives use the lyophilization method. Two of the methods pool plasma from either 11 or 1500 donors and provide an ABO universal product (FLYP and Bioplasma FDP), whereas the third producer use single donor quarantine plasma (LyoPlas N-w). The shelf life of dried plasma is 15–24 months stored in room temperature (below 25 °C), and the products shall be used immediately after reconstitution that takes only a few minutes. No increase in adverse events has been reported.<sup>9</sup> As during the WWII, dried plasma is currently mainly in use for prehospital transfusions both in military and civilian health services serving as a “bridge” to balanced transfusion for patients with life-threatening bleeding.

### Pathogen reduced plasma

During WWII, millions of dried plasma units were transfused to treat bleeding soldiers, but by the end of the war, it became apparent that the transfusion of plasma was related to the risk of viral infections such as hepatitis. The use of human plasma was therefore discouraged, and an effort to improve safety of plasma transfusion was initiated. Measures are now implemented to reduce the risk of transfusion transmitted disease, and these include screening of plasma donors for viral disease and implementation of pathogen reduction methods. Current methods for pathogen inactivation of plasma involve four major processes using solvent–detergent (SD), methylene blue (MB), amotosalen, and riboflavin as additives.<sup>10</sup> The latter three are used in combination with visible or ultraviolet light. Whereas the solvent–detergent treatment is performed in large centralized manufacturing centers for large pools of plasma, the other three methods are designed for localized production in a Blood Bank/Blood Center. All methods result in a reduction in protein values with the percent retention of FVIII activity in the range of 67–78% and fibrinogen of 65–84%. Protein S and alpha<sub>2</sub>-antiplasmin are lower following solvent–detergent treatment. Alterations in fibrinogen structure have been reported with methylene blue.<sup>2,10–13</sup> An overview of pathogen reduced plasma components is presented in Table 21.2.

### Cryoprecipitate for transfusion

Cryoprecipitate is a plasma component that is rich in cryoproteins, namely, FVIII, von Willebrand factor (VWF), FXIII, fibronectin, and fibrinogen. Cryoprecipitate is manufactured by slowly thawing of FFP overnight at 4 °C, which precipitates out the cryoproteins. After centrifugation, the cryoproteins are resuspended in a reduced (20–60 mL) plasma volume (see Table 21.3 for specifications). Cryoprecipitate can be produced in a single unit, derived from one donor, or it can be produced by pooling several units (e.g., in the UK pooled cryoprecipitate is prepared from five single donations), which have larger volume (200–280 mL) than a single unit and higher concentration of cryoproteins (i.e., 700 mg fibrinogen and 350 IU FVIII). Like FFP, cryoprecipitate is stored at ≤25 °C for a maximum of 36 months, and prior to use it requires thawing.<sup>4</sup>

**Table 21.2** Specifications of Different Fresh-Frozen Plasma.

	<b>Standard FFP</b>	<b>Solvent-Detergent (Octaplas LG)</b>	<b>Amotosalen Intercept</b>	<b>Vitamin B2 Mirasol</b>	<b>Methylene Blue**</b>
<b>Volume</b>	Varies between countries (ranges between 200 and 300 mL)	200 mL	200–300 mL (input plasma 385–650 mL)	170–360 mL	200–260 mL (50 mL neonatal size available)
<b>Virological Testing (Genomic Unless Stated)</b>	HIV, HBV, HCV, HEV all donations. HTLV new donors	All donations HIV, HBsAg and HCV by ELISA as well as HIV, HCV, HEV, HAV, HBV, and parvovirus B19 by PCR	Local routines	Local routines	HIV, HBV, HCV, HEV all donations
<b>Treatment Step</b>	None	1% TNBP 1% Triton X-100	150 µM amotosalen + UVA light 4 minutes	50 µM riboflavin + UV light 4–10 minutes	1 µM MB + visible light 30 minutes
<b>Removal Step for Residual Chemicals?</b>	N/A	YES <2 µg/mL TNBP <5 µg/mL Triton-X	YES	NO	YES <0.3 µmol/L MB
<b>Shelf Life—Frozen</b>	12 months to 3 years (depending on countries)	4 years	2 years	2 years	3 years
<b>Shelf Life at 4 °C Once Thawed</b>	24 hours (120 hours for unexpected major hemorrhage)	24 hours at 2–8 °C, 8 hours at 20–25 °C	24 hours	6 hours	24 hours
<b>Coagulation Factor Losses (Compared to Standard FFP)</b>	Over time all clotting factors reduce in extended thawed FFP	Batches tested for V, VIII, XI (all >0.50 IU/mL), protein C (>0.70 IU/mL), protein S (>0.30 IU/mL), antiplasmin (>0.20 IU/mL)	20–30% loss of FVIII and fibrinogen, others less affected	20–30% loss of FVIII, FXI and fibrinogen, others less affected	20–30% loss of FVIII, FXI and fibrinogen, others less affected
<b>Indications</b>	See text of this article	As for FFP	As for FFP	As for FFP	As for FFP Not TTP

\*\* Methylene blue specification are from UK data, although this component is not in use in the UK anymore.

HIV: human immunodeficiency virus; HBV: hepatitis B virus; HCV: hepatitis C virus; HEV: hepatitis E virus; HTLV: Human T-cell lymphotropic virus; HBsAg: hepatitis B surface antigen; HAV: hepatitis A virus; PCR: polymerase chain reaction; RCT: randomized control trial; TTP: thrombotic thrombocytopenic purpura; FFP: fresh-frozen plasma; SHOT: serious hazard of transfusion; HLA/HNA: human leukocyte antigen (HLA)/human neutrophil antigen (HNA).

Source: Adapted from Green *et al.*<sup>4</sup>

**Table 21.3** Typical Values for Cryoprecipitate in the UK and Australia.

	<b>UK<sup>4</sup></b>		<b>Australia<sup>8</sup></b>	
	<b>Single Cryoprecipitate</b>	<b>Pooled Cryoprecipitate</b>	<b>Whole-Blood-Derived FFP Cryoprecipitate</b>	<b>Apheresis-Derived FFP Cryoprecipitate</b>
Volume (mL)	49 ± 5	237 ± 28	36 ± 2	60 ± 2
FVIII (IU/unit)	108 ± 33	524 ± 130	151 ± 30	347 ± 57
Fibrinogen (Clauss)	0.43 ± 0.14 (g/unit)	1.67 ± 0.27 (g/unit)	357 ± 110 mg/unit	906 ± 268 mg/unit
Storage temperature	≤-25 °C	≤-25 °C	<-25 °C or below	<-25 °C or below
Shelf life—frozen	36 months	36 months	12 months	12 months
Shelf life once thawed	4 hours	4 hours	6 hours if it is a closed single unit  4 hours if it is an open system or units have been pooled	
Indications	Treatment of fibrinogen deficiency or dysfibrinogenemia when there is clinical bleeding, or disseminated intravascular coagulation, or prior to an invasive procedure.			

Data given as mean with SD.

Source: Adapted from Green *et al.*<sup>4\*</sup>

Cryoprecipitate was originally used as FVIII replacement therapy for hemophilia patients; however, currently cryoprecipitate is used as a standard concentrated source of fibrinogen in countries such as UK, Australia, United States, etc. Other countries use concentrates of fibrinogen. In the UK, a typical adult dose is two five-donor pools (equivalent to 10 single donor units). One such treatment administered to an average adult would typically raise the plasma fibrinogen level by about 1 g/L.<sup>14</sup> Larger doses of cryoprecipitate may be considered in patients with fibrinogen concentration <0.5 g/L and/or larger individuals. Cryoprecipitate should not be considered for transfusion solely as a more concentrated form of FFP (e.g., when there are concerns about fluid overload) as it predominantly contains cryoproteins.

The remaining product, following the removal of cryoprecipitate, is termed cryosupernatant, cryopoor plasma, or cryoprecipitate-depleted plasma.<sup>1</sup> It has been used in the treatment of TTP because of the theoretical benefit of its reduced content of von Willebrand factor, high-molecular-weight multimers, although the added benefit of cryosupernatant has not been proven. There is no current indication for cryoprecipitate-depleted plasma in the UK.<sup>4</sup>

Viral-inactivated purified fibrinogen concentrate is now available and may represent a safer concentrate for direct fibrinogen replacement in isolated deficiencies, for example inherited hypofibrinogenemia. This product is not licensed for broader clinical use in many countries; however, its use has been reported in other clinical situations, including trauma, cardiac surgery, and obstetrical hemorrhage.

## Clinical use of plasma transfusion

Clinical use of FFP has been maintained over the last two decades in many countries, although the defining characteristic of plasma transfusion is wide variation in transfusion rates. This variation in usage is seen both within and between countries. In a comparison of FFP use in five countries, the ratio of FFP units to red blood cell units transfused varied from 1:3.6 in the United States to 1:8.5 in France.<sup>15</sup> Studies have reported a wide variation in the use of FFP also among centers within countries and even within hospitals.<sup>16,17</sup> This variation is exemplified in multiple UK audits following transfusion practice for example adult patients undergoing cardiac surgery during a three-month period in 2010. In this audit, data on each patient were obtained from the Central Cardiac Audit Database and blood transfusion laboratories. The range of blood components usage, including plasma, was very wide across the different centers; for example, for patients undergoing CABG, the mean use of red cells was reported as nearly 3 units (2.98 units, range 0–32) and for FFP at nearly 2 units (1.98 units, range 0–22).<sup>18</sup> Although there may be some heterogeneity in the clinical characteristics of patients between different cardiac surgical centers in this audit, it seems unlikely to provide a full explanation for the differences between centers in transfusion rates.

FFP is given primarily for two indications: to stop bleeding (therapeutic) or to prevent bleeding (prophylactic). In addition, plasma is used as replacement fluid in patients undergoing therapeutic apheresis procedures. The core rationale for these uses is plasma as a source of procoagulants. The main specific recommendations for the transfusion of FFP have remained relatively consistent over the years, including (1) active bleeding as part of a massive transfusion protocol and/or due to acquired deficiencies of coagulation factors (such as in trauma, surgery, invasive procedures, etc.); (2) reversal of vitamin K deficiency related to vitamin K antagonists effect (such as warfarin) in patient with active bleeding, or prior to surgery or an invasive procedure (in conjunction with the use of prothrombin complex concentrates [PCCs]); (3) disseminated intravascular coagulopathy (DIC) or consumptive coagulopathy with active bleeding; (4) as replacement fluid in plasma exchange procedures in TTP; and (5) active bleeding due to congenital factor deficiency where there is no alternative therapies.<sup>4,19–22</sup> Variations of these indications will be found in most national guidelines. In a survey of Finnish hospitals, nearly 6000 FFP units were tracked indicating that FFP was transfused most often to surgery patients, especially cardiac surgery.<sup>23</sup> This finding is consistent with reports of FFP transfusions in other countries.<sup>17,24–26</sup> Uses of FFP that are considered inappropriate include the correction of hypoalbuminemia or nutritional support, and immunoglobulin replacement.

In 2020, an additional use of plasma was the focus of intense clinical and research interest.<sup>27</sup> During the COVID-19 pandemic, convalescent plasma (CP) was collected from recovered patients as a source of neutralizing anti-COVID-19 antibodies that may lower or eliminate the viral load in sick patients.<sup>28–30</sup> CP has been used previously in clinical studies for treating severe acute respiratory syndrome including SARS, MERS, avian influenza A (H5N1), and Ebola with some promising results, but many countries and groups provided CP in the context of randomized trial evaluations. Indeed, a staggeringly large number of trials have been registered, nearly 100, although there are many differences in protocol design, including the use of CP to treat adults or children in the intensive care unit (ICU) or in settings other than ICU or even as prophylaxis (for people in close contact with those confirmed to have COVID-19). Although recent studies indicate no effect of CP in unselected hos-

pitalized patients, it remains to be seen whether there is a benefit in patient groups such as those who are immunocompromised. Other factors will be relevant to the interpretation of the results when they become available, for example the different types of products (apheresis plasma and whole-blood-derived plasma) and the definitions of threshold anti-COVID antibody titers within the product. Another treatment option is to collect plasma to extract hyper immune immunoglobulins that may be infused in a concentrated form as passive immunization.

## Plasma transfusion in treatment of bleeding

For many years, national guidelines recommended that the transfusion of FFP for the management of bleeding was guided by conventional clotting tests. These recommendations were based on expert opinion rather than randomized control trials (RCTs). From 2005 onward, the initial transfusion of FFP during major hemorrhage was switched from laboratory-test-based protocols to empiric or ratio-driven protocols where FFP is issued based on a specified plasma to red cell component ratio before any clotting test result is available. Current guidelines now advocate that FFP transfusion should be given in a near-physiological ratio (1:1) with red blood cells and platelets in patients with massive hemorrhage.<sup>2,14,27,31</sup> This new approach came from observational studies in trauma showing that around one-quarter of all trauma patients who present with bleeding have a coagulopathy (defined as abnormal PT or APTT) that occurs early.<sup>32,33</sup> These studies have shown that the presence of acute traumatic coagulopathy is associated with increased risks of major hemorrhage, massive transfusion, and an increased risk of death. Therefore, the reversal of this coagulopathy with early balanced blood transfusion is an integral part of damage control resuscitation for major blood loss due to trauma.<sup>34</sup>

The role of higher and early doses of plasma and platelets has been addressed in the PROPPR randomized trial in North America, which compared 1:1:1 vs. 1:1:2 ratios of plasma, platelets and red cells.<sup>35</sup> For the composite main outcome of 24-hour and 30-day all-cause mortality, there were no significant differences between the groups, and this lack of a difference was also seen across most secondary outcomes. However, more patients in the 1:1:1 group achieved hemostasis and fewer experienced death due to exsanguination by 24 hours. The interpretation of this trial has been discussed previously;<sup>36</sup> however, the trial results have led in the implementation of a 1:1:1 ratio of plasma, platelet, and red cell transfusion in trauma bleeding patients in many countries.<sup>14</sup>

Another area of practice that is developing very fast is prehospital resuscitation support of bleeding patients in the “field” and outside the hospital setting. Evidence from the military, where evacuation time to definitive care may be prolonged, has provided evidence for pushing resuscitation with blood transfusion far forward and placing emphasis on early hemostatic resuscitation prior to arrival at definitive care. Two recent civilian prehospital RCTs have evaluated the effect of plasma transfusion versus standard of care (saline and/or red cell transfusion), giving conflicting results regarding the benefit of prehospital plasma in reducing mortality, with one trial (the COMBAT study) showing no difference in mortality at 28 days<sup>37</sup> whereas the other (the PAMPER study) showed significantly lower mortality at 30 days after plasma transfusion.<sup>38</sup> However, the transport times differed between the two studies, with median transport time for the COMBAT study being 19 (plasma group) and 16 minutes (standard group) compared to a median transport time of 42 (plasma group) and 40 minutes (standard group) in the PAMPER study. A post hoc analysis of

these two trials suggested that early plasma transfusion had the most benefit in patients in hemorrhagic shock with transport time over 20 minutes from a trauma center.<sup>39</sup> Neither studies observed any difference between the plasma group and the standard group with regard to safety. These results are consistent with findings from the military setting, showing improved survival with early transfusion of erythrocytes and/or plasma.<sup>40</sup>

In accordance with the ratio-driven transfusion strategy, whole blood for transfusion has been launched as an alternative to a 1:1:1 blood component strategy for red blood cells, plasma, and platelets.<sup>41–43</sup> Whole blood contains red cells, plasma, and platelets in a physiological ratio. It is an all-in-one solution to a balanced transfusion preferred by some over a blood-component-based strategy due to the logistical advantages and because it offers a more concentrated blood product.<sup>44</sup> Whole blood has been implemented in several hospitals and for prehospital transfusion both in military and civilian settings,<sup>45</sup> and several studies have been performed and are in progress evaluating the clinical effects of whole blood transfusion.<sup>46</sup> Whole blood is suggested as a preparedness product enabling a platelet containing transfusion alternative for use in smaller rural hospitals if platelets are not available in inventory or in case of blood shortages.<sup>27,47</sup>

### **Plasma in the treatment of patients with disseminated intravascular coagulation (DIC)**

Disseminated intravascular coagulation (DIC) is a syndrome characterized by the systemic activation of blood coagulation, but there is debate about definitions. DIC may occur as a complication of infections, solid cancers, hematologic malignancies, obstetric diseases, trauma, aneurysm, and liver diseases, among others, and each type of DIC presents characteristic features related to the underlying disorder. The most important treatment for DIC is the specific treatment of the underlying disorder. Blood component therapy with platelet or FFP transfusion should not be instituted on the basis of laboratory results alone but should be reserved for those with active bleeding.<sup>48</sup> The administration of FFP may be useful in patients with active bleeding with either prolonged PT/APTT (>1.5 times normal) or decreased fibrinogen (<1.5 g dL<sup>-1</sup>). It should also be considered in DIC patients requiring an invasive procedure with similar laboratory abnormalities.<sup>48</sup>

### **Plasma transfusion for prophylactic use**

In this section, we discuss the role of plasma transfusion to prevent bleeding (prophylactic) and the use of plasma transfusion in the absence of bleeding to correct abnormal clotting tests.

### **Plasma transfusion to correct abnormal clotting tests in the absence of bleeding**

The laboratory tests of aPTT and PT were developed to investigate coagulation factor deficiencies in patients with a bleeding history by providing an end assessment of thrombin generation by fibrin formation. The PT and aPTT results may be abnormal for a number of reasons not associated with bleeding risk, not least normal variation for some individuals. Some laboratories report the international normalized ratio (INR), which is based on PT and was developed to monitor warfarin therapy, by standardizing results to account for different sensitivities of thromboplastins. The INR can potentially quantify the coagulation status of other types of patient populations, like patients with liver disease, but the results should not be interpreted as having the same meaning in these patients, as for the INR patients receiving warfarin.<sup>49</sup>

In two early trials, evidence for a lack of benefit for prophylactic use of FFP was reported. Both were designed to carefully evaluate the effectiveness of FFP in a large group of patients and provided information about the sample sizes required to allow adequate power to detect clinically important differences between the groups of patients. In the first trial, the Northern Neonatal Nursing Initiative (NNNI) Trial Group randomized 776 neonates to FFP or to volume expanders (gelofusin or dextrose–saline) and did not show any differences in the prevention of intraventricular hemorrhage.<sup>50</sup> Allocation concealment and blinding of outcome assessors (to monitor clinically relevant long-term developmental outcomes) were reported. Of note, the study did not include the measurement of coagulation tests. In the other trial, the effectiveness of FFP was evaluated in patients with acute pancreatitis, and, in total, 275 patients were randomized to receive either FFP or a colloid solution. No evidence for the benefit of FFP was reported.<sup>51</sup>

A systematic review investigating whether a prolonged PT or elevated INR predicts bleeding during invasive diagnostic procedures published in 2005 found little evidence to support this common perception of a link between abnormal clotting tests and a higher bleeding risk.<sup>52</sup>

Patients with liver disease have a complex coagulopathy, with abnormalities of platelets, fibrinolysis, and inhibitors of coagulation as well as coagulation factor deficiency. Observational studies have demonstrated that the prolongation in clotting times is not necessarily indicative of bleeding risk in these individuals, particularly in the setting of common liver-related complications, such as variceal hemorrhage.<sup>53,54</sup> There is variable clinical practice in the use of FFP and cryoprecipitate for prophylaxis in patients with liver disease.<sup>55</sup>

A study by Abdel-Wahab and colleagues showed that the transfusion of plasma for mild abnormalities of coagulation values results in partial normalization of PT in a minority of patients and fails to correct the PT in 99% of patients.<sup>56</sup> Youssef and colleagues reported the effects of FFP transfusion in 100 patients with liver disease and found that it was difficult to correct abnormalities of coagulation tests unless large volumes of FFP were transfused and that the effects of transfusion were short-lived.<sup>57</sup>

A recent guideline from the British Society of Hematology states that PT and APTT do not reflect the true hemostatic status of patients with advanced liver disease and that abnormalities of PT and APTT need to be interpreted with caution in these patients. The guideline writing group found no good evidence to endorse the routine use of prophylactic FFP for correction of abnormal clotting tests in nonbleeding patients prior to interventions such as elective variceal banding or low-risk interventions.<sup>4</sup>

The risk–benefit ratio of a liberal FFP transfusion strategy for critically ill patients is often summarized as not favorable.<sup>58</sup> The Transfusion of Fresh Frozen Plasma in Nonbleeding ICU Patients (TOPIC) trial was a noninferiority, multicenter, randomized trial that investigated whether it is safe to withhold FFP transfusion to critically ill patients with abnormalities of coagulation tests undergoing an invasive procedure.<sup>59</sup> In the intervention group, prophylactic FFP transfusion prior to an invasive procedure was omitted compared to transfusion of a fixed dose (12 mL/kg) in the control group. Due to slow inclusion, the trial was stopped before the predefined target enrolment was attained, at which time 81 patients had been randomized for a total of 1 major and 13 minor bleeds. Preprocedural omission of FFP was not associated with increased occurrence of bleeding (relative risk, 1.17; 95% confidence interval [CI], 0.62–2.19;  $p=0.78$ ). FFP transfusions resulted in only a small reduction of international normalized ratio (INR), and to <1.5 in

only around half (54%) of the transfused patients. On the question of side effects, there were no differences in lung injury scores between the two groups, but there was an increased duration of mechanical ventilation in patients receiving FFP. In summary, although a small randomized study, the findings did not support a difference in complications of bleeding by FFP use as prophylaxis or not.<sup>59</sup>

A prospective pilot study of 119 intensive care patients also concluded that surgical tracheostomy in intensive care unit patients can be performed without bleeding complications in the case of normal thromboelastometry results (EXTEM CT) despite increased PT-INR.<sup>60</sup>

A systematic review that evaluated prophylactic use of FFP to either no FFP or no plasma product after cardiopulmonary bypass (CPB) showed no evidence of consistent significant beneficial effect on blood loss or transfusion requirement with the use of FFP, and no significant difference on other outcomes.<sup>61</sup> The hemostatic changes related to CPB are multifactorial, and not solely related to coagulation factor deficiency, and perhaps it is not surprising that FFP use prophylactically fails to provide benefit.

In conclusion, there is no evidence to support routine prophylactic use of FFP in nonbleeding patients with abnormal standard coagulation test preprocedure. The impact on commonly used doses of FFP to correct clotting results or reduce bleeding risk is also recognized to be very limited, particularly when the PT ratio or INR is between 1.5 and 1.9.<sup>4,61,62</sup>

### Reversal of warfarin effect

In patients with acquired vitamin K deficiency and the absence of bleeding, primary treatment should be initiated with oral/IV vitamin K.<sup>4</sup> Prothrombin complex concentrates (PCCs) are considered the product of choice in several countries for reversal of warfarin over-anticoagulation in patients who are bleeding or undergo urgent surgery or invasive procedures.<sup>63,64</sup> PCCs are produced by fractionation of pooled plasma and contain coagulation factors II, IX, X, and VII (no VII in the three-factor PCC) at a significantly higher concentration than FFP, as well as the natural anticoagulants (proteins C and S). A noninferiority RCT comparing the safety and efficacy of a four-factor PCC vs. FFP ( $n = 202$ ) in patients with elevated INR ( $\geq 2.0$ ) and experiencing an acute major bleeding event reported a faster correction in the INR in patients receiving PCC than with transfusion of FFP.<sup>65</sup> There were no differences in hemostatic efficacy or safety in patients receiving PCC or FFP. A multi-center RCT that compared FFP (20 mL/kg) vs. four-factor PCC (30 IU/kg) in patients with intracranial hemorrhage was terminated earlier than planned due to safety concerns seen with FFP.<sup>66</sup> Of the 50 patients that received study drug (23 FFP and 27 PCC), two patients in the FFP group vs. 18 in the PCC group reached the primary end point (i.e., of  $\leq$ INR 1.2 within three hours of treatment initiation,  $p = 0.0003$ ). However, a total of 13 patients died: 8 in the FFP group (5 from hematoma expansion, all occurring within 48 hours after symptom onset) and 5 in the PCC group (none from hematoma expansion). There were 43 serious adverse events (20 in the FFP group and 23 in the PCC group): six serious adverse events were judged to be FFP related and two PCC related. The results suggest that for the treatment of vitamin K antagonist-related intracranial hemorrhage, PCC should be administered instead of FFP.<sup>66</sup> Further studies should be undertaken to investigate whether the faster improvements in coagulation screening tests translate into clinical benefit in specific populations and if there are any increased risks of thromboembolic complications.

### Therapeutic plasma exchange

FFP is frequently used as a replacement fluid in patients undergoing therapeutic apheresis procedures.<sup>67</sup> Evidence-based guidelines with grading and categorization of apheresis indications are published by The American Society for Apheresis (ASFA) guideline.<sup>68</sup> Plasma exchange with FFP is the first-line treatment of choice for TTP, providing a source of ADAMTS13 (A Disintegrin And Metalloprotease with ThromboSpondin type 1 motif 13).<sup>22</sup> TTP is a medical emergency. Due to high risk of preventable early deaths in TTP, treatment with therapeutic plasma exchange should be initiated as soon as possible, and preferably within 4–8 hours regardless of time of day at presentation if a patient presents with a microangiopathic hemolytic anemia and thrombocytopenia in the absence of any other identifiable clinical cause.

In relation to the COVID-19 pandemic, discussions have been undertaken on the role of therapeutic plasma exchange in the treatment of the cytokine release syndrome in COVID-19. Removal of these cytokines by therapeutic plasma exchange prior to end-organ damage may potentially improve clinical outcomes. As per the ASFA-2019 guidelines, TPE for patients with infections and multi-organ failure due to various causes falls under Category III Grade 2B, meaning that the optimum role of apheresis therapy in treating these patients is not established and that the current moderate quality of peer-reviewed evidence only supports an overall weak recommendation for this approach. TPE for COVID-19 patients with MOF may also be considered under this category.<sup>69</sup>

### Clinical use of cryoprecipitate

Cryoprecipitate is a source of concentrated fibrinogen, and its use is largely focused on patients with clinical bleeding and low-fibrinogen concentrations. A common approach is that if fibrinogen level is  $< 1.5$  g/L, fibrinogen should be supplemented.<sup>14</sup> In these patients with a critically low-fibrinogen level, the concentration of fibrinogen in FFP is insufficient to achieve the rapid rise in levels required. Clinical data do not clearly support one form of concentrated fibrinogen replacement therapy over another, whether cryoprecipitate or fibrinogen concentrate. Fibrinogen is the key final component of the clotting pathway and forms fibrin insoluble protein that is the foundation of a stable clot. Fibrinogen is one of the earliest coagulation proteins to fall in major bleeding,<sup>70</sup> and when levels fall, patients have a reduced ability to form clots and may bleed for longer periods.

Trauma hemorrhage remains the clinical setting where much of the research on fibrinogen replacement is being conducted. Low levels of fibrinogen and increased breakdown are key components of the early coagulopathy in trauma, and low-fibrinogen levels on admission to hospital are independent predictors of early mortality in trauma patients.<sup>70</sup> Fibrin strands that form in a low-fibrinogen environment are more susceptible to clot breakdown (fibrinolysis), and this process is both overactivated and ubiquitous in major trauma.<sup>71</sup> Fibrinogen supplementation using concentrates may improve outcomes for trauma hemorrhage, by improving clot strength and reducing blood loss.<sup>72,73</sup> In an uncontrolled observational study of 131 hemorrhagic patients, mortality rates fell by 14% after fibrinogen treatment.<sup>74</sup> Two observational cohort trauma studies have also reported reduction of mortality in patients receiving higher fibrinogen content during massive transfusion therapy.<sup>75,76</sup> Despite very high doses of fibrinogen (up to 12 g), plasma levels did not increase beyond the normal range in healthy subjects, and there were no reported adverse events.<sup>74</sup>

A recent trial that compared fibrinogen concentrate with cryoprecipitate in patients undergoing elective aortic surgery who develop clinically significant bleeding and hyperfibrinogenemia after cardiopulmonary bypass showed no significant differences for adverse events, and its results indicated that fibrinogen concentrate was noninferior to cryoprecipitate with regard to the number of blood components transfused in a 24-hour period post bypass.<sup>77</sup> There is a paucity of other good-quality clinical or cost-effectiveness comparative research between fibrinogen concentrate and cryoprecipitate in many clinical settings.

### The side effects of plasma and cryoprecipitate transfusion

Crucial to recommendations in guidelines for FFP transfusion practice is the need for a clear understanding of the risk of harm. As with all blood components, FFP is not without risk.<sup>78–81</sup> More immediate and serious complications of FFP are transfusion-related acute lung injury (TRALI) and transfusion-related circulatory overload (TACO), although there are ongoing issues of reporting and diagnosing these conditions that make accurate estimation of prevalence difficult.<sup>82</sup> In the UK, an association between cases of TRALI and female donors has been identified through the Serious Hazards of Transfusion hemovigilance scheme, and this has resulted in the policy of using male donors only for the production of plasma components, with significant reduction in the incidence of TRALI.<sup>1</sup> In other jurisdictions, requirements on negative tests for anti-HLA and/or anti-HNA antibodies are applied for single donor plasma. Fluid overload is another important complication associated with the transfusion of larger doses of FFP in an attempt to reverse abnormal coagulation tests, particularly in patients undergoing cardiac surgery or for critically ill elderly patients. Other risks are transfusion-transmitted infections, including an unquantifiable risk of prion disease. Allergic reactions to FFP are relatively common, and they can be extremely troublesome or, rarely, life-threatening.

Understanding the risks of FFP transfusion is important when considering its use as prophylaxis. A prophylactic policy should only be justified if the risk of bleeding is greater than the risk of harmful effects. Without evidence of benefit, such policy aimed at preventing (uncommon) bleeding complications could involve transfusing FFP to a large number of patients, many of whom might not bleed even if prophylactic FFP were not given.

Dara *et al.* reported a single-center retrospective cohort study of FFP use in medical ICU patients.<sup>83</sup> They identified patients in whom an INR  $\geq 1.5$  was found during ICU stay and evaluated FFP use in the subgroup who were not actively bleeding. In addition to variability in FFP transfusion practice, the authors observed that

patients who received FFP had a similar rate of hemorrhage to matched cases, but had a higher incidence of “acute lung injury” during the 48 hours after transfusion (18% vs. 4%;  $p = 0.02$ ). This association raises the possibility that critically ill patients, many with concurrent inflammatory problems, may be more susceptible to TRALI after receiving plasma-rich blood products, although distinguishing TRALI from other clinical problems such as volume overload remains problematic. These findings do not prove cause and effect, but again emphasize a need for concern about inappropriate use of FFP, in view of the real risk of harm.<sup>58</sup>

Risks from cryoprecipitate transfusion are generally considered small but include those common to FFP (i.e., allergic reactions, infection, transfusion-associated acute lung injury, and transfusion-associated circulatory overload).<sup>4,84</sup>

### Conclusions

FFP and cryoprecipitate are important sources of procoagulants for the treatment of patients with bleeding. When administered as prophylaxis, an understanding of the full risks of FFP and cryoprecipitate transfusion is particularly important. A prophylactic policy should only be justified if the risk of bleeding is greater than the risk of harmful effects, and these components should not be routinely used to correct abnormal clotting tests in the absence of bleeding when the risk of bleeding is low.

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## CHAPTER 22

# The purification of plasma proteins for therapeutic use

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### Introduction

It has been noted that blood plasma is the largest human proteome containing approximately 20,000 proteins plus splicing and post-translational variants.<sup>1,2</sup> As it is part of the circulation system, plasma contains other tissue proteomes as subsets through its contact with all organs of the body and their diverse cellular components. In addition, it contains specific plasma cells involved in the immune response and homeostasis. Proteins found in plasma can constitute proteins secreted by solid tissues that act in plasma; immunoglobulins (Ig); “long-distance” receptor ligands, such as hormones; “local” receptor ligands, such as cytokines and growth factors; and tissue leakage products.

The true plasma proteins are considered to be those that carry out their functions in circulation.<sup>3</sup> These constitute a number of proteins predominantly produced by the liver and immunoglobulins from the bone marrow.<sup>4,5</sup> The hepatic-derived plasma proteins encompass key proteins that constitute:

- The coagulation pathway and its regulation; these include the clotting factors (fibrinogen [factor I], prothrombin [factor II], and factors V, VII, VIII, IX, X, XI, XII, and XIII) and the regulatory factors or protease inhibitors (antithrombin III, heparin cofactor H, and proteins C, S, and Z).
- Components of the immune response and reaction to foreign bodies via the classic and nonclassic pathway, consisting of the complement factors and mannose binding lectin.
- Acute phase reactants, such as  $\alpha_1$ -acid glycoprotein, C-reactive protein, serum amyloid protein, serum amyloid A, and  $\alpha_2$ -HS glycoprotein, constitute part of the body's response to injury and infections; increased production of several hundred-fold can occur.
- Plasma proteinase inhibitors, which regulate the action of key proteolysis enzymes such as plasmin cathepsin G, elastase, and kallikrein and include  $\alpha_1$ -antichymotrypsin,  $\alpha_2$ -macroglobulin, inter- $\alpha$ -trypsin inhibitor, C1 inhibitor, and  $\alpha_1$ -antitrypsin.
- Carrier proteins, which are involved in the transport of iron, copper, hemoglobin, heme, bilirubin, and fatty acids; these include albumin, transferrin, ceruloplasmin, transthyretin, retinal binding protein, haptoglobin, hemopexin, and vitamin D binding protein.

- Biochemical regulators involved in immune cell regulation such as interleukins, interferons, and growth factors, for example, tumor necrosis factor and transforming growth factor  $\beta$ .

The immunoglobulins, produced in the bone marrow, exist as a number of subtypes—IgG, IgM, IgA, IgE, and IgD. IgG and IgM have the highest concentration and play the prime role in immune surveillance, the sequestration of foreign bodies, and the initiation of subsequent immune cellular and humoral responses.<sup>6</sup>

With increased understanding of the biochemical basis of disease, researchers see plasma proteins as possible therapeutic agents in several conditions either as a means of supplementation in cases of deficiency or to regulate biochemical pathways to achieve desired therapeutic outcomes. The biochemistry, clinical use, and production of these proteins have been reviewed recently.<sup>7,8</sup> Of the many identified plasma proteins, 20 therapeutic products have been developed and are in clinical use, with approximately another five in various stages of development.<sup>9–32</sup> These are presented in Table 22.1.

The production of plasma-derived medicinal products (PDMPs) has led to the development of a large global industry involving the complex activities of collecting blood and plasma donations and applying bioprocessing procedures to produce products with validated efficacy and pathogen safety.<sup>33</sup>

It is generally agreed that the start of plasma fractionation can be traced to the work of Edwin J. Cohn in his Harvard Laboratory in the 1940s, where he developed a cold ethanol-based precipitation process for the purification of albumin to be used as a resuscitative fluid in theatres of war.<sup>34</sup>

This chapter describes the manufacture of plasma proteins for therapeutic use and makes extensive use of divergent examples to illustrate the range of approaches that can be taken. In addition, it covers the relevant pathogen removal methodologies that ensure product safety and important information relating to specifications of the final product. This information will be of use to the reader involved in the assessment, purchase, or use of plasma products, who is often confronted with literature on products containing unfamiliar concepts and terminology relating to plasma fractionation, viral removal procedures, and product specifications. An increased understanding of the production of plasma protein therapeutics will facilitate communication with manufacturers of plasma products and aid in the assessment of existing products and the exploration of possibilities for future development.

**Table 22.1** Plasma Proteins with Established Clinical Use or Under Active Investigation

Protein or Therapeutic Product	Established Clinical Use
Albumin Immunoglobulins: Intravenous and subcutaneous	Fluid replacement in trauma, burns, sepsis, and cirrhosis Replacement therapy in primary and secondary immunodeficiency, immunomodulation in certain autoimmune diseases including immune thrombocytopenia purpura, Kawasaki disease, Guillain–Barré syndrome, autoimmune polyneuropathy, and myasthenia gravis Treatment of hepatitis A, hepatitis B, cytomegalovirus, varicella-zoster, and tetanus infections
Immunoglobulin hyperimmune to viral and bacterial antigens	
Anti RH(D) immunoglobulin	Prevention of Rh(D) isoimmunization due to fetus-maternal rhesus D incompatibility, treatment of immune thrombocytopenia purpura
Factor VIII	Replacement in hemophilia A-factor VIII deficiency; induction of immune tolerance for antifactor VIII antibodies Treatment of vWF deficiency
Von Willebrand factor (vWF)	Replacement in hemophilia B-factor IX deficiency
Factor IX	Treatment of patients with inhibitory antibodies to factor VIII or factor IX
Activated prothrombin complex concentrate (aPCC) Composed predominantly of prothrombin and factors VIII, VII, VIIa, IX, X, Xa; and protein C. Brand name: Factor VIII Inhibitor Bypassing Activity (FEIBA®)	Treatment of the rare conditions of factor VII and factor X deficiencies, reversal of the anticoagulant activity of warfarin and prevention of massive bleeding, often in combination with fibrinogen
Prothrombin complex concentrate (PCC); factors II, VII, IX, and X; and regulatory proteins: protein C, S, and Z	Replacement in congenital fibrinogen deficiency and in acquired deficiency following massive bleeding
Fibrinogen	Treatment of Factor XI deficiency; incidence: 1:10 <sup>6</sup>
Factor XI	Treatment of Factor VIII deficiency; incidence: 1:2 × 10 <sup>6</sup>
Factor XIII	Treatment of Factor X deficiency; incidence: 1:1 × 10 <sup>6</sup>
Factor X	Used in surgery as a sealant to achieve hemostasis, as a surgical glue and to promote wound healing
Fibrin glue (fibrinogen and thrombin components)	Treatment of alpha <sub>1</sub> -proteinase inhibitor deficiency
Alpha <sub>1</sub> -proteinase inhibitor	Treatment of acquired and hereditary deficiency of Antithrombin III
Antithrombin III	Treatment of hereditary angioedema
C1-esterase inhibitor	Treatment of purpura fulminans and venous thrombosis in patients with severe congenital protein C deficiency
Protein C	Treatment of hemoglobinemia or hemoglobinuria due to hemolysis after burn, trauma, blood transfusion or open-heart surgery
Haptoglobin	Use in Ukraine and Russia has been as an adjunct therapy in cancer therapy, emergency medicine indications, and infection; outcomes thought to be enhanced by the antioxidant and ferroxidase activity of ceruloplasmin
Ceruloplasmin	
<b>Under Investigation</b>	
Plasmin	Potential Clinical Applications Dissolution of clots by direct application in peripheral arterial occlusion, stroke, deep vein thrombosis, myocardial infarction, and clotted intravascular devices
Reconstituted plasma-derived high-density lipoprotein (derived from apolipoprotein A1)	Atherosclerotic plaque stabilization in acute coronary syndrome through reduction in lipid content and other anti-inflammatory and antithrombotic mechanisms
Transferrin	Treatment of hypotransferrinemia, enhancing erythropoiesis, and reducing anemia in β-thalassemia.
Plasminogen	Used in hematological stem cell transplantation to sequester free iron binding of iron reduces free-iron-mediated ischemia/reperfusion injury in transplanted organs
Hemopexin	Treatment of ligneous conjunctivitis related to plasminogen deficiency Removal of free heme arising from hemolysis in sickle cell disease

## Key components of the manufacture of plasma protein therapeutics

### Plasma for fractionation

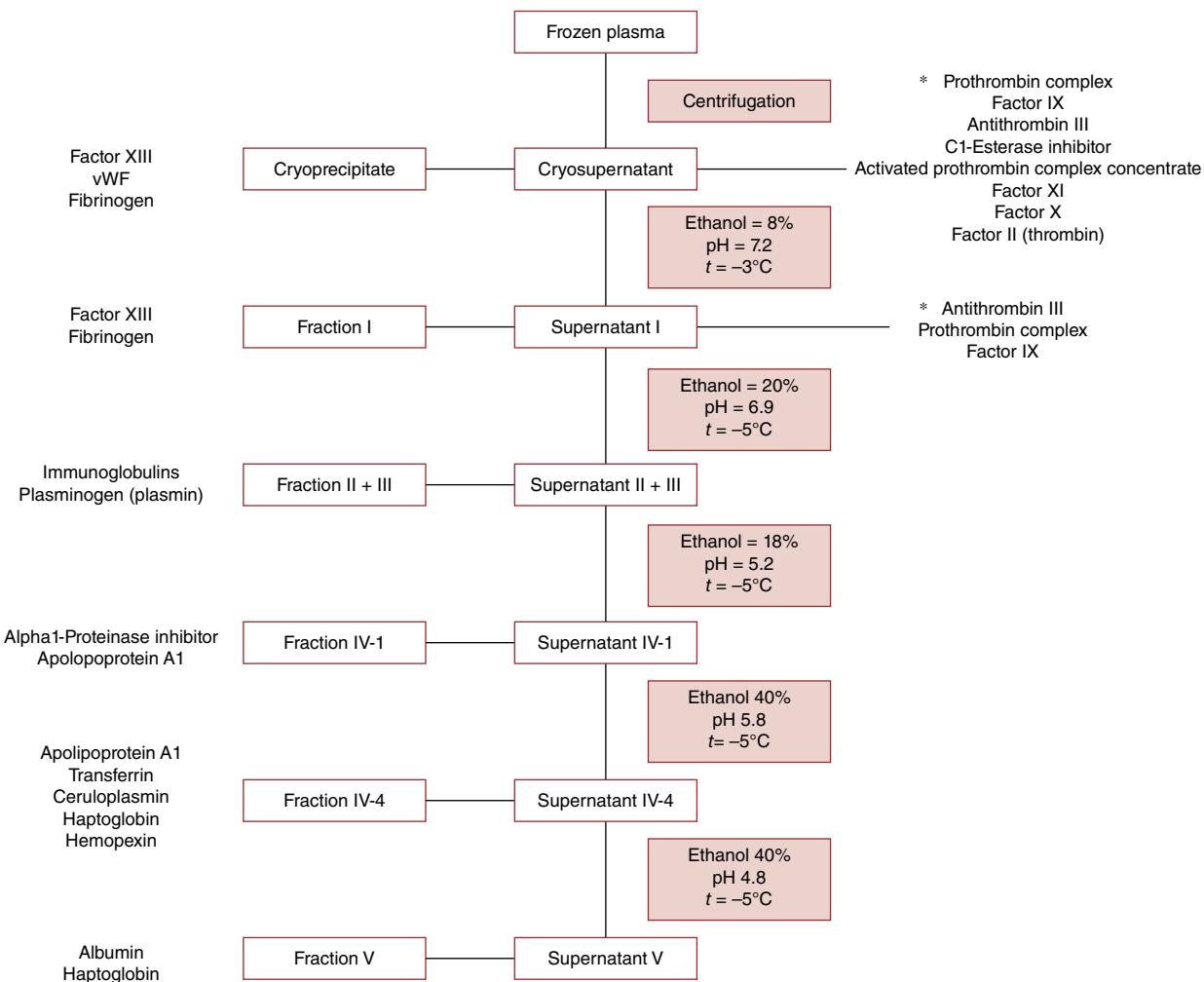
The manufacture of plasma-derived medicinal products commences with the collection of plasma. This is a logistically and technically complex activity due to the need to ensure product quality and safety.<sup>35</sup> The viral safety of plasma collected for fractionation is ensured by what has been referred to as a “Five Layer Safety.”<sup>36</sup> These layers are donor screening, blood testing, donor referral, quarantine, and investigation. Details of these activities are provided in Chapters 4, 5 and 44.

Plasma used in the United States for manufacture is either recovered plasma (plasma derived from whole blood collection) or source plasma (collected by plasmapheresis). No specific monograph exists in the United States which details the handling conditions to be applied to collected plasma. However, the pharmacopoeia of the European Union (European Pharmacopoeia) specifically details the freezing conditions applicable for labile proteins and recommends that plasma to be used in the manufacture of labile

proteins needs to be frozen within 24 hours of collection, and that the rate of freezing should be such that –25 °C is attained in 12 hours of being placed in the freezing apparatus. For the recovery of nonlabile proteins,<sup>37</sup> the plasma must be frozen to –20 °C or below within 24 hours of collection. During storage and transportation, the temperature of the plasma can exceed –20 °C for not more than a total of 72 hours, it must not transiently exceed –15 °C on more than one occasion and at no time must the temperature exceed –5 °C.

### Cold ethanol fractionation

The cold ethanol fractionation procedure that continues to have a pivotal role in plasma fractionation was developed by Cohn in the 1940s. It is based on the differential precipitation of plasma proteins by manipulation of ethanol concentration and pH of a low-ionic-strength solution maintained at a subzero temperature.<sup>34</sup> The original process, known as the *Cohn method*, is presented in Figure 22.1 and shows the conditions for the generation of specific fractions. Also shown is the recovery of cryoprecipitate from freeze-thawed plasma by centrifugation, typically incorporated in the processing of plasma. In addition, the source of current therapeutic proteins or



**Figure 22.1** The cold ethanol fractionation process for plasma (Cohn method 6) with the fractions indicated that are the source of various therapeutic proteins. Proteins that are recovered from the cryosupernatant and Supernatant I fractions require specific adsorption using chromatographic resins (denoted by \*).

those under development, listed in Table 22.1, is detailed. An example is considered to specifically illustrate the principle of the Cohn process: if one takes Supernatant I, adding ethanol to a final concentration of 25% (v/v) and adjusting the solution to pH 6.9, while ensuring that the ionic strength and temperature remain at approximately  $I = 0.09$  and  $-5^\circ\text{C}$ , respectively, result in the generation of the immunoglobulin-enriched Fraction II + III and Supernatant II + III.

It is important to have an understanding of the cold ethanol fractionation process as the terminology associated with the process is often used in literature associated with various plasma-derived products. Each fraction is an enriched, albeit crude, source of various plasma proteins that can be further purified to generate a therapeutic product. How this is achieved is covered in the subsequent sections. Prior to this, however, a description of viral removal procedures commonly used in manufacturing processes is undertaken. These need to be incorporated in any manufacturing process to ensure the viral safety of the therapeutic product.

### Chromatographic procedures

The cold ethanol fractionation process can generate highly purified albumin and immunoglobulin products.<sup>34,38</sup> Today, albumin is still predominately purified by the cold ethanol fractionation procedure,

and this was the case until the late 1990s to produce immunoglobulin products. The purification of other therapeutic proteins from the other enriched fractions has only been able to be achieved by the application of chromatographic techniques.<sup>39</sup>

Details of the purification of the major plasma-derived therapeutic proteins are provided in this chapter. However, an appreciation and understanding of these processes requires knowledge of the principles of chromatography and the orthogonal approaches that need to be applied to obtain highly purified preparations.

Chromatographic techniques allow the purification of proteins because of their differences in molecular weight, charge, hydrophobicity, and specific affinity for ligands. This involves the use of size exclusion, ion exchange, hydrophobic interaction (HIC), and affinity chromatography, respectively.<sup>40–43</sup> Chromatography is typically performed with columns packed with resin beads of about 50–90  $\mu\text{m}$  in diameter and derivatized with particular functional groups to allow the separation to be performed.

Size exclusion separation occurs as proteins percolate through the beads. Smaller proteins enter the pores of the beads, and hence their passage through the column is retarded, leading to the separation of proteins on the basis of molecular weight, with higher molecular weight proteins eluting first followed by the smaller species.

Ion exchange chromatographic resins exist in either the anion (positively charged) or cation (negatively charged) forms. Typical ion exchange ligands include diethylaminoethyl (DEAE), quaternary amino ethyl (QAE), quaternary ammonium (Q), carboxymethyl (CM), sulfopropyl (SP), and methyl sulfonate (S) for anion and cation exchange chromatography, respectively. As proteins are zwitterion molecules, the net charge of the molecule can be modulated by changing the pH of the protein environment. Proteins have different pI values—the pH at which the net charge of the protein is zero.<sup>43</sup> Therefore, the net charge of a protein depends on pH, and this determines the degree of interaction with an ion exchange resin. Interaction can be further modulated by changes in ionic strength. Thus, by utilizing conditions that promote differential binding to ion exchange resins, coupled with defined elution conditions—again involving specific conditions of pH and conductivity, the fractionation of a mixture of proteins can be achieved.

Proteins exhibit differences in their hydrophobic profile, reflecting differences in the composition of amino acids with hydrophobic (nonpolar) or hydrophilic (polar) properties.<sup>43</sup> This difference is exploited in hydrophobic interaction chromatography (HIC) and mixed-mode chromatography. Solution conditions can be manipulated so that the more hydrophobic proteins are retained on a column while less hydrophobic proteins flow through.

Proteins exhibit unique surface epitopes and functional domains.<sup>43</sup> Immobilized antibodies and specific ligands on chromatographic resins, which can specifically interact with these parts of proteins, can be the basis of affinity chromatography for the purification of proteins from complex mixtures. Immobilized monoclonal antibodies, heparin, and metal affinity ligands are used in the purification of several plasma proteins.<sup>39</sup>

### **Pathogen reduction of plasma-derived medicinal products**

Since the occurrence of human immunodeficiency virus (HIV) transmission by transfusion of blood components and plasma-derived medicinal products (PDMPs) in the early 1980s, the plasma fractionation industry has invested a significant effort to vastly improve the safety of PDMPs through implementation of the complementary measures of donor selection, plasma testing, and strategic manufacturing.

PDMPs are manufactured from relatively large pools of human plasma (~2000–4000 L). Intermediates from multiple pools of plasma may be combined such that a final container may contain proteins from more than 50,000 individual donations. Compared to blood components derived from single individuals, the pooling of many donations may increase the risk that the starting material is contaminated by an unknown or untested-for virus. However, judicious qualification of donors, coupled with serological and nucleic acid testing (NAT) of plasma donations for relevant blood-borne viruses, effectively screens out donations containing high titers of viruses known to have been transmitted by blood or plasma products in the past. Moreover, the pooling process provides a dilution factor that also contributes to safety. Importantly, the plasma pools can also be tested for viruses by NAT methods as a redundant practice to minimize the risk that plasma for further manufacture is contaminated.

By 1994, plasma fractionation companies had made remarkable progress, as indicated by the absence of disease transmission of hepatitis viruses or HIV by any product manufactured by American or European biological producers. After the introduction of second-generation serological testing for, and exclusion of, hepatitis C virus

(HCV) antibodies from plasma for further manufacture, the loss of inherent anti-HCV protection in immune globulin products unintentionally allowed for instances of HCV transmission.<sup>44</sup> However, the occurrence of further transmission was prevented by the introduction of NAT testing of plasma donations and plasma manufacturing pools, as well as the inclusion of effective and robust virus reduction steps into the manufacturing processes of immune globulin for intravenous administration (IVIG).<sup>45</sup>

The manufacturing processes of all PDMPs contain steps specifically designed to have a high capacity to inactivate or remove viruses. Such capacity is imparted to a process by incorporation of, typically, two effective and robust virus reduction steps that are orthogonal and complementary to one another in their modes of action. Taken together, donor selection, plasma testing, and manufacturing concepts have been highly successful in ensuring the safety of PDMPs against known and (re)emerging viruses.

### **Incorporation of virus reduction capacity into biomanufacturing processes**

A manufacturing process step can be intentionally designed with a mechanism to inactivate viruses (such as pasteurization developed by Norbert Heimburger<sup>46</sup> or solvent/detergent treatment developed by Bernard Horowitz)<sup>47</sup> or remove viruses from the process stream (e.g., filtration). Such dedicated mechanisms are relied upon to confer effective and robust virus reduction capacity. Alternatively, a step primarily designed to purify the target protein may also possess virus reduction capacity via partitioning of viruses along with impurities. Because purification steps are designed to isolate the protein of interest, these partitioning steps often offer only limited virus reduction capacity. Careful assessments are required to determine the extent that such purification steps contribute to the overall virus reduction capacity of a product's manufacturing process.

Successful incorporation of virus reduction capacity into biomanufacturing processes requires that the functional activity of the target therapeutic protein be maintained. Furthermore, it must be demonstrated that neoantigens, which could lead to immunogenicity, have not been introduced into the product stream.<sup>48,49</sup> Amino acid analysis, cleavage with proteolytic enzymes, circulatory survival in animal models, and measurement of sedimentation and diffusion coefficients, viscosity, circular dichroism, and optical rotary dispersion are examples of analytics used to ensure the integrity of the target biotherapeutic.<sup>50</sup>

### **Validation of the capacity of a manufacturing process to inactivate and/or remove viruses**

A successful virus reduction strategy also requires that steps be formally validated for their capacity to inactivate and/or remove viruses. Virus validation studies must be performed by trained staff in dedicated, properly equipped laboratories kept separately from manufacturing facilities so as not to contaminate the production line.

In a virus validation study, a selected step (or a series of steps) of the manufacturing process is studied to assess and quantitate how effectively it can inactivate or remove a broad range of viruses. Experiments are carried out using a small-scale version of the commercial manufacturing process with aliquots of starting material (production intermediate) obtained from the commercial-scale process. The production-scale manufacturing process is closely mimicked in the small-scale model by tightly controlling key operating parameters. The suitability of the small-scale model is confirmed by comparing key performance characteristics and by demonstrating that the biochemical characteristics of the resultant

output material from the small-scale model are comparable to those of the production-scale process.

A virus validation experiment is performed by deliberately spiking a small volume of high-titer virus into an aliquot of product intermediate derived from the commercial manufacturing process. The virus-spiked product intermediate is then run through the qualified small-scale model of the commercial manufacturing process, and samples are removed for subsequent assay. A process step capable of removing  $\geq 4 \log_{10}$  of infectious virus particles is considered effective when the manufacturing process step can be reliably performed and is insensitive to modifications within the specifications of the manufacturing process.<sup>51</sup>

In addition to providing an accurate reflection of the full-scale manufacturing procedure, small-scale versions of the production process are used to study the robustness of the virus clearance capacity. These robustness experiments evaluate the production parameters that may have an influence on virus inactivation and/or removal. Examples of relevant production parameters include protein concentration, the concentration of precipitating agents, temperature, pH, reaction time, amount of stabilizer, and the amount of chemical inactivating agents. For chromatographic steps, protein concentration, flow rate, washing and elution volume, and resin reuse should be assessed. Robustness experiments are designed to assess the impact of extreme production conditions on virus reduction capacity. As such, they are performed for given parameters using operating conditions at the boundaries of their ranges or even beyond the specifications of routine production. The goal of these experiments is to demonstrate that the capacity of a process to remove viruses is robust across the ranges of operating parameters at which the manufacturing process is licensed.

Viruses chosen for validation studies should closely resemble the viruses that may potentially be present in contaminated plasma (i.e., transfusion-relevant viruses). To test the ability of the manufacturing process to remove viruses in general, the chosen test viruses should also exhibit a wide range of physicochemical properties. For these studies, strains of viruses should be chosen that replicate to high titers in cell culture and can be assayed in an effective, sensitive, and reliable manner via *in vitro* infectivity methods.

Virus validation studies should be performed with HIV and hepatitis A virus (HAV) as relevant viruses. For those blood-borne viruses that cannot be propagated in cell culture systems, model viruses must be used. Model viruses for HCV include, for example, bovine viral diarrhea virus (BVDV) (a flavivirus) or Sindbis virus (SINV) (a togavirus). A specific model virus for hepatitis B virus (HBV) is not available; therefore, nonspecific model viruses must be used. In situations where a novel inactivation method is under test, duck hepatitis B virus could also be used as a surrogate for HBV. For parvovirus B19 (B19V), currently only experimental cell culture systems are available; therefore, animal parvoviruses, e.g., canine parvovirus (CPV), minute virus of mice (MVM), or porcine parvovirus (PPV), are used as model viruses. In the case of interactions between human immunoglobulins with viruses used in virus validation studies, the use of model viruses may be essential: HAV can be replaced by porcine or bovine enteroviruses or encephalomyocarditis virus (EMCV).<sup>51,52</sup>

## **Virus inactivation**

### **Heat treatment in aqueous solution (pasteurization)**

Heating in aqueous solution at 60 °C for 10 hours or greater can be a highly effective method for the inactivation of both enveloped and nonenveloped viruses. The effectiveness of inactivation is dependent

upon the composition of the solution and the concentration of stabilizers that are used to protect proteins and minimize neoantigen formation; stabilizers can also protect viruses (especially nonenveloped virus). Stabilizer concentrations are carefully chosen to find conditions that maximize retention of biological activity of the therapeutic molecule while maximizing virus inactivation. The impact of stabilizers and temperature on the virus inactivation capacity for a wide range of viruses must be carefully validated.<sup>53</sup>

Pasteurization has proven to be an effective virus inactivation method for albumin products for several decades. The pharmacopoeia method for albumin requires formulation with sodium caprylate alone or with *N*-acetyl tryptophan as a stabilizer prior to sterile filtration, filling, and treatment at 60 °C for 10–11 hours in the final product container. The method has since been successfully applied to a range of other plasma protein therapeutics including immunoglobulins, fibrinogen, alpha<sub>1</sub>-proteinase inhibitor, haptoglobin, C1 esterase inhibitor, and even labile molecules such as factor VIII concentrates.<sup>54,55</sup> This is only possible by the use of high concentrations of glycine and sucrose or selected salts.<sup>54</sup> This approach was developed by Heimburger in the late 1970s, resulting in a plasma-derived factor VIII/vWF product with no proven cases of virus transmissions over more than 30 years.<sup>56</sup>

## **Dry heat**

For lyophilized PDMPs, dry heat treatment, which involves heating at 80 °C for 72 hours or 100 °C for 30 minutes, is also an effective virus inactivation step. Higher residual moisture in the lyophilized product can enhance the inactivation of some viruses, but such conditions can increase protein aggregation while decreasing a product's stability. However, even at residual moisture levels as low as 0.3%, effective inactivation of most viruses can be obtained. In a commercial manufacturing process, the residual moisture of the lyophilized product is controlled, and the process is validated to ensure that the moisture of each vial remains between predefined limits informed by viral validation studies.<sup>57</sup>

## **Vapor heat**

Lyophilized (intermediate) products are heated at defined (relatively high) residual moisture (e.g., 60 °C for 10 hours followed by 80 °C for 1 hour). Low residual moisture levels of dry heat treatment do not interfere with the success of this method because the product intermediate is further processed to the final product.

## **Solvent/detergent (S/D) treatment**

Organic S/D treatment acts by disrupting the lipid membrane of enveloped viruses, rendering them unable to bind to and infect cells. This method has been shown to be extremely robust and effective in inactivating a broad range of enveloped viruses, including WNV, HIV, HBV, and HCV.<sup>58</sup> This method does not inactivate nonenveloped viruses. An example of conditions used for virus inactivation is 0.3% tri (*n*-butyl) phosphate (TNBP) and 1% ionic detergent (usually either Tween 80 or Triton X-100) at approximately 24 °C for 4–6 hours. It is even possible to treat some preparations at 4 °C; however, a longer contact time is required to achieve equivalent inactivation to that obtained at higher temperatures.<sup>59</sup> Following addition of S/D to the product, the resultant intermediate must be filtered to eliminate virus trapped in gross aggregates. Continuous mixing is generally carried out, and homogeneity of the S/D reagents throughout the treatment step is ensured at the commercial scale through validation studies. As every droplet must be treated, solutions are often transferred from a formulation tank to

the incubation tank to ensure that material on the ports, lid, or surfaces of the first tank is subject to the treatment. Following treatment, the S/D reagents are removed by downstream unit operations such as chromatographic or precipitation steps.

Despite Triton X-100 being a highly effective detergent for inactivation of enveloped viruses, it is considered ecologically unsustainable given its slow degradation rate and that its degradant, 4-*tert*-octylphenol, has endocrine disrupting properties for aquatic organisms.<sup>60</sup> For this reason, the European Commission has placed Triton X-100 on the REACH Annex XIV authorization list, meaning that it cannot be supplied to the market without specific authorization.<sup>61</sup> Manufacturers of therapeutic products using Triton X-100 have therefore looked to replace it with alternative detergents such as Tween 80 or have investigated the use of novel, eco-friendly detergents.<sup>60–62</sup>

### Octanoic acid (caprylate) treatment

Octanoic acid treatment works by disrupting the lipid membrane of enveloped viruses, rendering them unable to bind to and infect cells.<sup>63</sup> Similar to S/D treatment, this method is not effective against nonenveloped viruses. The rapidity and effectiveness of inactivation are dependent upon the amount of free nonionized octanoic acid present; thus, octanoic acid concentration, pH, temperature, and protein concentration are all critical variables that need to be controlled during treatment.<sup>64,65</sup>

### Low pH

Low pH (approximately pH 4) has been shown to be an effective method for some immune globulin preparations to inactivate enveloped viruses and certain nonenveloped viruses such as human parvovirus B19V.<sup>66,67</sup> The effectiveness and robustness of a low-pH inactivation treatment are highly dependent on parameters including pH, time, temperature, and the type and concentration of excipients.

### UV irradiation

Ultraviolet-C (UV-C) irradiation has long been known to be virucidal and microbiocidal;<sup>68</sup> however, its application as a viral inactivation method in the manufacture of protein therapeutics has been limited, especially with regard to uniform irradiation processes.<sup>69</sup> UV-C irradiation acts by damaging the nucleic acids of viruses, mainly through the formation of cyclobutane pyrimidine and pyrimidine pyrimidone dimers.<sup>70</sup> Because viruses lack the ability to repair these modifications, dimer formation prevents their ability to infect cells.<sup>71,72</sup> Although it is widely used for water treatment, its use in the manufacture of protein therapeutics has been modest

owing to concerns that it may also modify proteins.<sup>73</sup> A serum protein solution, BISEKO (manufactured by Biotest for several decades), includes a combined beta-propiolactone–UV-C irradiation treatment step.<sup>74</sup> More recently UV-C irradiation has been employed for the manufacturing of trimodulin, an IgM-containing immunoglobulin preparation, investigated in a clinical phase II study<sup>75</sup> and a COVID-19 treatment trial.<sup>76</sup>

UV-C irradiation has shown to be effective against a broad range of viruses, including small nonenveloped viruses,<sup>73,77,78</sup> and has also been investigated in a continuous flow format in which plasma protein activity was preserved.<sup>79</sup> Continuous-flow UV-C irradiation has been further developed into the UVivatec® device, employing a spiral tube around a UV-C irradiation source.<sup>80,81</sup> The geometry of the spiral flow in the reactor generates Dean vortices, thereby ensuring homogeneous mixing and uniform exposure to the UV-C rays.<sup>82</sup>

UV-C irradiation can be an option to provide inactivation of small, nonenveloped viruses for proteins that may be too large or preparations that are too complex for efficient virus filtration.

### Dedicated virus removal

#### Virus filtration

**Virus filtration**, also called *nanofiltration* (Figure 22.2), is specifically designed to remove viruses.<sup>83</sup> It does so by removing virus particles based on size while allowing the flow through of the desired protein. Large protein molecules or easily aggregated proteins pose a challenge for this method. Virus filtration using small-pore (~20 nm) or parvo-virus-retentive filters is generally a robust and effective means for removing viruses as small as 18–24 nm from process streams. Successful filtration can depend not only on pore size but also on hydrodynamic forces, adsorption of the virus to the filter surface, and removal of virus that has aggregated within antigen–antibody or lipid complexes.<sup>84</sup> Virus filtration is considered gentle; however, appropriate testing during process development must ensure that shear forces do not damage the protein of interest. Following integration into manufacturing processes over 20 years ago, virus filtration has proven to be a robust method that is complementary to other virus inactivation methods.<sup>83</sup>

### Contributing virus removal by protein purification

#### Ethanol, polyethylene glycol (PEG), and caprylate precipitation

At the low temperatures and pH ranges typically used in plasma fractionation processes, the inactivation property of ethanol against enveloped viruses is minimal. However, cold ethanol fractionation of plasma can contribute to virus safety by partitioning viruses away from the therapeutic protein of interest.<sup>85</sup> Prior to the incorporation

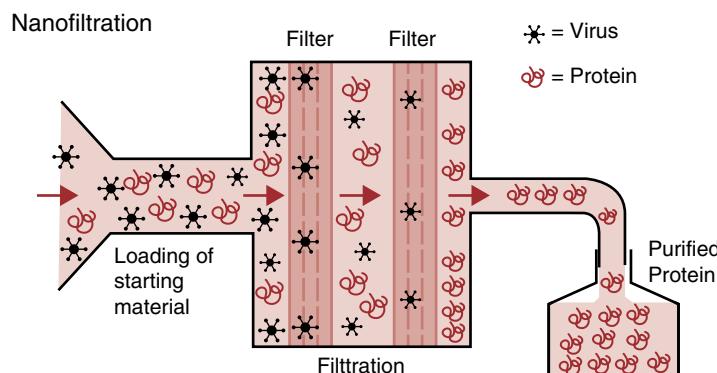


Figure 22.2 A diagram of virus removal by virus filtration nanopore size exclusion.

of dedicated virus reduction steps into manufacturing processes, partitioning during fractionation and purification, along with antibody neutralization in plasma pools, was largely responsible for ensuring the safety of immune globulin products. Comparable principles related to ethanol precipitation steps apply to other precipitation principles such as octanoic acid (caprylate) precipitation, PEG precipitation, glycine and NaCl precipitation, and ammonium sulfate precipitation. Although octanoic acid can rapidly inactivate enveloped viruses, under some process conditions nonenveloped viruses and certain enveloped viruses have been shown to partition with the precipitate (rather than be inactivated) by octanoic acid.<sup>67,86,87</sup> Virus removal by precipitation and depth filtration is virus specific, but it can significantly contribute to the overall safety of the final product.

### Chromatography

Ion exchange, gel filtration, hydrophobic interaction, or affinity chromatography can remove viruses.<sup>88,89</sup> When the resin of the chromatography column is reused, efficient sanitization of resins (and internal column hardware) is a necessary step to avoid batch-to-batch contamination.

### Prion safety

Prions, the causative agents of human transmissible spongiform encephalopathy (TSE) such as Creutzfeldt–Jakob disease (CJD) and variant (v)CJD, cannot be inactivated under conditions used for the manufacture of PDMPs without negatively impacting the qualities of the therapeutic protein. However, manufacturing processes have been shown to contain process steps that have the potential to significantly reduce prion agent infectivity.<sup>90</sup> The prion removal capacity of a manufacturing process can be measured in small-scale laboratory investigative studies and model infectious agents of TSE using principles consistent with virus validation studies. In such investigative studies, a variety of commonly used manufacturing steps (virus filtration [nanofiltration], octanoic acid and caprylate precipitation, PEG precipitation, and affinity chromatography) have been shown to significantly reduce TSE infectivity. These studies provide reasonable assurance that low levels of prions, if present, would be removed by the manufacturing process. There has been one putative report of vCJD (not CJD) transmission of a medium-purity factor VIII concentrate to a hemophiliac patient who did not develop clinical vCJD.<sup>91</sup> The hemophiliac had multiple prion risk exposures, including exposure to 8Y (BPL) factor VIII concentrate manufactured from UK-sourced plasma, which in at least one lot contained plasma from a preclinical vCJD donor. It should also be noted that the prion removal capacity of the 8Y process was also relatively low versus that of other PDMPs ( $3.3 \log_{10}$ ).<sup>92</sup> Importantly, even with heightened surveillance measures in place, there have been no other reports of clinical or nonclinical vCJD (or CJD) transmission by PDMPs manufactured by products that were manufactured from high-prion-risk UK-sourced plasma.

### Manufacturing controls and assessment of final product for virus safety

Each manufacturer must carry out process-specific validation for the licensed manufacturing process used to produce a plasma-derived therapy. The manufacturing process conditions and in-process monitoring for virus inactivation/removal steps must be clearly defined and justified. Segregation of intermediates pre- and postvirus reduction must be ensured by the use of closed systems

and/or different manufacturing zones with dedicated equipment, air-handling systems, gowning, and restricted personnel movement. Virus reduction is further ensured by confirmation of the production records that the conditions validated for the process were rigidly adhered to and that opportunities for cross-contamination and recontamination are prevented.<sup>52</sup>

In general, it is not appropriate to test the final product for viral markers as such tests are not reliable and are difficult to interpret. Testing of final products by NAT is not suitable to demonstrate a safe product due to statistical methods of sampling (Poisson distribution); furthermore, NAT detects genomic sequences and not infectivity of a virus. Virus inactivation techniques such as S/D treatment, octanoic acid treatment, dry heat treatment, and pasteurization disrupt the envelope and/or capsid proteins of the virus but can leave nucleic acid fragments that may be detected by NAT methods but have no bearing on the virus safety of the final product.<sup>52</sup> An assessment of virus safety in the finished product is a regulatory requirement by some authorities.

## Manufacturing processes for plasma-derived medicinal products

In this section, specific processes used in the recovery of all the major plasma proteins for therapeutic use are described. The focus is on illustrating the range of techniques that can be used in the manufacture of these products and exposing the reader to a wide range of concepts underpinning the fractionation of plasma proteins. In addition, the expected pharmacopeial specifications of the final product, which ensure desired product safety and efficacy, are detailed.

### Albumin

The cold-ethanol fractionation process remains the dominant process for the manufacture of albumin (Figure 22.1).<sup>34</sup> Details on the production of albumin by the cold-ethanol fractionation process in a modern facility and the required quality attributes expected of the product have been reviewed elsewhere.<sup>19</sup>

As previously described, the cold-ethanol fractionation process involves manipulation of ethanol concentration and pH of solutions at low ionic strength, while maintaining subzero temperatures, to achieve differential precipitation of proteins. The recovery of albumin involves the generation of Supernatant I, II + III, IV-I, IV-4, and finally the precipitation of albumin as Fraction V at 40% (v/v) ethanol, pH 5.8, with temperature maintained at  $-5^{\circ}\text{C}$ . A variation to the cold-ethanol fractionation process developed by Cohn was reported by Kistler and Nitschmann in 1962.<sup>93</sup> The aim was to maximize albumin yield and decrease the use of ethanol. Precipitate A, the equivalent of Fraction II + III in the Cohn process, is produced with 19% (v/v) ethanol, pH 5.85, and allows the recovery of the albumin-containing Supernatant A. This then progresses through the generation of Supernatant IV (40% v/v ethanol, pH 5.85,  $-8^{\circ}\text{C}$ ) and Precipitate C (40% v/v ethanol, pH 4.8,  $-8^{\circ}\text{C}$ ). Resuspension of Precipitate C and recovery of Supernatant D (10% v/v ethanol, pH 4.6,  $-3^{\circ}\text{C}$ ) provide the final purified albumin. This process was initially used by the Swiss manufacturer ZLB and continues to be used following its incorporation into CSL Behring.<sup>33</sup> The albumin recovered by ethanol fractionation is highly purified, although trace amounts of residual plasma proteins—typically, haptoglobin, ceruloplasmin,  $\alpha_2$ -macroglobulin,  $\alpha_1$ -acid glycoprotein, hemopexin, and transferrin—can be detected<sup>94,95</sup> (CSL data on file).

A small number of manufacturers have incorporated chromatographic steps into their albumin purification processes. This

includes the fully chromatographic method developed by CSL Behring (Australia) and a hybrid method that was developed by BioProducts Laboratories (BPL). The chromatographic purification method employed by CSL Behring (Australia) was developed in the 1990s for the Albumex product and was based on the method of Curling *et al.*<sup>94,96,97</sup> The process was initially developed for the purification of albumin from Supernatant II + III but was later adapted for the processing of Supernatant I.<sup>98</sup> The process incorporates three chromatographic steps—anion and cation exchange chromatography with DEAE and CM Sepharose<sup>\*</sup>, respectively, and size exclusion chromatography using Sephadex<sup>†</sup> S200 resin. This last step markedly contributes to the purity of the recovered albumin, removing residual proteins and aggregates. The BPL method for Zenalb takes the Fraction V intermediate and utilizes a DEAE anion exchange resin to further reduce trace levels of residual plasma proteins.<sup>99</sup> The purified albumin is formulated, and bulk pasteurized before filling and a second pasteurization step in the vial. The Albumex and Zenalb processes are shown in Figure 22.3.

Chromatographically purified albumin is purer than that derived by cold-ethanol fractionation and exhibits a lower aggregate content.<sup>94</sup> This increased purity has been associated with a decreased rate of adverse reactions for patients infused with chromatographically purified albumin.<sup>98</sup>

The cold-ethanol fractionation process for the manufacture of albumin contributes to the viral safety of the product through partitioning virus away from the product stream.<sup>100–103</sup> With the alternative chromatographic process, similar partitioning is achieved at the chromatographic steps.<sup>104,105</sup> In addition, a specific viral inactivation step is included in the manufacturing process—pasteurization at 60 °C for 10 hours.<sup>100–103</sup> This has been part of the production of albumin from the very beginning and undoubtedly accounts for the fact that there has never been a viral transmission from an albumin product.<sup>106</sup> The pasteurization of albumin was possible due to the finding that *N*-acetyl tryptophanate and sodium octanoate (caprylate), or sodium octanoate alone, stabilized the molecule during heating.<sup>107,108</sup>

The European Pharmacopoeia (EP) and US regulations (CFR21) stipulate that pasteurization be performed in the final container following dispensing in order to completely remove the risk of recontamination.<sup>109,110</sup> Reflecting advances in bioprocessing execution and control, CSL Behring (Australia), in addition to developing a novel chromatographic manufacturing process, obtained approval from the Australian regulatory authority to implement bulk pasteurization of its albumin product prior to dispensing.<sup>94</sup> Albumex<sup>\*</sup> has an additional viral inactivation step consisting of incubation at low pH in the presence of caprylic acid at 30 °C for 10 hours.<sup>111</sup> This step was introduced to add further viral safety to the product and reflected the regulatory expectations that plasma protein products have at least two dedicated viral inactivation or removal steps.<sup>112</sup>

The specifications of the albumin products are governed by EP and US Pharmacopoeia and National Formulary (USP) or CFR pharmacopeial requirements, which provide limits for physical properties, biological safety, purity, excipients, and contaminants.<sup>109,110,113</sup> Purified albumin solutions are supplied by various manufacturers as 4, 4.5, 5, 20, and 25% (w/v) solutions and typically exhibit a shelf life of up to five years at room temperature (25–30 °C).<sup>19</sup> It is a requirement that the solution must be clear and can be almost colorless, yellow, amber, or green (EP). The variation in color reflects differences in the presence of residual heme as well as colored protein impurities such as haptoglobin, ceruloplasmin, transferrin, and hemopexin.<sup>95,114</sup> While chromatographically purified albumin has lower levels of colored protein impurities than cold-ethanol purified albumin, it contains higher levels of albumin-bound bilirubin (yellow) and its oxidized derivative biliverdin (green), which contribute to the color.<sup>114</sup>

The product should have a pH (at 20 °C) of 6.7–7.3 (EP) or 6.4–7.4 (USP). Osmolality should be equivalent to that of plasma (USP). With respect to excipients, sodium should not exceed 160 mmol/L (EP) or 130–160 mmol/L (USP). Sodium caprylate (sodium octanoate) should be 0.16 mmol/g albumin if it is the single stabilizer (CFR21). In preparation where dual stabilizers, sodium caprylate and *N*-acetyl tryptophanate, are used, the concentration of each should be 0.08 mmol/g albumin (CFR21).

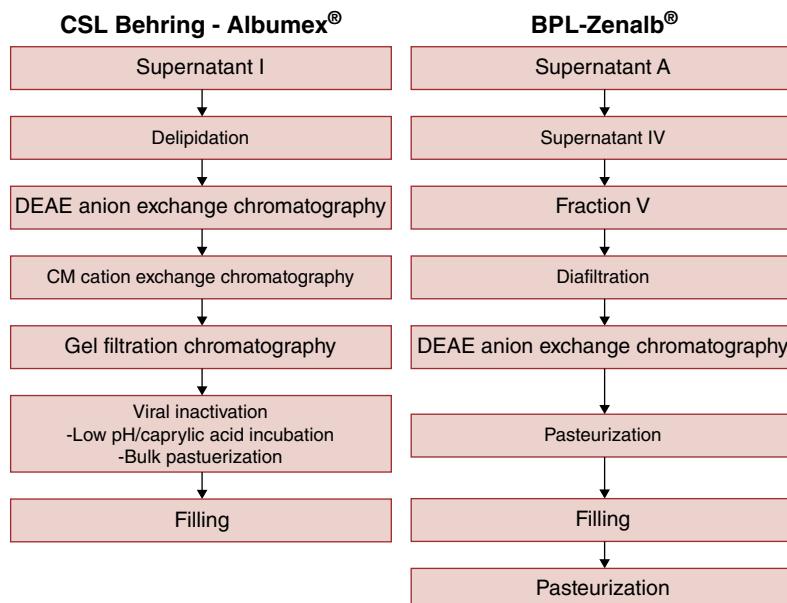


Figure 22.3 Chromatographic processes for the purification of albumin—Albumex<sup>\*</sup> (CSL Behring, Australia) and Zenalb<sup>\*</sup> (BioProducts Laboratories).

Albumin purity should be at least 95% (EP) or 96% (USP), with aggregate content not more than 10% of total protein. Heme levels as determined by adsorption at 403 nm should not exceed 0.15 AU (EP).

Prekallikrein activator (PKA) activity should not be more than 35 IU/mL (EP). This is an important specification as PKA (also known as factor XIIa or Hageman factor fragment), if present, can lead to a clinical hypotensive reaction.<sup>115</sup>

Contaminant levels, of which potassium and aluminum are specified, should be less than 0.05 mmol/g albumin (EP) or 2 meq/L (USP) and 200 µg/L (EP), respectively. The limit for aluminum was introduced into the British Pharmacopoeia in 1993 to ensure albumin safety for use in renal dialysis and with premature babies.<sup>99</sup> Another concern was the accumulation of aluminum with the treatment of burn patients.<sup>116</sup> The source of aluminum is from the glass containers used for the product. Leaching of aluminum is abetted by even very low concentrations of citrate (<0.1 mmol/L), which originates from the anticoagulant used during plasma collection.<sup>117</sup> Through optimization of diafiltration processes used in manufacturing, to minimize citrate content, current albumin products meet the required aluminum pharmacopeial limit.<sup>19,118</sup>

The solution should be sterile, nonpyrogenic, and with endotoxin levels not more than 0.5 EU/mL (EP/USP). The pharmacopoeias also specify that the final product be incubated for a period prior to final inspection to provide additional assurance of product sterility. CFR21 requires incubation for at least 14 days at 20–35 °C, and the EP states not less than 14 days at 30–32 °C, or not less than four weeks at 20–25 °C.

## Immunoglobulins

The Cohn process also served as the starting point for the purification of immunoglobulins.<sup>34</sup> Oncley developed a process for the recovery of purified immunoglobulins from Cohn Fraction II + III.<sup>38</sup> In the process, the immunoglobulin fraction is resuspended and reprecipitated with 20% v/v ethanol, at pH 7.6 (modifications exist with pH 6.7), with the temperature maintained at –5 °C. The recovered precipitate is then resuspended, and the solution adjusted to 17% v/v ethanol, at pH 5.2, with the temperature maintained at –6 °C. This results in the precipitation of IgM and other impurities (Fraction III), while the IgG remains in the supernatant (Supernatant III). IgG is then recovered as Fraction II by precipitation with 25% v/v ethanol, pH 7.4, with the temperature maintained at –5 °C.

A modification of the Oncley process was developed by Kistler and Nitschmann in 1962.<sup>93</sup> The aim was to increase the yield of IgG and reduce ethanol use. In this process, Precipitate A (corresponding to Cohn Fraction I + II + III) is obtained at 19% v/v ethanol, pH 5.85, with the temperature maintained at –5 °C. Following the resuspension of the precipitate in water, the solution is adjusted to pH 5.1 and ethanol is added to 12% v/v while maintaining the temperature at –5 °C. This results in the precipitation of impurities, including IgM (Precipitate B), with IgG remaining in the supernatant (Supernatant B) (equivalent of Cohn Supernatant III). The IgG is recovered by precipitation (Precipitate G) at 25% v/v ethanol, pH 7.0, with the temperature maintained at –7 °C. This precipitate corresponds to Cohn Fraction II. The above-mentioned processes, with various modifications, for many years, have accounted for the bulk of commercially produced immunoglobulins.<sup>119,120</sup> The Kistler-Nitschmann process was particularly identified with the ZLB (later CSL Behring) facility in Bern, for the production of Sandoglobulin.<sup>121</sup>

The development of new immunoglobulin manufacturing processes from the mid-1990s has reflected the desire of manufacturers

to improve product characteristics and safety. This has led to the increase in protein concentration in the final formulation in order to increase the convenience of use, the introduction of viral inactivation steps to improve safety, and the introduction of procedures to improve yield and manufacturing efficiency.<sup>119,120,122</sup> A number of companies have developed hybrid processes where the crude IgG precipitate generated by cold-ethanol precipitation (Fraction II + III or Precipitate A) is subjected to further chromatographic processing to recover the purified immunoglobulin.<sup>20,119,120,122,123</sup> CSL Behring (Australia) developed a unique completely chromatographic procedure, and in 2000, its use commenced for the production of immunoglobulin for the Australian market and other toll customers.<sup>124–126</sup> A key feature of this process was the increased yield in IgG that was achieved when compared to the cold ethanol-based process.<sup>127</sup>

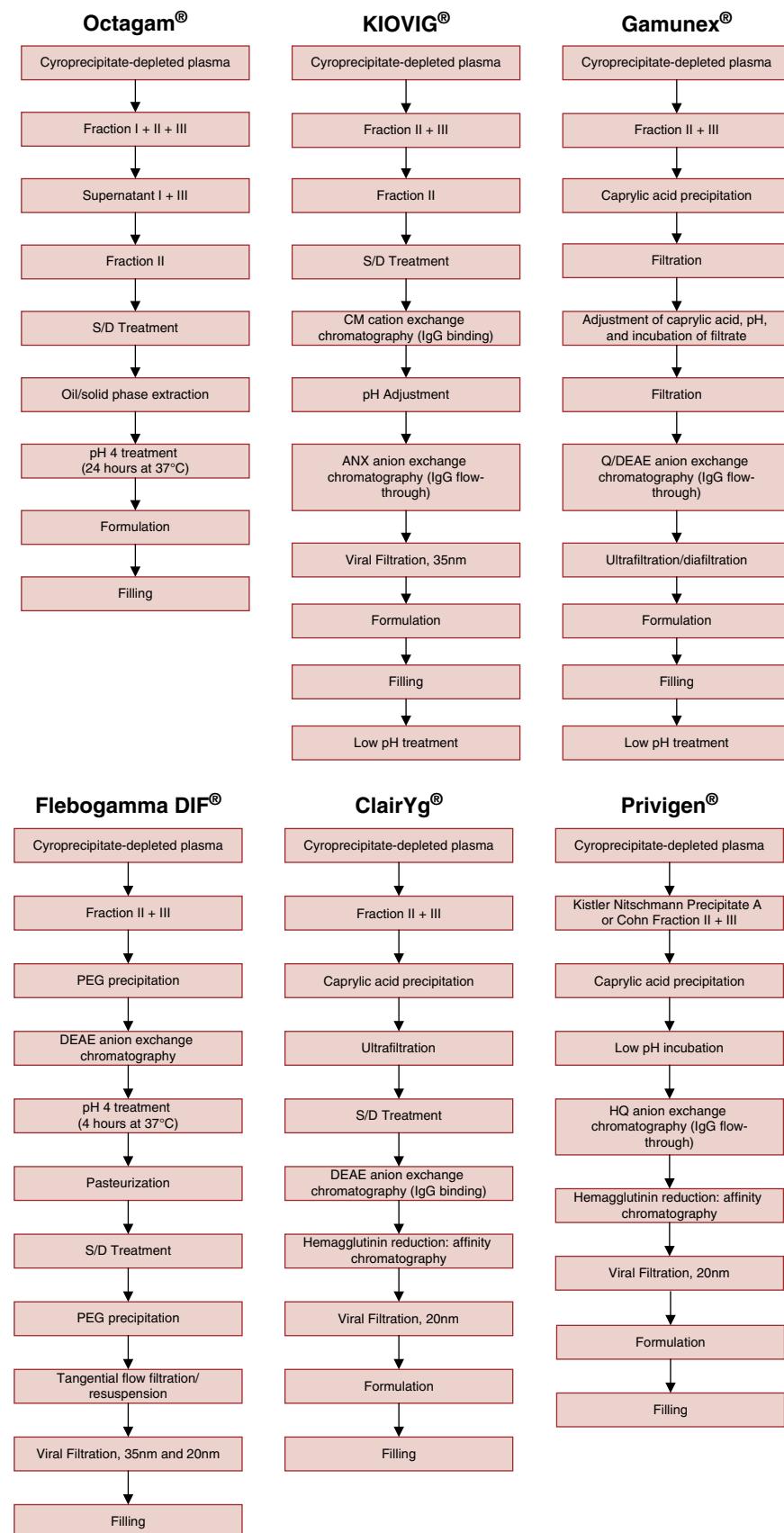
In the following, a brief description is presented for the manufacturing processes of all the major immunoglobulin products currently produced and the expected pharmacopeial specifications of immunoglobulin products. This area has also been reviewed extensively elsewhere.<sup>20</sup>

A few products are purified essentially by the complete application of the Oncley ethanol fractionation process. In the manufacture of Octogam<sup>®</sup> (Octapharma), purified IgG is recovered as Fraction II (Figure 22.4). In addition to the viral partitioning that occurs during the fractionation process, there is solvent-detergent (S/D) treatment and low-pH incubation, as described above, for viral inactivation. The oil/solid phase extraction step is a means of removing the S/D used during the viral inactivation procedure.<sup>20,128,129</sup> Octapharma has recently registered a new immunoglobulin product, Panzyga, which incorporates an ion exchange chromatography step to increase product purity and a virus filtration step.<sup>130</sup>

KIOVIG<sup>®</sup> or Gammagard<sup>®</sup> produced by Takeda (European and US trade names, respectively) is also produced by the Oncley process resulting in the generation of purified IgG as Fraction II (Figure 22.4).<sup>131</sup> The purified immunoglobulin is then subjected to three viral inactivation or removal procedures—S/D treatment, viral filtration with a 35 nm filter, and low-pH formulation and incubation of the dispensed product. The cation exchange chromatography step is used to bind the immunoglobulin and thereby allows the removal of the S/D used in the viral inactivation procedure. Passage through the anion exchange column retains impurities and results in further purification of the immunoglobulins.

The manufacture of Gammunex<sup>®</sup> (Grifols) is one of the several hybrid manufacturing processes that involve the generation of a crude IgG fraction by ethanol precipitation followed by further purification by chromatography (Figure 22.4). Cohn Fraction II + III is suspended and caprylic acid added to precipitate impurities. The caprylic acid concentration is adjusted in the recovered filtrate and incubated at 25 °C for one hour. This constitutes the first viral inactivation procedure in the manufacturing process. The generation of pure IgG is achieved by sequential passage through a strong (Q) and a weak (DEAE) anion exchange chromatographic column. Following buffer exchange, concentration, and formulation, where pH is adjusted approximately 4.25, the product is dispensed and then incubated at 28 °C for 21 days. This constitutes the second viral inactivation step.

In the manufacture of Flebogamma DIF<sup>®</sup>, Cohn Fraction II + III is resuspended, partially purified by precipitation of impurities by the addition of polyethylene glycol (PEG), and then further purified by anion exchange chromatography. The purified immunoglobulin



**Figure 22.4** Comparison of IgG manufacturing processes. Source: Based on Teschner *et al.*<sup>131</sup> and Roch *et al.*<sup>133</sup>

is subjected to three dedicated viral removal or inactivation procedures: pasteurization, S/D treatment, and viral filtration through 20 nm filters. The PEG precipitation of the resuspended Cohn Fraction II + III is also associated with significant viral clearance. The second PEG precipitation after the low-pH treatment, pasteurization, and S/D treatment serves to remove immunoglobulin aggregates and facilitates the passage of the solution in the subsequent viral filtration step<sup>132</sup> (Figure 22.4).

The purification of Clair Y g<sup>+</sup> (LFB) involves the use of Cohn Fraction I + II + III. Following solubilization, impurities are removed by caprylic acid precipitation. The filtrate is ultrafiltered to remove caprylic acid, and the retentate is subjected to S/D treatment. The immunoglobulin is then captured on an anion exchange column allowing the removal of the S/D. The eluted immunoglobulin is then passed through an affinity resin to reduce the anti-A and anti-B isoagglutinin titer. The immunoglobulin then undergoes viral filtration, the second viral removal step, prior to formulation and filling (Figure 22.4).<sup>133</sup>

The manufacture of Privigen<sup>®</sup> (CSL Behring) is also a hybrid manufacturing process that involves the generation of a crude IgG fraction by ethanol precipitation followed by further purification by chromatography.<sup>134</sup> Kistler–Nitschmann precipitate A or Cohn Fraction II + III is resuspended, and caprylic acid addition is used to precipitate impurities. The recovered partially purified immunoglobulin solution is subjected to low-pH incubation in the presence of a detergent as part of the first viral inactivation procedure. Final purification of the immunoglobulins is achieved by passage through a Q anion exchange chromatographic column under conditions where impurities (predominantly IgM and IgA) are bound and IgG flows through. The purified product is in turn passed over an affinity column to decrease the anti-A and anti-B hemagglutinin titers.<sup>135</sup> The recovered immunoglobulin solution is then subjected to viral filtration, the second viral removal procedure in the process, prior to formulation and dispensing (Figure 22.4).

Gammimune N<sup>®</sup> (Miles Cutter/Bayer) in 1992 was the first immunoglobulin for intravenous use to be available as a 10% (w/v) formulation.<sup>136</sup> There has been a consistent trend over the last 20 years of moving from a final protein concentration of 5 or 6% (w/v) to 10% (w/v). The increased protein concentration reduces the volume that needs to be infused and, hence, is more convenient for the patient.<sup>137</sup> Today, all the major immunoglobulin products on the market which have been described here are available at the 10% w/v formulation.<sup>138</sup> A number of manufacturers have developed immunoglobulin products at protein concentrations of 16–20% w/v (Hizentra<sup>®</sup>-CSL Behring; Cuvitru<sup>®</sup>-Takeda; Cutaquig<sup>®</sup>-Octapharma; Xembify<sup>®</sup>-Grifols), which are intended for subcutaneous administration.<sup>139–142</sup>

A key challenge in the development of an immunoglobulin preparation suitable for intravenous use identified a means of preventing the development of anticomplementary activity in the product arising from the formation of aggregates. Early success in removing the aggregates associated with anticomplementary activity was achieved in 1962 by Barandum and Isliker through mild proteolysis with pepsin at pH 4.0.<sup>143</sup> Improvements in processing, such as the use of ultrafiltration and diafiltration at pH 4.0 for the removal of ethanol from Supernatant III and for the concentration of IgG, instead of precipitation and lyophilization, minimize aggregate formation and help generate products that have good clinical tolerability.<sup>144</sup> It was then shown that the formulation of the immunoglobulin at approximately pH 4.25 generated a liquid stable product with low anticomplementary activity.<sup>145,146</sup> The stability of

immunoglobulin at low pH is due to the fact that this is below their isoelectric point and results in noninteracting positively charged molecules. Consequently, many immunoglobulin products are now formulated at less than pH 5.0.<sup>138,145,147</sup>

Excipients are used in the formulation of immunoglobulins. These serve to stabilize the immunoglobulin molecules in solution by minimizing protein–protein interactions and to ensure the clinically acceptable tonicity of the product.<sup>148,149</sup> Many immunoglobulin products in fact can be stored at room temperature for up to 36 months.<sup>138,150</sup> Currently used excipients comprising of amino acids, sugars, and sugar alcohols have a long history of use and are known to be well tolerated.<sup>148</sup> Glycine is most commonly used, but proline, maltose, sorbitol, and mannitol are also used. Low concentrations of sodium chloride and polysorbate detergent may also be added to adjust tonicity and enhance stability.<sup>151–155</sup>

Although there are differences in the manufacturing processes for immunoglobulins and differences in final formulation, there is no evidence that they significantly correlate with differences in product efficacy and tolerability.<sup>115–156</sup> As registered products, these immunoglobulin products meet the standards as prescribed by relevant regulatory authorities.

The European Pharmacopoeia has a comprehensive monograph on immunoglobulins for intravenous use.<sup>157</sup> The product is expected to be sterile and free of pyrogens and endotoxins. A liquid formulation is expected to be clear or slightly opalescent, colorless, or pale yellow. The allowable pH is 4.0–7.4. Osmolality must be greater than 240 mOsmol/kg. The minimum allowable protein concentration of a formulation should not be less than 30 g/L (3% w/v), and the purity is expected to be greater than 95%. Aggregate content must not exceed 3% of total protein. This is an important quality attribute as aggregates typically result in complement activation-mediated adverse reactions. Any propensity for this is monitored through the measurement of anticomplementary activity, with the specification set at <1 CH<sub>50</sub>/mg IgG.

The allowable maximum limit of prekallikrein activator (PKA) (factor XIIa), which can mediate the formation of the vasoactive peptide bradykinin from kininogen and lead to hypotensive reactions, is set at 35 IU/mL.<sup>158</sup>

Hemagglutinins, anti-A and anti-B antibodies to red cell antigens, can result in hemolytic reactions with the infusion of immunoglobulins. Therefore, the EP stipulates a maximum allowable titer of 1:64. Given that high-volume administration of immunoglobulins is required in some indications, specifically those associated with achieving immune modulation, manufacturers are introducing specified hemagglutinin removal steps in manufacturing their process to lower the titer in their products.<sup>133,156</sup> Therefore, the titer of some products will be considerably lower than this limit.

The EP does not define a maximum level of IgA allowed in immunoglobulin products, but the product must comply with the level stated on the label. The range for the products described here is 5–200 µg/mL.<sup>138</sup> Knowledge of the IgA content of an immunoglobulin product is important for a clinician when confronted with an IgA deficient patient who could exhibit an anaphylactic reaction if infused with a higher IgA concentration immunoglobulin product.<sup>159</sup>

Although not prescribed in the EP, immunoglobulin products are expected to exhibit a subclass distribution comparable to that normally found in plasma.<sup>159</sup> Despite a number of manufacturing processes resulting in partial depletion of IgG<sub>3</sub> and IgG<sub>4</sub>, there is no evidence that this affects the efficacy of the product.<sup>20</sup> In addition, the EP requires that the immunoglobulin purification process

maintains the integrity of the Fc portion of the immunoglobulin molecule—as this is key to its immunomodulatory role through interaction with the Fc receptor and its effector role in complement activation.<sup>159</sup> Monitoring of Fc function is not a product release requirement but is assessed during process validation. An immunoglobulin product should exhibit an Fc function of >60% of that of an EP standard.

The EP also stipulates that the antibody titer to hepatitis B should be at least 0.5 IU/g IgG, and the United States also includes minimum antibody levels for measles, diphtheria, and polio.<sup>160</sup> There is no limit for antibodies to hepatitis A, but regulatory authorities expect levels to be above 10 IU/mL. Maintaining a consistent level of antibodies to these targets is challenging as there has been a consistent decline in antibody titers in plasma due to increased vaccination and decreased disease prevalence.<sup>20,161,162</sup>

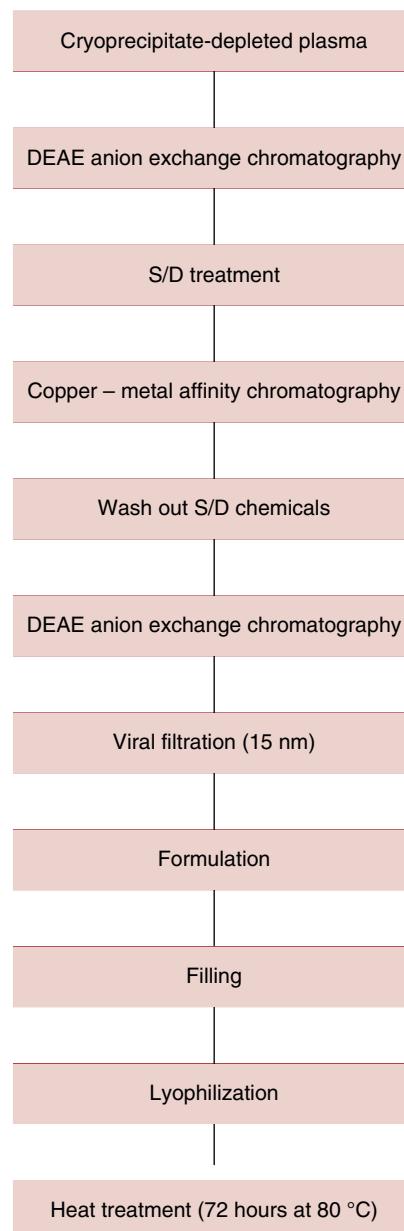
In 2010, increased incidences of thromboembolic events were reported in patients using Octogam.<sup>163</sup> Analysis showed this was due to increased levels of factor XIa caused by a process change. Following this, the regulatory agencies requested that manufacturers confirm that their processes have the capability of removing thrombogenic activity and generate a product low in procoagulant activity.<sup>157,164,165</sup> Procoagulants have been shown to be partitioned away from the product during purification and inactivated by pasteurization and exposure to low pH.<sup>163–165</sup> The commercially available immunoglobulin products typically have very low to nondetectable levels of factor XIa.<sup>166,167</sup>

### Factor XIII

The development of manufacturing processes for factor XIII has been extensively reviewed.<sup>15</sup> The focus here is on describing the production of a major commercial factor XIII product, Fibrogammaglobin P<sup>®</sup> (in Europe) and Corifac<sup>®</sup> (in the United States), as undertaken by CSL Behring. The process utilizes Cohn Fraction I precipitate derived from cryoprecipitate-depleted plasma. Following resolubilization and removal of fibrinogen by treatment with aluminum hydroxide and Vitacel<sup>®</sup> (a cellulose fiber), the clarified solution is subjected to DEAE anion exchange chromatography. The purified factor XIII is then subjected to two viral inactivation and removal procedures—pasteurization (60 °C for 10 hours) and viral filtration with 20 nm filters. Following formulation to the target potency and with the addition of albumin, glucose, and sodium chloride, the solution is sterile filtered, dispensed, and lyophilized.<sup>168,169</sup> There is no monograph for factor XIII product, but these products have been extensively characterized with respect to purity, function, and factor XIII integrity. This includes factor XIII activity, specific activity, factor XIII subunit A content, fibrinogen clotting time, PKA, and activated factor XIII.<sup>15</sup>

### Factor X

The manufacture of a therapeutic factor X produced by BPL (UK) is shown in Figure 22.5. The key steps in the process are adsorption of factor X from cryoprecipitate-depleted plasma by DEAE anion exchange chromatography. Further purification is achieved by metal affinity chromatography with a copper chelate resin. Viral removal is achieved by S/D treatment, viral filtration with 15 nm filters, and finally the lyophilized product is subjected to heat treatment at 80 °C for 72 hours.<sup>15</sup> The product has been shown to comply with required toxicity, thrombogenicity, and immunogenicity requirements. In addition, in vitro tests confirm that the product is potent, highly purified, and free of potentially hazardous residues.

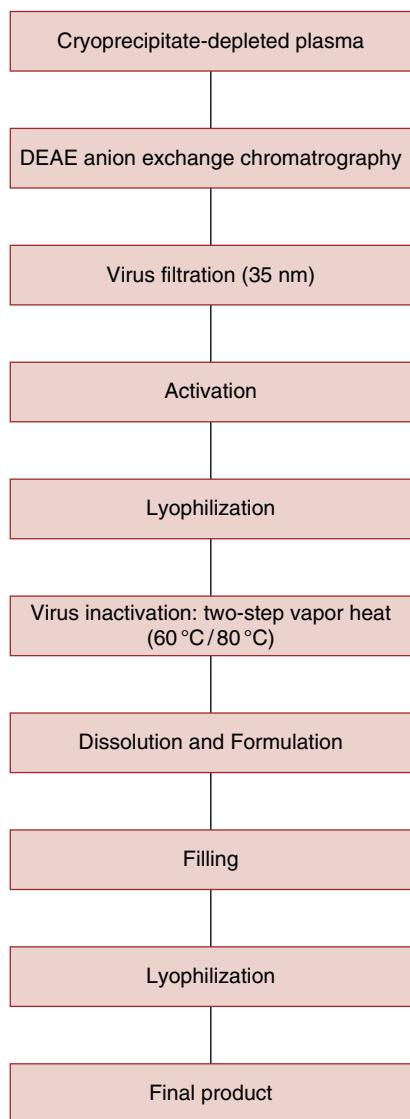


**Figure 22.5** Manufacturing process for factor X (BPL).

The testing includes the determination of factor X potency, specific activity, the presence of factor II, factor IX, proteins C and S, and NaPTT.<sup>15</sup>

### Activated prothrombin complex concentrate (aPCC)

aPCC, known as factor VIII inhibitor bypassing activity (FEIBA<sup>®</sup>), is manufactured by Takeda. Details on the development, production, mechanism of action, and clinical use of the product have been reviewed elsewhere.<sup>11</sup> The manufacturing process is shown in Figure 22.6. It involves the purification of prothrombin complex factors (II, VII, IX, and X) from cryoprecipitate-depleted plasma by DEAE anion exchange chromatography. The recovered material undergoes viral filtration with a 35 nm filter. The factors are then contact activated, generating activated Factor VII and some activated



**Figure 22.6** Manufacturing process for factor VIII bypassing activity (FEIBA®) (Takeda).

factor X. These activated factors, together with prothrombin, are thought to be important for the clinical efficacy of the product.<sup>11,170</sup> The product is lyophilized and undergoes a second viral inactivation treatment involving vapor heat treatment at 60 and 80 °C for at least 8.5 hours and 1 hour, respectively. The product is dissolved and then formulated with sodium citrate and sodium chloride, and dispensed at either 500, 1000, or 2500 arbitrary FEIBA units before lyophilization. One unit of activity is defined as the amount of product that shortens the activated partial thromboplastin time (aPTT) of a high-titer factor VIII inhibitor reference plasma to 50% of the blank value. The final product is a lyophilized preparation.<sup>171</sup>

#### Alpha<sub>1</sub>-proteinase inhibitor

The manufacturing processes for the four major alpha<sub>1</sub>-proteinase inhibitor (API) products, Aralast NP® (Takeda), Zemaira® (CSL Behring), Prolastin C® (Grifols), and Glassia® (Kamada) are presented in Figure 22.7.<sup>172–176</sup> A comprehensive examination of these manufacturing processes is available elsewhere.<sup>23</sup>

Cohn Fraction IV-I is the source material for all products. The Aralast NP® process utilizes a unique step involving an extended thaw of the Fraction IV-I paste and dissolution in pH 6.0 buffer that allows the recovery of an API-enriched precipitate. Following dissolution, all processes include an initial “activation hold” step at approximately pH 9 and at elevated temperature (approximately 45 °C). This results in increased recovery of functional protein, which could be related to refolding of denatured protein. The commencement of the purification process typically involves polyethylene glycol (PEG) precipitation of impurities or destabilization of unwanted proteins by reduction with dithiothreitol (DTT) and adsorption with fumed silica (Aerosil®). This treatment takes advantage of the fact that there are no disulfide bonds in the API molecule.<sup>177</sup> All processes utilize anion exchange chromatography for further purification. Further polishing is achieved by adsorption with bentonite (aluminum phyllosilicate clay), hydrophobic interaction chromatography, or cation exchange chromatography. The Aralast NP®, Glassia®, and Prolastin-C® processes all incorporate S/D treatment as the first viral inactivation procedure. Pasteurization is used in the Zemaira® process. All processes include viral filtration as the second viral removal step. Aralast NP®, Zemaira®, and Prolastin C® are presented as lyophilized final products, whereas Glassia® is a liquid product.<sup>23</sup>

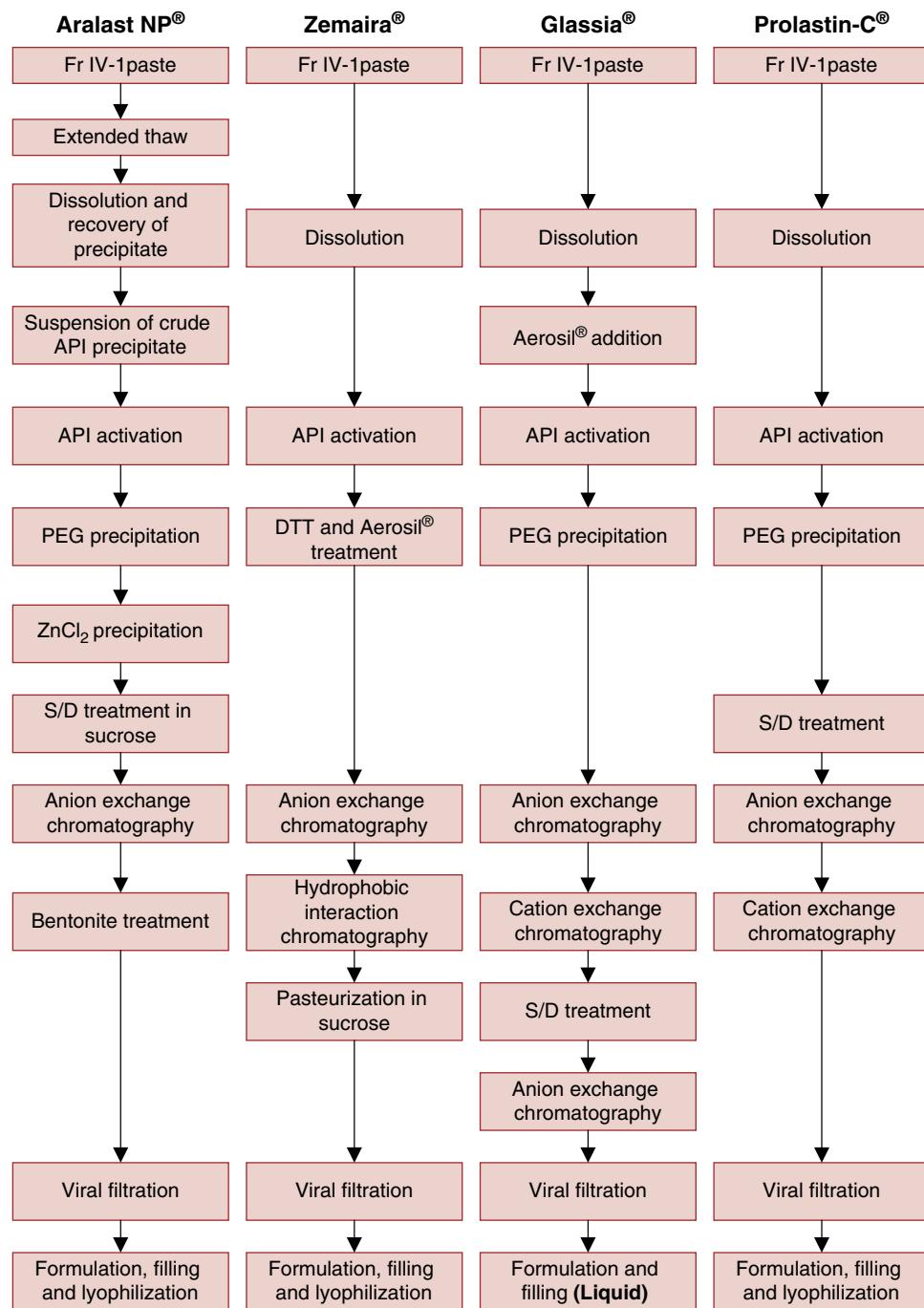
The EP prescribes certain product qualities for API preparations.<sup>178</sup> The specific activity should not be less than 0.35 mg of active API per mg of total protein. The ratio of API activity to antigen should not be less than 0.7. With respect to appearance, the liquid preparation should be clear or slightly opalescent, colorless, or pale yellow, pale green, or pale brown; the freeze-dried preparation should be a powder or solid friable mass, hygroscopic, and white, pale yellow, or pale brown.

It is required that the pH should be in the range of 6.5–7.8, the preparation should be completely soluble, and osmolality should be greater than 240 mOsmol/kg. The product must be sterile and free of pyrogens and bacterial endotoxins.

#### Factor XI

The manufacture of factor XI is undertaken by LFB Biotechnologies (France).<sup>14</sup> Cryoprecipitate-depleted plasma (cryosupernatant) is passed through a negatively charged filter to capture the factor XI. It is then eluted with 1 M NaCl containing antithrombin III (ATIII), which inhibits any proteolytic activity that could degrade the product. Following buffer exchange by ultrafiltration, the solution is subjected to viral inactivation treatment with S/D. The solution is passed through a sulfate (S) cation exchange chromatographic resin to capture the protein and allow the removal of the virus-inactivating chemicals. The factor XI is eluted and formulated with heparin and ATIII before sterile filtration, dispensing, and lyophilization. The product is highly purified, containing only trace amounts of fibrinogen, albumin, C1-esterase inhibitor, fibronectin, alpha<sub>2</sub>-macroglobulin, and IgG. The levels of other coagulation factors, kinin system components, and proteases are also low or nondetectable.<sup>179</sup>

The EP required that the freeze-dried final product should be white or almost white powder or friable solid, and it should completely dissolve within 10 minutes following reconstitution. The potency must be within 80–120% of that stated on the label. The reconstituted product should have a pH of 6.8–7.4 and have a minimum osmolality of 240 mOsmol/kg. Heparin and ATIII added as stabilizers must comply with the level stated on the label. Ensuring the absence of activated coagulation factors, the NaPTT coagulation time should not be less than 150 seconds. There should not be



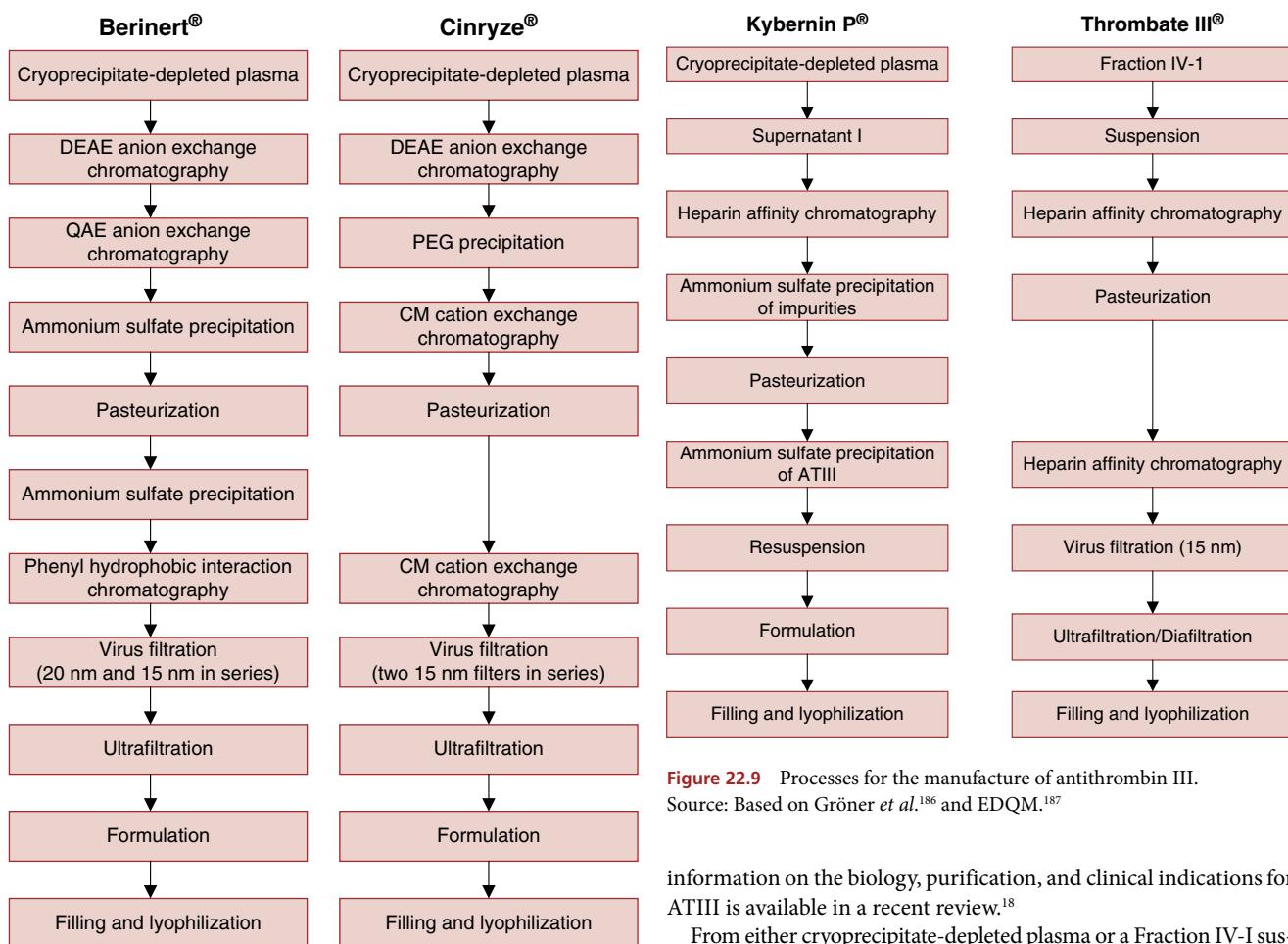
**Figure 22.7** Comparison of alpha<sub>1</sub>-proteinase inhibitor manufacturing processes. Source: Based on Hwang *et al.*<sup>172</sup>; Matthiessen *et al.*<sup>173</sup>; Kee *et al.*<sup>174</sup>; Lebing *et al.*<sup>175</sup> and Bonitz *et al.*<sup>176</sup>

any significant presence of anti-A and anti-B hemagglutinins, with no agglutination occurring at a 1:64 dilution of the product. The product must be sterile and free of bacterial endotoxins.<sup>180</sup>

### C1-esterase inhibitor

The biology, purification, and clinical use of C1-esterase inhibitor has been comprehensively reviewed elsewhere.<sup>24</sup> This section focuses on describing the manufacturing processes of two of the major C1-esterase inhibitor products on the market: Berinert<sup>®</sup> (CSL Behring) and Cinryze<sup>®</sup> (Takeda).<sup>24,181,182</sup> As can be seen in Figure 22.8,

the processes are quite distinct in the use of chromatographic processes. In the production of Berinert<sup>®</sup>, cryoprecipitate-depleted plasma is initially passed through a DEAE anion exchange column, under conditions in which the C1-esterase inhibitor flows through and subsequently captured on a QAE anion exchange resin. Further purification is achieved by ammonium sulfate precipitation steps and hydrophobic interaction chromatography (HIC). In the Cinryze<sup>®</sup> process, the C1-esterase inhibitor is captured from the starting material by anion exchange chromatography, and further purification is achieved by PEG precipitation and two separate



**Figure 22.8** Processes for the manufacture of C1-esterase inhibitor.

cation exchange chromatography steps. In both processes, viral removal involves pasteurization and virus filtration requiring two filters in series.<sup>181,183</sup> Following virus filtration, the products are formulated, dispensed, and lyophilized.

There are no pharmacopeial requirements for C1-esterase inhibitor concentrates. However, as with other lyophilized therapeutic protein products, it must have an acceptable appearance in the lyophilized state and must comply with registered residual moisture content. The product must readily dissolve and present as a colorless and clear solution with clinically acceptable osmolality and insoluble particle count. It must be sterile and free of bacterial endotoxins. Manufacturers determine and report the potency of the product.<sup>184</sup> The integrity of the product is determined by monomer content, which is a quality control release specification. Thus, for example, Berinert® must have a monomer content of >89%. Recently, a biochemical and purity comparison of four commercially available C1-esterase inhibitor products (Berinert®, Cinryze®, Cetor®, and Ruconest® [transgenic product]) was published with a view that the work would contribute to the establishment of regulatory requirements for determining purity and setting allowable threshold levels.<sup>185</sup>

### Antithrombin III

As examples of manufacturing processes for Antithrombin III (ATIII), the processes for Kybernin P® (CSL Behring) and Thrombate III® (Grifols) are described and shown in Figure 22.9.<sup>186,187</sup> Further

**Figure 22.9** Processes for the manufacture of antithrombin III.  
Source: Based on Gröner *et al.*<sup>186</sup> and EDQM.<sup>187</sup>

information on the biology, purification, and clinical indications for ATIII is available in a recent review.<sup>18</sup>

From either cryoprecipitate-depleted plasma or a Fraction IV-I suspension, ATIII is captured by heparin affinity chromatography. The Kybernin P® process then uses sequential ammonium sulfate steps to remove impurities and to concentrate the ATIII by precipitation. All processes incorporate pasteurization as a viral inactivation step. In the Kybernin P® process, a subsequent ammonium precipitation of ATIII allows the removal of pasteurization stabilizers and the production of a concentrated solution, which is formulated, dispensed, and lyophilized. In the Thrombate III® process, further purification is achieved by heparin affinity chromatography. In addition to pasteurization, the Thrombate III® process incorporates virus filtration. The recovered filtrate is then concentrated and diafiltered prior to formulation, dispensing, and lyophilization. The Thrombate III® process incorporates two dedicated viral inactivation and removal steps, while the Kybernin P® process has only one dedicated viral inactivation step—pasteurization. Despite this, additional viral clearance in the Kybernin P® process is achieved by precipitation at the first ammonium sulfate precipitation step.<sup>186</sup>

The EP prescribes a number of product specifications.<sup>188</sup> At least a 60% fraction of the product must bind heparin. The specific activity of the product should not be less than 3 IU ATIII per mg of protein (excluding albumin excipient). The lyophilized product should be a white or almost white, hygroscopic, friable solid or powder. The water content must be within limits. The product should completely dissolve within 10 minutes with gentle swirling, giving a clear or slightly turbid, colorless or almost colorless solution. The limits for pH and osmolality are 6.0–7.5 and >240 mOsmol/kg, respectively. The maximum allowed heparin content is 0.1 IU per IU ATIII. The product must be sterile and free of bacterial endotoxins.

## Fibrinogen

The manufacturing processes for the two main fibrinogen products Riastap® (US) or Haemocomplettan® (Europe) produced by CSL Behring and Fibryga® produced by Octapharma are shown in Figure 22.10. Both processes utilize cryoprecipitate as the starting intermediate. The Riastap® manufacturing process is based on a series of glycine-based precipitations—a procedure initially described by Bromback and Bromich in 1956.<sup>189,190</sup> The cryoprecipitate extract is initially adsorbed with aluminum hydroxide, and then the fibrinogen is precipitated with high-concentration glycine. The reconstituted fibrinogen is then pasteurized. Following the addition of glycine to promote precipitation of residual proteins, a purified fibrinogen is recovered by precipitation by increasing the concentration of glycine. The pure fibrinogen precipitate is redissolved, concentrated and diafiltered, formulated, dispensed, and lyophilized. For the Fibryga® process, the resuspended cryoprecipitate is also treated with aluminum hydroxide prior to the first virus inactivation step utilizing solvent/detergent. The fibrinogen is further purified via two ion exchange chromatography steps and a precipitation step. The second dedicated virus clearance step involves virus filtration, before the product is formulated, filled, and lyophilized.<sup>191</sup> A third fibrinogen product, FibClot® produced by LFB, is also commercially available.

The EP requires that the lyophilized product should be white or pale yellow in a hygroscopic powder or friable solid; that the water content is within approval limits; and that it dissolves within 30 minutes at room temperature, forming an almost colorless, slightly opalescent solution. The solution should contain not less than 10 g/L fibrinogen with a pH of 6.5–7.5 and a minimum osmolality of 240 mOsmol/kg. The product should be sterile and free of bacterial endotoxins.<sup>192</sup>

## Prothrombin complex concentrate

The manufacture of commercially available prothrombin complex concentrates (PCC) should ensure the generation of a product with a balanced content of vitamin-K-dependent factors (II, VII, IX, and X) and the presence of physiologically relevant amounts of the regulatory inhibitory proteins, proteins C, S, and Z.<sup>12</sup> A comparison of different PCC products has shown that the PCC products had similar levels of coagulation factors, but there were differences in purity and content of the inhibitory proteins.<sup>193</sup>

To specifically illustrate the principles of the manufacture of PCC, the process used for the production of Kcentra® (CSL Behring) (Beriplex® in Europe) and Octaplex® (Octapharma)—two commercial products in wide use—will be examined (Figure 22.11). The manufacture of Kcentra® is initiated by the adsorption of the PCC factors from cryoprecipitate-depleted plasma with DEAE anion exchange resin.

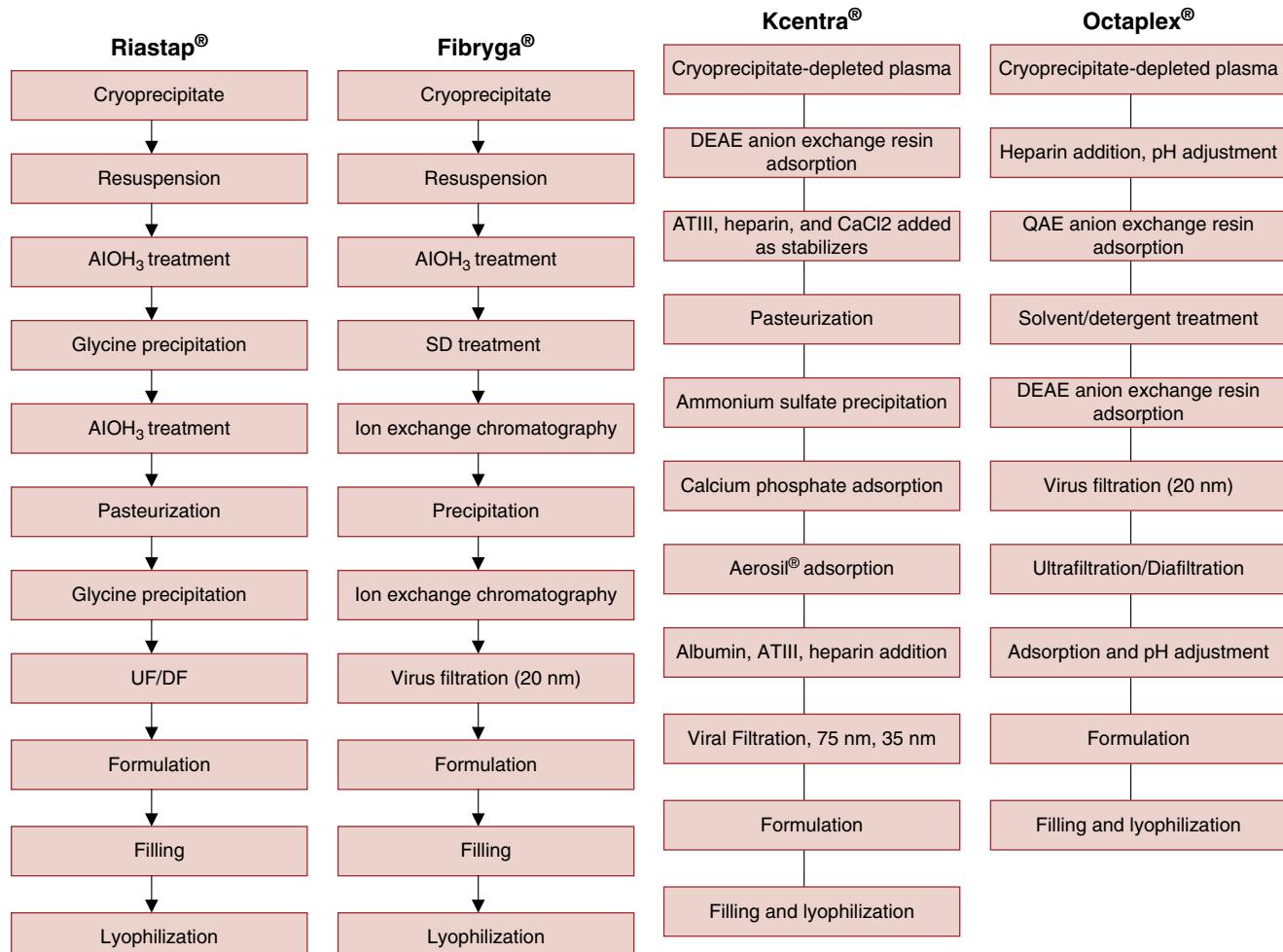


Figure 22.10 Comparison of the manufacture of two fibrinogen products.

Figure 22.11 Comparison of the manufacture of two prothrombin complex concentrate products.

The factors are eluted and stabilized by the addition of ATIII, heparin, and  $\text{CaCl}_2$ . The eluate is pasteurized to ensure viral safety. Further purification involves ammonium precipitation of impurities, binding to calcium phosphate, followed by elution and Aerosil® (fumed silica) adsorption of the eluate. The recovered solution is formulated by the addition of albumin, ATIII, and heparin to stabilize the active components, and then subjected to viral filtration through two 20 nm filters. Following dilution to a targeted factor IX concentration and the adjustment of the final albumin concentration, the product is dispensed and lyophilized.<sup>194</sup> In the Octoplex® process, PCC factors are adsorbed from cryoprecipitate-depleted plasma with a Q anion exchange chromatographic resin following pH addition and heparin addition to prevent factor activation. The recovered eluate undergoes S/D treatment for viral inactivation. Subsequent binding to a DEAE anion exchange resin allows the washing away of the viral inactivation chemicals and provides further purification. The product is then subjected to viral filtration with a 20 nm filter. Following ultrafiltration, the product is formulated with the addition of heparin (but no albumin) and then dispensed and lyophilized.<sup>12,195</sup>

There is an EP monograph for PCC products.<sup>196</sup> It stipulates that a product must contain factor IX together with variable amounts of factor II, VII, and X. The preparation may contain stabilizers (e.g., albumin, heparin, and ATIII). The lyophilized product should be a white or slightly colored, very hygroscopic powder or friable solid. Water content should be within approved limits. The preparation must dissolve completely within 10 minutes with gentle swirling. The potency of the reconstituted preparation should not be less than 20 IU of factor IX per milliliter. The pH of the solution should be between 6.5 and 7.5, and osmolality should not be less than 240 mOsmol/kg. If the content of any of the other factors is stated, it should not be less than 80% or more than 120% of the stated range. The specific activity of factor IX should not be less than 0.6 IU/mg of total protein (before addition of any stabilizer). The presence of any activated coagulation factors should not result in the coagulation time in an NaPTT assay to be less than 150 seconds. If heparin has been used in the formulation, the amount should comply with that on the label and not be more than 0.5 IU per IU of factor IX. The preparation must be sterile and free of bacterial endotoxins.

## Factor IX

The approaches taken for the purification of high-purity factor IX have been extensively documented elsewhere.<sup>13,197</sup> Predominantly, the process commences with the initial capture of prothrombin complex factors (factors II, VII, IX, and X). The isolation of factor IX can then involve the use of ion exchange or affinity chromatography, or adsorption with insoluble metal salts. The manufacturing processes incorporate specific viral removal procedures, usually S/D treatment and viral filtration.<sup>13,197</sup>

Details of the manufacture of three products—Alphanine® (Grifols), Betafact® (LFB), and Berinin P® (CSL Behring)—are shown in Table 22.2. It can be seen that all products involve initial adsorption of factor IX (and the other PCC factors II, IX, and X) from cryoprecipitate-depleted plasma by DEAE anion exchange chromatography. In the manufacture of Alphanine®, final purification involves adsorption to a barium citrate precipitate, followed by two sequential dextran sulfate affinity chromatography steps. Viral removal is achieved by S/D treatment and viral filtration.<sup>13,198</sup> The recovery of purified factor IX in the Betafact® process involves further purification by DEAE anion exchange chromatography and heparin affinity chromatography. Viral removal is achieved by S/D treatment and viral filtration.<sup>13,199</sup>

In the Berinin P® process, the eluate obtained from DEAE anion exchange chromatography undergoes a viral inactivation procedure—pasteurization—and then it is partially purified by ammonium sulfate precipitation, prior to recovery of factor IX by calcium phosphate adsorption and final purification by heparin affinity chromatography (CSL data on file). The products are then formulated, dispensed, and lyophilized.

The required properties of a purified factor IX product are detailed by an EP monograph.<sup>200</sup> The lyophilized product should be a white or pale-yellow hygroscopic powder or friable solid. The preparation should completely dissolve with gentle swirling within 10 minutes. The reconstituted product should have a pH between 6.5 and 7.5, and a minimum osmolality of 240 mOsmol/kg. Potency should not be less than 20 IU/mL, and specific activity should not be less than 50 IU/mg of total protein. Any presence of activated factors should not cause the coagulation time in the NaPTT test to exceed 150 seconds. If heparin has been used in the formulation, it should be stated on the label but in all cases should not exceed 0.5 IU of heparin per IU of factor IX. The product should be sterile and free of bacterial endotoxins.

## von Willebrand factor

Haemate P®/Humate P® (CSL Behring) was one of the first developed intermediate-purity factor VIII-vWF products. Since its licensing in Germany in 1981 for the treatment of hemophilia A, it has also been used to this day for the treatment of von Willebrand disease.<sup>201</sup> The manufacture of the product is from cryoprecipitate and involves initial adsorption of impurities from the reconstituted solution with aluminum hydroxide and then purification of the factor VIII-vWF complex by a series of glycine and sodium-chloride-mediated precipitation steps. Pasteurization is incorporated as a viral inactivation step<sup>197</sup> (CSL data on file).

In the late 1980s, new purification processes were developed incorporating chromatographic procedures to improve the efficiency

**Table 22.2** Examples of Manufacturing Processes for Factor IX.

Brand Name (Manufacturer)	Purification Process	Specific Viral Removal Steps
Alphanine® (Grifols)	DEAE anion exchange chromatography Barium citrate adsorption Dextran sulfate affinity chromatography (x 2)	Solvent/detergent treatment Viral filtration (15 nm)
Betafact® (LFB)	DEAE anion exchange chromatography (x 2) Heparin affinity chromatography	Solvent/detergent Viral filtration (15 nm)
Berinin P® (CSL Behring)	DEAE anion exchange chromatography Ammonium sulfate precipitation of residues $\text{Ca}_2\text{PO}_4$ adsorption/elution Heparin affinity chromatography	Pasteurization (60 °C, 10 h)

Source: CSL Behring.

**Table 22.3** Examples of Manufacturing Processes for von Willebrand Factor

Brand Name (Manufacturer)	Purification Process	Specific Viral Removal Steps
Haemate P® /Humate P® (CSL Behring)	Aluminum hydroxide treatment Glycine precipitation Sodium chloride precipitation NaCl/glycine precipitation	Pasteurization (60 °C for 10 hours)
Alphanate® (Grifols) Wilate® (Octapharma) Wilfactin® (LFB)	Polyethylene glycol precipitation Heparin affinity chromatography DEAE anion exchange chromatography Size exclusion chromatography Alumina gel treatment DEAE anion exchange chromatography (x 2) Gelatin affinity chromatography	Solvent/detergent treatment. Dry heat (80 °C for 72 hours) Solvent/detergent treatment. Dry heat (100 °C for 2 hours) Solvent/detergent treatment Viral filtration, 35 nm Dry heat (80 °C for 72 hours)

of manufacture, improve product purity, and facilitate the introduction of viral inactivation procedures. A detailed examination of the chromatographic approaches that can be used to purify vWF, and an overview of the manufacturing processes and the viral removal procedures utilized for registered vWF products have been published elsewhere.<sup>10,197</sup> They show that a range of chromatographic procedures are used, consisting of anion exchange, size exclusion, and affinity chromatography. Viral removal procedures include S/D treatment, pasteurization, dry heat treatment, and viral filtration.

The purification processes and viral removal procedures for some specific vWF products are shown in Table 22.3. The starting material in all cases is cryoprecipitate. The manufacture of Alphanate<sup>\*</sup> involves an initial purification involving PEG precipitation to remove impurities, followed by heparin affinity chromatography to recover the purified vWF.<sup>10,202</sup> The manufacture of Wilate<sup>\*</sup> and Wilfactin<sup>\*</sup> involves the use of size exclusion chromatography and gelatin affinity chromatography, respectively, to further purify the DEAE anion exchange chromatographic fraction obtained through the processing of a cryoprecipitate solution.<sup>10,203,204</sup> The manufacturing processes incorporate S/D treatment and dry heat as viral inactivation steps. In the Wilfactin<sup>\*</sup> process, viral filtration is included.

The manufacturing process for a vWF product must deliver a consistent product with respect vWF multimer composition. The EP requires that during process development the quantitative analysis of the product must be undertaken using electrophoretic and densitometric techniques to confirm that the product closely approximates the multimer distribution of a plasma reference preparation.<sup>205–207</sup> The dispensed product must contain not less than 20 IU/mL of vWF, and the measured value must be within 20% of that stated on the label. Factor VIII must be tested if there is greater than 10 IU of factor VIII per 100 IU of vWF. The measured value must be within 40% of the stated value. vWF potency must only be measured by the ristocetin cofactor assay. vWF products are characterized on the basis of specific activity and a vWF activity–FVIII ratio. For the former, it must be greater than 1 U vWF/mg of total protein excluding any protein stabilizer. A value greater than 80 U vWF/mg protein is usual, although products with lower specific activity exist.<sup>197</sup> The vWF activity–FVIII ratio is not prescribed but must comply with the approved limit for the product with the competent authority. Values for the various products are from 0.75 to 2.4. The exception is Wilfactin, where the ratio is approximately 60.<sup>197</sup> This reflects the design of the manufacturing process that reduces the copurification of factor VIII, thereby producing a predominantly vWF product.<sup>203</sup>

With respect to other product characteristics, the EP requires that the lyophilized final product should be a white or pale yellow, hygroscopic powder or friable solid with a water content that is

within the approved limits. The product should completely dissolve with gentle swirling within 10 minutes, giving a clear or slightly opalescent, colorless yellow solution. Some products allow for filtration to remove flakes or particles present after reconstitution. If this is the case, it must be shown in validation studies that there is no impact on product potency. The reconstituted product must have a pH between 6.5 and 7.5, an osmolality greater than 240 mmol/kg, and no anti-A- or anti-B-mediated agglutination at a 1:64 dilution at a defined dilution of the reconstituted preparation. The product must be sterile and endotoxin free.<sup>207</sup>

### Factor VIII

The production of cryoprecipitate for clinical use by Judith Pool in 1965 was a pivotal event in the treatment of hemophilia as it provided a high-potency product and an alternative to what hitherto had been used—fresh-frozen plasma.<sup>208</sup> In the following years, purification processes were developed that further improved the purity and viral safety of factor VIII products. Haemate P/Humate P<sup>\*</sup>, in Germany in 1981, was the first registered intermediate-purity product containing a dedicated viral inactivation procedure. The product contained both factor VIII and vWF, and it became a recognized therapeutic for both hemophilia A and von Willebrand disease.<sup>201</sup> The purification process increased the specific activity of vWF from 1 IU/mg of protein for cryoprecipitate to approximately 38 IU/mg of protein in Haemate P/Humate P<sup>\*</sup>.<sup>197</sup>

In subsequent years, the application of chromatographic steps further increased the purity of factor VIII products.<sup>9</sup> Commonly referred to as *high-purity products*, the specific activity was typically greater than 100 IU/mg and for some products as high as 180 IU/mg.<sup>9,197</sup> The different manufacturing processes resulted in a variation in the vWF content, which in some cases limited their use to the treatment of hemophilia A and the exclusion of von Willebrand disease. With the development of these new processes, the opportunity was also taken to introduce two specific viral removal procedures. Typically, these were S/D treatment and dry-heat treatment of the lyophilized final product. Vapor heat and viral filtration have also been applied.<sup>9,197</sup> The use of monoclonal affinity chromatography allowed the generation of factor VIII concentrates with higher specific activity: >3000 IU/mg. These products, of course, do not have vWF.<sup>9,197</sup>

Table 22.4 presents the manufacturing process of a number of commercially available factor VIII products. The aim is to specifically present details to illustrate the range of processes that have been used to purify factor VIII and ensure viral safety. In the manufacture of the high-purity products Beriate P<sup>\*</sup> (CSL Behring), Factane<sup>\*</sup> (LFB), and Immunate<sup>\*</sup> (Takeda), there is typically an initial Al(OH)<sub>3</sub> adsorption step. This serves to remove

**Table 22.4** Representative Manufacturing Processes for Factor VIII

Brand Name (Manufacturer)	Purification Process	Specific Viral Removal Steps	Specific Activity (IU/mg)
<b>Intermediate-Purity Product</b>			
Haemate P®/Humate P® (CSL Behring)	Al(OH) <sub>3</sub> treatment Glycine precipitation Sodium chloride precipitation NaCl/glycine precipitation	Pasteurization (60 °C for 10 hours)	38
<b>High-Purity Products</b>			
Beriate P® (CSL Behring)	Al(OH) <sub>3</sub> adsorption Al(OH) <sub>3</sub> /QAE anion exchange resin adsorption	Pasteurization	170
Factane® (LFB)	DEAE anion exchange chromatography Al(OH) <sub>3</sub> adsorption DEAE anion exchange chromatography FVIII/vWF dissociation–0.3 M CaCl <sub>2</sub> FVIII/vWF reassociation	Viral filtration, 20 nm Viral filtration, 35 nm, 15 nm	>100
Immunate® (Takeda)	Al(OH) <sub>3</sub> adsorption DEAE anion exchange chromatography	Solvent/detergent treatment	75
Alphanate® (Grifols)	PEG precipitation Heparin affinity chromatography	Vapor heat (60 °C for 10 hours, 7–8% moisture, 190 bar) Solvent/detergent treatment Dry heat (80 °C for 72 hours)	>100
<b>Very-High-Purity Products</b>			
Hemofil M® (Takeda)	Recovery of cryoprecipitate Antifactor VIII affinity chromatography QAE anion exchange chromatography	Solvent/detergent treatment Viral filtration, 20 nm	>3000

vitamin-K-dependent prothrombin complex factors. Further purification then occurs by anion exchange chromatography.<sup>209–211</sup> Factane® has a unique viral filtration step involving passing the product through a 15 nm filter. It had been thought that the factor VIII–vWF complex was too large to be able to be successfully filtered. However, this was achieved by dissociating the complex prior to filtration by exposure to calcium chloride and then reassociating the complex by its removal from the filtrate. The manufacturing process for Alphanate® differs in that there is an initial PEG precipitation step that is effective in removing fibrinogen and fibronectin. Final purification utilizes heparin affinity chromatography.<sup>212–214</sup>

The examples of the manufacture of very-high-purity factor VIII products offer some interesting and distinct differences. In the case of Hemofil M®, following a cold precipitation clarification step of the cryoprecipitate suspension, the solution undergoes S/D treatment to effect viral inactivation. Factor VIII purification is achieved by antifactor VIII immunoaffinity chromatography. This step is very effective in achieving the removal of fibrinogen, fibronectin, and vWF. There is then a viral filtration step. A subsequent Q anion exchange chromatographic step binds factor VIII eluted from the affinity column and allows any leached monoclonal antibody affinity ligand to flow through.<sup>215,216</sup>

All factor VIII products are lyophilized. Various excipients are used and can include amino acids, sugar alcohols, sugars, and salts. In some cases, albumin is also included.<sup>197</sup>

The required properties of factor VIII are detailed in the EP.<sup>217</sup> The specific activity should not be less than 1 IU of factor VIII per milligram of total protein before the addition of any protein stabilizer. But, as has been noted, with modern purified products this is readily achieved. The potency of the preparation as stated on the label should result in not less than 20 IU factor VIII per milliliter. With respect to the appearance of the lyophilized preparation, water content, solubility, pH, osmolality of the reconstituted material, anti-A and anti-B hemagglutinin titer, sterility and endotoxin content, the requirements previously described for the vWF product apply.

## Summary and outlook

The production of plasma protein-based therapeutic products is a complex process commencing with the collection of plasma by a system incorporating donor selection, viral testing, and quality assurance measures that ensure the viral safety of the plasma for fractionation.<sup>35</sup> Starting with the work of Cohn, and through the subsequent application of knowledge of protein chemistry, bio-processing technology, and virology, processes have been developed for the production of therapeutic products for a range of clinical indications, which meet the high standards of safety and efficacy prescribed by the regulatory authorities.<sup>7</sup>

Today, the production of plasma protein therapeutic products is a large global industry.<sup>33,218</sup> Despite the development of many recombinant alternatives, the production of products by fractionation of plasma remains cost-effective and continues to grow, particularly for albumin and immunoglobulins.<sup>219,220</sup> Albumin is required in large amounts, and immunoglobulins have unique biological properties, which make it commercially unattractive or scientifically unjustifiable to produce these products by recombinant means, and therefore they will most likely continue to be produced from plasma for the foreseeable future.<sup>19,219,221</sup>

Plasma fractionators are continuing to explore the clinical utility of additional plasma proteins and improve existing products. Proteins with therapeutic potential which are being investigated are shown in Table 22.1. Plasmin and reconstituted HDL are the most advanced in the clinical development pathway.<sup>25,26</sup> With respect to existing products, the focus is on improving formulations and addressing issues that can lead to adverse reactions. As the plasma raw material is limited and expensive, plasma fractionators are also focused on increasing process recoveries for the various plasma-derived therapeutic products.<sup>222</sup>

The pathogen safety of manufactured PDMPs has been dramatically enhanced since the 1980s through the implementation of NAT technologies that effectively screen out units with high virus titers. Additionally, the incorporation of multiple orthogonal pathogen inactivation and removal steps into the manufacturing processes has greatly enhanced the safety of the final products against both

known and emerging/reemerging viruses. Vigilance, assessment, and verification studies to assess the effectiveness of the virus reduction technologies against emerging pathogens, especially small nonenveloped viruses, are ongoing responsibilities of the manufacturers of plasma-derived therapies. Although the safety of plasma-derived therapies has an excellent history over the last two decades, the development of robust technologies (i.e., 20-nm virus filtration) and the incorporation of these technologies into new and existing manufacturing processes continue.

The developed countries are well served for fractionation capacity. Many developing countries such as China and Brazil are extending and improving their capabilities.<sup>31</sup> However, there are many developing countries that do not have adequate access to plasma protein products and cannot afford to purchase the requirements for their population.<sup>223</sup> Arguments for and against the establishment of a localized fractionation facility have been discussed elsewhere.<sup>222,224</sup> The adoption of improvements in bioprocessing approaches could make the design and establishment of a fractionation facility less complex, cheaper, and hence feasible for construction and operation in a developing country. However, in addition to establishing fractionation capacity, supporting infrastructure is required for facilities to enable plasma collection and NAT testing of donations. Given the complexity and expense of developing these capabilities, developing countries must decide whether it is worthwhile to establish their own facilities or simply obtain the plasma-derived therapeutics on the commercial market.

The plasma fractionation industry will continue to deliver unique therapeutics essential for human health. It is likely that the future will be as eventful as the period from Cohn to the present day.

## Acknowledgments

The authors acknowledge the efforts of Joe Bertolini and Nathan Roth for the preparation of the chapters in the previous edition of Rossi's Principles of Transfusion Medicine (Chapters 27 and 56). The current authors have refined the original chapters with recent developments in the plasma protein therapies and other relevant information.

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A full reference list for this chapter is available at: <http://www.wiley.com/go/simon/Rossi6>

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## CHAPTER 23

# Immunoglobulin products

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Plasma-derived immunoglobulin (Ig) is used for an increasingly wide range of autoimmune and inflammatory diseases in addition to its traditional uses for the prevention of infection and for antibody replacement and augmentation therapy in immune deficiency diseases<sup>1–3</sup> (Figures 23.1 and 23.2). Increased recognition and treatment of immune deficiencies in the developing world have added to the global demand for IgG. However, the major reason for the continued strong growth in demand for IgG products is the increased use of “high-dose” therapy, particularly in autoimmune neurologic diseases.<sup>1–3</sup> Long-term treatment of adults with doses in the range of 1–2 g/kg or even more per month is the major contributor to the continuously growing global utilization of Ig products, which exceeded 230 metric tons in 2020 (Figures 23.1 and 23.2).<sup>4</sup> Indeed, the demand for Ig is the major driver of the continuously increasing demand for plasma and growth in fractionation capacity.

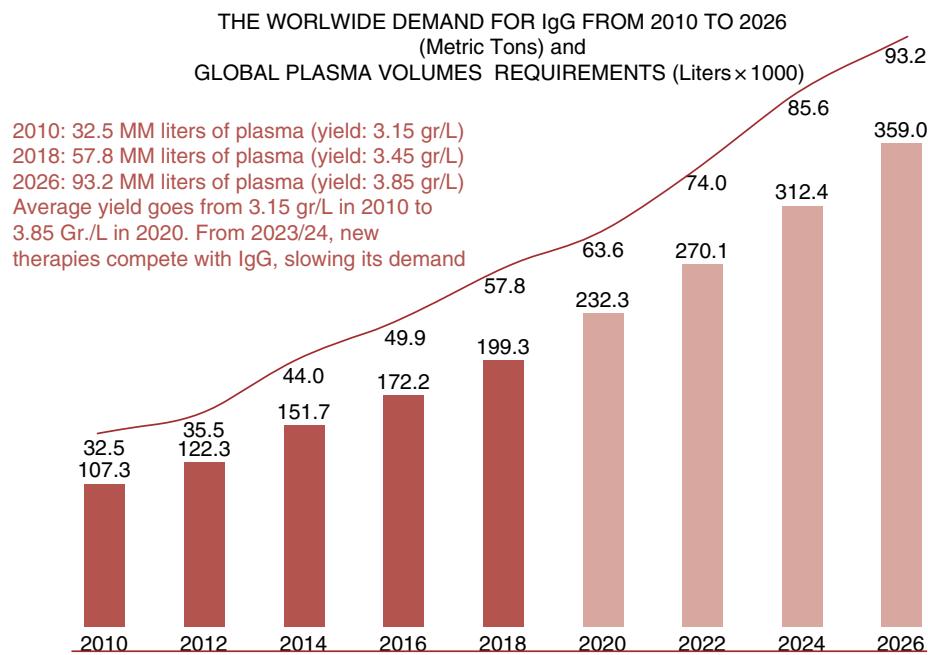
### Structure and origin of Ig molecules

In order to understand the many uses for serum immunoglobulin preparations, it is necessary to understand the major difference between immunoglobulins and most other plasma proteins: diversity.<sup>5–8</sup> The prototypic immunoglobulin molecule is a tetramer composed of two identical heavy chains, each with a molecular mass of approximately 55 kD, and two identical light chains of 22 kD, giving an overall molecular mass of 155 kD.<sup>5,7</sup> There are four major classes, or *isotypes*, of immunoglobulins in plasma: IgA, IgE, IgG, and IgM, with IgG accounting for 75% of all of the immunoglobulin in plasma. These classes are defined by their heavy chains  $\alpha$ ,  $\epsilon$ ,  $\gamma$ , and  $\mu$ , respectively. Gamma heavy chains are actually a family of four types,  $\gamma 1$ – $\gamma 4$ , which are grossly related but contain differences that result in different effector functions (Table 23.1). Depending on which chain a particular IgG molecule contains, it is assigned to “subclass” 1–4. Besides IgA, IgE, IgG, and IgM, there is a fifth class, IgD, composed of molecules with  $\delta$  heavy chains. These play an important role in B-cell differentiation, but are only a minor component of the total plasma Ig pool. There are two types of light chains,  $\kappa$  and  $\lambda$ . Only one type is used in any individual Ig molecule. In addition, there are loci in the  $\gamma$  and  $\kappa$  chain genes at which different individuals may have different alleles or markers called Gm1–17

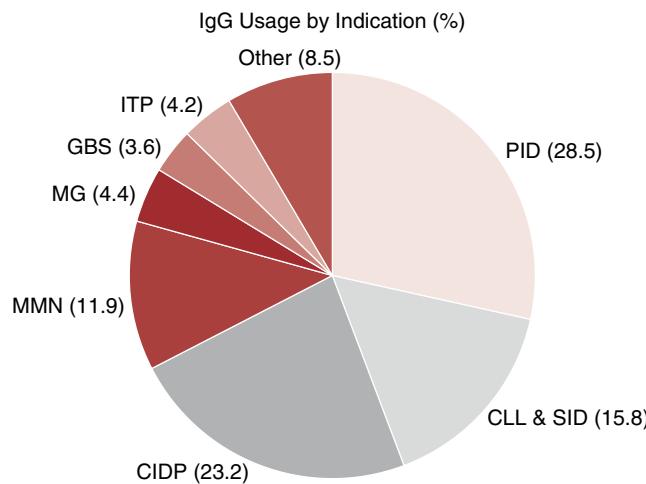
and Km1–3. These are useful as markers termed *allotypes* in genetic studies, but their clinical significance is not known.

The four chains are arranged into a pair of dimers linked together with varying numbers of interchain disulfide bonds and sugar chains at characteristic positions in each class or subclass. Immunoglobulin molecules are generally depicted as having a Y-like structure (Figure 23.3).<sup>5,7</sup> Each light chain contains a single variable region and a single constant region, whereas each heavy chain also has a single variable region, but three to four constant regions. The junction between the first and second constant regions of the heavy chain is considered a “hinge” that gives some flexibility to the arms. The variable regions of the light and heavy chains are aligned together to form the antigen binding sites, whereas the heavy chain constant domains align to form a “handle,” which binds receptors and other proteins that facilitate the effector functions and determine the metabolic fate of each class of molecules. IgA and IgM may contain an extra *joining* (J) chain, which binds to the heavy chains and holds together two of the four-chain units in the former, and five in the latter.

Ig molecules are cleaved into characteristic fragments by proteolytic enzymes such as papain or pepsin (Figure 23.3). The dimer containing the second and third constant domains of each of the heavy chains from all members of a class is crystallizable, and it has therefore been termed Fc. In contrast, because of the diversity of the variable regions, the fragments containing the antigen-binding sites are not crystallizable. With papain, the cleavage occurs toward the amino terminus side of the “hinge,” and two separate but identical antigen-binding fragments (Fab') are released. With pepsin, cleavage occurs toward the carboxyl side of the hinge, and a single fragment with two identical antigen-binding sites (F[ab']<sub>2</sub>) is produced. The antigen-binding specificity of each immunoglobulin molecule is determined by the sequences of several short stretches of amino acids in the N-terminal domain of each heavy and light chain, which are called *hypervariable* or *complementarity-determining* regions. Each person's immune system can make approximately 10<sup>12</sup> different antibody specificities. This remarkable diversity initially arises by *rearranging* or splicing together multiple DNA segments for different variable and constant region domains at slightly different junctions, to make a single antibody gene unique to that



**Figure 23.1** Global demand for IgG products (IVIG and SCIG) (Bars) and estimated volume of plasma (in millions of liters) necessary to produce that amount of IgG (upper line and numbers). Source: IFPA, Ref. 4.



**Figure 23.2** Use of IgG by Indication: UK, 2019–2020. Data from Ref. 3. PID: primary immune deficiency. CLL&SID: chronic lymphocytic leukemia and secondary immune deficiencies. CIDP: chronic inflammatory demyelinating polyneuropathy. MMN: multifocal motor neuropathy. MG: myasthenia gravis. GBS: Guillain-Barre syndrome. ITP: immune thrombocytopenic purpura. “Other” includes inflammatory myopathies

particular immature B cell and its progeny.<sup>5–8</sup> Upon exposure to an antigen, a naive B-lymphocyte with a complementary binding site is selected and stimulated to proliferate, forming a germinal center in a lymph node or the spleen. Among the progeny of that B cell, in the appropriate milieu of antigen, helper T cells, and cytokines, some cells undergo further cutting and splicing of their heavy chain genes, resulting in isotype switching. Somatic hypermutation can also occur in their variable region genes,<sup>5,6</sup> resulting in *affinity maturation*. At any point, some cells may stop differentiating and become plasma cells, which are totally devoted to producing antibodies of a single isotype and specificity. Other cells may

**Table 23.1** Mechanisms of Action of IgG

**Dependent on Antigen-Binding (Fab') Region**

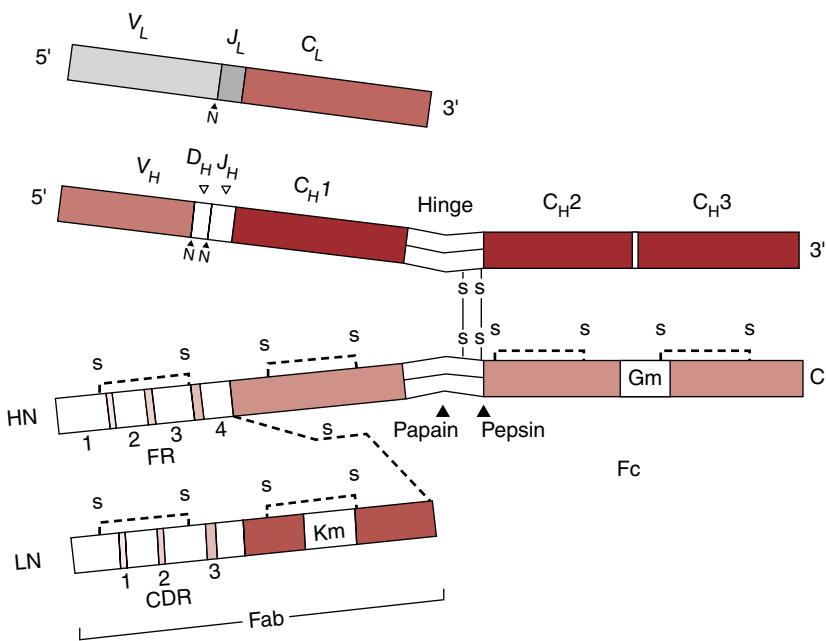
- Precipitation, agglutination, and neutralization of toxins and antigens
- Sensitizing targets for phagocytosis, complement, and cell-mediated cytology
- Neutralize viral adhesion
- Neutralizing superantigens
- Elimination of complement-activating circulating immune complexes
- Neutralizing autoantibodies (anti-id)

**Dependent on Fc Interactions with Effectors/Receptors**

- Binding to cellular receptors and activating phagocytosis and cell-mediated cytology
- Binding C1q and activating complement
- Increasing catabolism of autoantibodies by saturating FcRn
- Inhibiting C3 deposition or further activation
- Downregulation of B- and T-cell function
- Cytokine regulation
- Fc receptor blockage, altering phagocyte function

become quiescent “memory” cells, which can be rapidly stimulated on re-exposure to antigen. Upon initial exposure to an antigen or vaccine, the “primary” antibody response usually consists of IgM and relatively low-affinity IgG. Upon re-exposure, however, or during progression of an infection, class switching and affinity maturation will result in an increased ratio of IgG to IgM and an increase in the *avidity*: a product of the affinity, specificity, and quantity or “titer” of the overall antibody response. High-avidity IgG production is characteristic of “secondary” responses to vaccines and convalescent sera following infections.

The unique combination of light and heavy chain hypervariable regions that form the antigen-binding sites of any given antibody molecule may, in turn, seem “new” to the body and can serve as an antigen itself. Each unique antigen-binding site is called an “idiotype” because it belongs only to that specificity. An antibody that recognizes or blocks the antigen-binding site of another antibody is therefore called an “anti-idiotype” (anti-id).<sup>5,8</sup> Thus, the circulating



**Figure 23.3** Diagram of IgG structure: a single IgG molecule consists of two heavy chains and two light chains. The upper pair shows regions of the proteins encoded by different DNA segments: 5': 5' end of the DNA segment coding for that part of the protein; 3': 3' end of the DNA segment. V<sub>L</sub>, light chain variable region; J<sub>L</sub>, light chain joining region; C<sub>L</sub>, light chain constant region; V<sub>H</sub>, heavy chain variable region; D<sub>H</sub>, heavy chain diversity region; J<sub>H</sub>, heavy chain joining region; C<sub>H1</sub>, first heavy chain constant region; C<sub>H2</sub>, second heavy chain constant region; C<sub>H3</sub>, third heavy chain constant region. The lower pair shows structural features of the protein chains: HN and LN, amino terminus of heavy and light chains, respectively; C, carboxy terminus; S–S, disulfide bonds. The hinge region is shown in white. Sites of cleavage by papain and pepsin are shown by arrows above labels. Papain cleavage results in the formation of two monovalent F(ab) fragments and a disulfide-linked Fc fragment. Pepsin cleavage yields a bivalent disulfide-linked F(ab')<sup>2</sup> fragment. CDRs, complementarity determining regions (1–3, also known as *hypervariable regions*). FRs, framework regions (1–4), which are conserved. Note that FRs and CDRs are found in both heavy and light chains, as illustrated by white versus colored regions, but the labels are attached to only one chain each for readability. Gm is an allotypic region on the heavy chain; Km is an allotypic region on the κ-light chain. Source: Schroeder *et al.* (2010).<sup>5</sup> Reproduced with permission of Elsevier.

pool of IgG contains thousands or even millions of different antibody and anti-id specificities (Figure 23.4). Anti-ids are believed to be important in regulating B- and T-cell responses, forming a major component of the “network” hypothesis for which Jerne received a Nobel Prize in 1984.<sup>8</sup> Anti-idiotype neutralization of autoantibodies may be a major mechanism by which therapeutic IgG ameliorates autoimmune diseases (discussed further in this chapter).

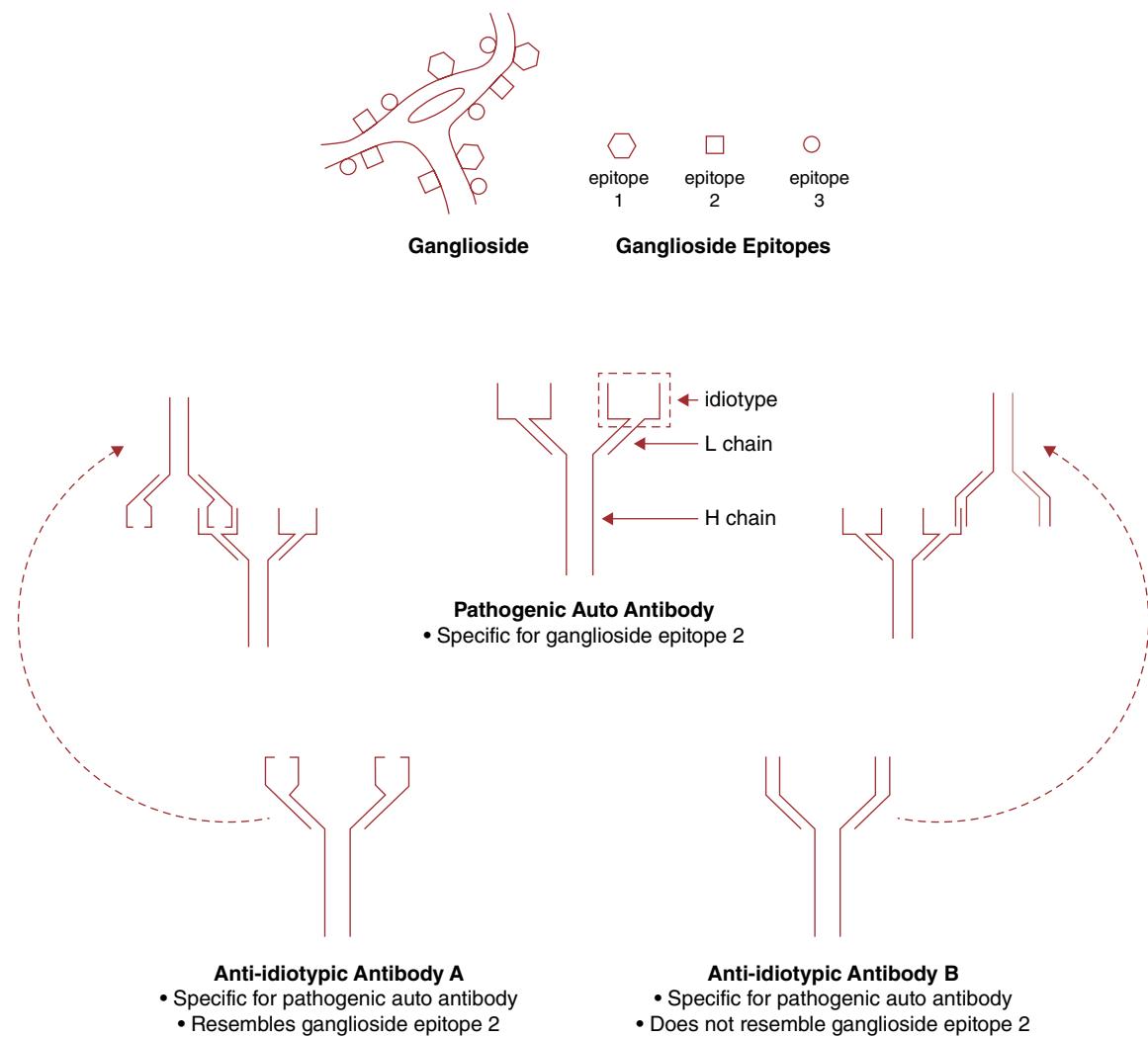
### A short history of commercial IgG production

Although sera from vaccinated animals had been used for passive immunity since the late 1800s, and convalescent human sera and placental extracts were used in the 1920s and 1930s, large-scale purification of immunoglobulins from plasma did not begin until the 1940s. With a grant from the US Navy, Edwin Cohn, a physical biochemist at Harvard University, developed a process for fractionating plasma using sequential precipitations with increasing concentrations of cold ethanol to produce large amounts of purified, stable albumin (fraction V) for the treatment of shock on the battlefield during World War II.<sup>9</sup> The globulins containing most of the antibody activity were found in fractions II and III, which also contained clotting factors and lipoproteins. Oncley modified the procedure by manipulating the pH, ionic strength, and ethanol concentration, and succeeded in recovering most of the 7s gamma globulins (now recognized as IgG) in fraction II, with the clotting factors and faster-sedimenting globulins in fraction III. During the 1940s, intramuscular (IM) injections of fraction II *immune serum*

*globulin* (ISG) were studied for the prevention of hepatitis and measles. Janeway *et al.* in Boston and Barandun in Switzerland attempted to give ISG intravenously, but their efforts were abandoned because of severe immediate reactions, including hypotension, chills, and fever.<sup>9,10</sup> IM injections of 16% plasma-derived ISG were routinely used for antibody replacement in immune deficiency patients and for prophylaxis against infectious disease until the 1980s, but the doses that could be tolerated by this route were limited, and efficacy in immune deficiencies was far from ideal.

Studies through the 1960s and 1970s suggested that the majority of vasomotor reactions and other immediate adverse effects (AEs) that accompanied the early trials of intravenous (IV) administration of ISG were attributable to complement activation by aggregates of the 7s globulins. Barandun *et al.* showed that these could be removed by ultracentrifugation or dissociated by treatment with low concentrations of pepsin, incubation at pH 4, and/or reduction and alkylation of disulfide bonds.<sup>10</sup> Other protein modifications such as S-sulfonylation and treatment with β-propiolactone were also studied. The first commercial IVIGs were pepsin- or plasmin-treated preparations that contained fragmented IgG molecules that sedimented slower than intact IgG (i.e., at 5–6.5 s) and had shortened half-lives and decreased effector function compared to the IgG in the IM preparations.

By the late 1970s, combined treatments including mild reduction and alkylation, reconstitution in 0.3 M glycine, and/or treatment at pH 4 with low concentrations of pepsin were introduced to prevent aggregate formation while leaving the IgG molecules intact. These



**Figure 23.4** Idiotypic antibodies produced against different determinants on antigen—in this case, ganglioside sugars in a bacterial lipo-oligosaccharide and different anti-idiotypes produced in response to a single idiotypic antibody. Source: Berger et al. (2013).<sup>63</sup> Reproduced with permission of Wiley.

processes eventually led to the first preparations that could safely be given by the IV route: Gammimune® and Sandoglobulin®, respectively. The most important contribution to the tolerability of these preparations, however, was arguably the use of high concentrations of sugars as stabilizers: maltose in Gammimune and sucrose in Sandoglobulin.

Despite minimizing complement-binding aggregates, early IVIG preparations still frequently caused hypotension and/or vasodilatation and increased capillary permeability, which were shown to be associated with the presence of contaminating amounts of proteins such as prekallikrein activator and kallikrein itself.<sup>10</sup> Contamination with factor XIa was also found to be responsible for procoagulant activity in many ISG and early IVIG preparations.<sup>11</sup> In addition, other studies showed that isolated Fc fragments, IgG aggregates, and antigen–antibody complexes induced secretion of prostaglandin E<sub>2</sub> from monocytes, suggesting that this mediator might also be contributing to pain and other adverse effects of early IVIG preparations.<sup>12</sup>

In 1981, the World Health Organization published a set of “Desirable Characteristics of IVIG Preparations”:<sup>13</sup>

- IVIG should be extracted from a pool of at least 1000 individual donors.
- IVIG should contain as little IgA as possible.

- The IgG should be modified biochemically as little as possible and possess opsonizing and complement-fixing activities.
- IVIG should be free from preservatives or stabilizers that might accumulate in vivo.

These characteristics were not fully achieved until the most recent generation of IVIG products was developed in the new millennium. Because there is no suitable alternative to polyclonal human IgG for antibody replacement therapy in immunodeficient patients, and because of the critical role of IVIG in treating a number of other diseases, the WHO includes IgG in its “Model List of Essential Medicines.”<sup>14</sup>

### Current IgG products

Additional methods to increase the yield of IgG per liter of plasma, improve the convenience of administration, and minimize AEs resulted in liquid IVIG preparations that are >95% pure IgG and are readily available in most countries. Many manufacturers now utilize modified purification procedures which only employ a single ethanol precipitation step and substitute precipitation with fatty acids such as caprylate, or medium-chain alcohols, together with depth filtration, for the serial Cohn–Oncley ethanol steps.<sup>15</sup>

Anion-exchange column chromatography is used to improve the purity by decreasing the concentrations of IgA and potentially vasoactive and/or thrombogenic protein contaminants, and some manufacturers also add cation-exchange chromatography to further purify the IgG. The use of ion-exchange chromatography, and other refinements in fractionation and purification processes, has allowed the recovery of IgG from fractions which had previously been separated from the predominant IgG-containing Cohn fraction II, increasing the yield of IgG per liter of plasma.

To improve the convenience of preparing and administering IVIG, most manufacturers have developed IVIG preparations that are available as 10% liquids and can be kept at room temperature for at least part of their shelf life.<sup>16</sup> Currently, three IV products—Gammagard<sup>®</sup> (Takeda), Gamunex<sup>®</sup> (Grifols), and Privigen<sup>®</sup> (CSL Behring)—dominate the US market. Gammagard and Gamunex are stabilized with glycine, and Privigen is stabilized with L-proline. All are available as 10% liquids, none contain any sugars as stabilizers, and none contain more than 50 mcg/mL IgA. IM ISG is rarely used in the United States, but Gammagard, Gamunex, and Gammaked<sup>®</sup> are labeled for subcutaneous administration, and CSL Behring markets a 20% proline-stabilized product, Hizentra<sup>®</sup>, specifically for subcutaneous use. In addition, Cuvitru<sup>®</sup> (20%), Xembify<sup>®</sup> (20%), and Cutiug<sup>®</sup> (16.5%) are subcutaneous products marketed in the United States by Takeda, Grifols, and Octapharma, respectively. Other manufacturers also have IV and SC products in the US market. Interested readers should consult current sources such as UpToDate<sup>®</sup>, or websites maintained by the US Food and Drug Administration (FDA), Immune Deficiency Foundation (IDF), or major distributors to track what is available at any given point in time.

### **Prevention of pathogen transmission**

Although clotting factor concentrates prepared by Cohn fractionation were known to have transmitted what is now recognized as hepatitis B virus, fraction II ISG had been given to hundreds of children for measles prophylaxis with only one case of apparent transmission of that virus. This was likely due to the presence of antibodies that neutralized possible virus in the product. The subsequent widespread use of fraction II ISG provided confidence that this product carried little risk of transmission of blood-borne infectious agents. Unfortunately, that turned out to be false confidence. Even after the recognition of AIDS, complacency persisted because HIV (then termed HTLV III) was inactivated and HIV and hepatitis-virus–antibody complexes were partitioned out by the ethanol precipitation procedures.<sup>17</sup> In the late 1980s, reports of “non-A, non-B” hepatitis (now termed *hepatitis C*, caused by hepatitis C virus [HCV]) began to appear among recipients of IVIG<sup>18</sup> and other plasma products. Solvent–detergent (SD) treatment and other dedicated steps were added to disrupt the lipid envelopes and/or coat proteins; and to inactivate, partition, or remove by nanofiltration nonenveloped viruses, to ensure the safety of these products (see Chapter 22). To date, there have been no reports of virus or prion transmission by current IgG products, but we must continue to be vigilant because of the threat of emerging pathogens.

### **Pharmacokinetics and metabolism of IgG**

#### **Conventional-dose IVIG therapy (for immune deficiencies)**

As recognition of primary immune deficiencies increased in the 1950s, a study by the British Medical Research Council resulted in

the recommendation that most patients with hypogammaglobulinemia could be successfully treated with 25 mg/kg/week of IM ISG.<sup>19</sup> Within a few years after IVIG became available in the early 1980s, this was increased to 400–500 mg/kg at intervals of 3–4 weeks, which kept the serum IgG levels in most recipients at or above the lower limit of normal, approximately 500 mg/dL, at the “trough” just before the next dose was given.<sup>20,21</sup> An intravenous bolus of 400 mg/kg of IgG given intravenously immediately raises the serum IgG level by about 1000–1200 mg/dL. The levels then drop by 40–50% over 48–72 hours, because IgG is distributed into the total extracellular fluid volume, of which only about 50% is intravascular.<sup>20,22</sup> After this equilibration phase, the IgG is catabolized with first-order kinetics and a half-life of 21–30 days, which differs in different individuals. Dosing is usually repeated every 3–4 weeks.<sup>21</sup> A major reason for this relatively long half-life of IgG, compared to IgA, IgM, and other plasma proteins, is the presence of a specific, saturable receptor on the surface of endothelial cells termed FcRn (n for neonate) because it is also found in the placenta and is responsible for the transfer of IgG into the fetus.<sup>23,24</sup> IgG, which binds to FcRn, is internalized into recycling endosomes, in which it is protected from lysosomal degradation, returned to the apical surface, and exocytosed back into the circulation.<sup>24</sup> Because IgG is distributed intra- and extravascularly but not sequestered into other compartments such as intracellular or lipophilic pools, *two compartment (i.e., intra- and extravascular) models* are traditionally used to describe its kinetics, although *single-compartment models* are sometimes used as an alternative. Early studies with radioactively labeled IgG showed that after equilibration 45–50% of the IgG remained intravascular.<sup>22</sup> Although the ratio of total extracellular fluid volume to body mass index (BMI) is relatively constant across a range of BMIs from 15 to 40,<sup>25</sup> many authorities limit the maximum dose of IVIG to 100 g in a single infusion or suggest that dosing in very obese individuals should be based on “ideal” rather than actual body weight.<sup>26</sup>

### **Subcutaneous IgG therapy**

Although first introduced in the late 1970s as an alternative to IM injections of ISG,<sup>27</sup> the use of small portable pumps to deliver concentrated IgG solutions subcutaneously has increased in popularity and is now employed by more than half of the PID patients in several countries. There are two fundamental differences between the subcutaneous route of administration of IgG (SCIG) and the intravenous route (IVIG), which lead to most of the practical differences between the two routes. The first of these is the lack of a requirement for venous access with SCIG; the second is the relatively slow adsorption of SCIG into the intravascular compartment, which was described in detail more than a hundred years ago.<sup>28</sup> In contrast, a trained professional is usually required to administer IVIG, and rapid infusion may contribute to systemic adverse events (AEs).<sup>27</sup> The doses of IgG that are practical to give by the subcutaneous (SC) route are smaller than those by the IV route, so SCIG is usually given weekly or even more frequently. Studies have shown little difference in the steady-state serum IgG levels if the same total dose of SCIG is fractionated into smaller doses given at intervals from daily to once every two weeks, and the approved US labels for most subcutaneous products allow dosing intervals from 1 to 7 days, and some labels allow dosing once every 14 days.<sup>29–30</sup>

These differences in kinetics of absorption result in several other effects that may lead to a preference for one route versus the other in a variety of different individual situations. With SCIG, the initial direction of the movement of IgG is opposite that of

IVIG: the IgG must first diffuse from a subcutaneous depot into lymphatics, from which it reaches the bloodstream indirectly via the thoracic duct.<sup>27,28</sup> Equilibration of SCIG from its injection sites into the total intravascular and extracellular fluid space requires about the same amount of time as equilibration of IVIG out of the intravascular compartment.<sup>22,28,31–32</sup> Thus, with SCIG, the intravascular IgG concentration increases more gradually, peaking at 36–72 hours after the end of an infusion. Most other features of SCIG are consequences of this difference in kinetics. For example, many of the systemic AEs of IVIG are related to the rate of the infusion and resolve when the infusion is slowed. The  $C_{\max}$  achieved with SCIG is, on average, only 61% of the peak achieved with IV infusions.<sup>27,31</sup> Studies comparing IVIG and SCIG in PID patients have shown the mean peak serum  $C_{\max}$  of 2303 mg/dL immediately after IV infusions.<sup>31–34</sup> In contrast, the mean peak with SCIG was 1410 mg/dL, and the interval ( $t_{\max}$ ) between beginning an SCIG infusion and the peak IgG concentration was 62.6 hours (2.61 days).<sup>31–34</sup> The slower rate of rise toward the peak and the truncation of its height are believed to be responsible for the much lower incidence of systemic AEs with SCIG. The safety and tolerability of SCIG has been confirmed in multiple studies and is consistent with observations that many AEs of IVIG infusions are rate related.<sup>27,33,35,36</sup> No differences have been reported in the 30–35-day half-life ( $t_{1/2}$ ) of current IgG preparations given by the subcutaneous versus IV routes.<sup>22</sup> With weekly SCIG, there are only about four days between the  $t_{\max}$  of one dose and the administration of the next dose, suggesting that only about 10–20% of the administered IgG is metabolized before the serum level starts to rise again. In contrast, with IVIG dosing intervals of 3–4 weeks, approximately 36–48% of the IgG may be metabolized by the time the next dose is due. These differences in the dosing intervals used in most SCIG-versus-IVIG regimens result in increased trough ( $C_{\min}$ ) serum IgG levels with SCIG. Pooled data from seven studies in which equivalent monthly IgG doses were given as weekly SCIG infusions versus IVIG infusions every 21–28 days showed that trough serum IgG levels were 10–20% (mean, 12.7%) higher with weekly SCIG.<sup>31,37</sup> After 6–12 weekly infusions, SCIG results in near-steady-state IgG levels, with differences between  $C_{\min}$  and  $C_{\max} \leq 5\text{--}10\%$  of the overall mean. (This steady state can also be achieved by “loading” the patient with five or six consecutive daily infusions of what will then be the weekly SCIG dose.)<sup>38</sup> In contrast, with IVIG, the trough-to-peak difference is often  $\geq 100\%$  of the overall mean.<sup>22,27,31</sup>

Another approach to subcutaneous IgG therapy involves the use of recombinant human hyaluronidase to temporarily depolymerize the hyaluronan chains that crosslink the subcutaneous tissue.<sup>39</sup> This increases the dispersion and absorption of medications delivered into the subcutaneous space and allows full monthly doses of 10% IVIG (in the range of 200–500 mL) to be given as a single SC infusion into a subcutaneous site.<sup>39,40</sup> The  $C_{\max}$  is between that of standard SCIG and an IV bolus, but the  $C_{\min}$  (trough) is not changed from that experienced with conventional 3–4 weekly IVIG dosing. The combination product, containing one vial of recombinant hyaluronidase and one of 10% Gammagard, is marketed as HyQvia® by Shire (Takeda). In clinical studies in immune-deficient patients, HyQvia was well-tolerated and efficacious.<sup>39,40</sup>

The area under the curve (AUC) of serum concentration versus time of a drug after a single intravenous infusion is defined as 100% bioavailability.<sup>41,42</sup> The bioavailability of the drug when given by any other route is generally lower, so this is not unexpected with subcutaneous versus IV administration of IgG, and it is also found

with monoclonal IgG antibodies and therapeutic fusion proteins containing its Fc domains.<sup>42</sup> In licensing studies of SCIG, the US FDA mandated determination of the bioavailability of SCIG as compared to IVIG and calculation of the dose adjustment which would be necessary to achieve an AUC with SCIG equal to that previously measured with IVIG in the same patients. Multiple studies and different lines of evidence demonstrated that the bioavailability of SCIG is about two-thirds of that of IVIG regardless of the preparations being compared.<sup>41</sup> Interestingly, and in close agreement with the results of the SCIG licensing trials, the results of pooled analyses including 500 subjects and 20 different IgG preparations show that the slopes of regression lines for IgG level versus monthly dose indicate an increment in the steady-state serum IgG level of 87 mg/dL for every 100 mg/kg increase in monthly SCIG dose compared to an increment of 121 mg/dL in the trough level of IgG for every 100 mg/kg increment in monthly IVIG dose, suggesting a bioavailability of 71.9%.<sup>43,44</sup> The decreased bioavailability may involve degradation in the tissues and/or local binding in the intercellular matrix but seems to be a general property of IgG. The European Medicines Agency does not require dose adjustment for SCIG as compared to IVIG, and analyses of large payor databases in the United States suggest that equivalent or even slightly lower total monthly doses of SCIG versus IVIG are actually given to PIDD patients.<sup>45</sup>

### **High-dose IVIG therapy for autoimmune and inflammatory diseases**

The initial dose of IVIG used for most autoimmune/inflammatory diseases is 2 g/kg given over 2–5 days, followed by maintenance doses of 1–2 g/kg every 3–4 weeks. This regimen is based on the serendipitous 1981 observation that five consecutive daily repetitions of the monthly dose of IVIG for immune deficiencies at that time (400 mg/kg) normalized the platelet counts in immune-deficient patients who also had immune thrombocytopenia.<sup>46,47</sup> Infusion of 2 g/kg of IVIG increases the serum IgG level greater than fourfold, from pretreatment means of 700–1060 mg/dL to peaks well over 3000 mg/dL,<sup>47</sup> and IgG levels as high as 5000–7000 mg/dL have been reported.<sup>48,49</sup> These extremely high levels may contribute to vaso-occlusive AEs due to hyperviscosity in some patients.<sup>48,49</sup> The distribution phase of the IVIG is not expected to be altered by the IgG level, but if serum levels are sufficient to saturate FcRn, the catabolic rate may be increased.

### **Drug interactions**

Standard polyclonal IVIG is not known to bind or influence the distribution or metabolism of small molecules, neither does conventional drug therapy alter the levels or metabolism of IgG. Although not formally studied, high-dose IVIG might be expected to increase the catabolism of IgG monoclonal antibodies and fusion proteins containing the Fc of IgG, due to saturation of FcRn,<sup>24,42</sup> as explained further in this chapter. IgG therapy, whether given by the IV or SC route, may decrease the antigenicity of live virus vaccines, such as measles, mumps, and rubella (MMR), and varicella, so it is recommended that a period of six months be allowed to elapse between the last IgG therapy and administration of any of these vaccines.<sup>50</sup> If a patient has not been immunized and has received IgG therapy more than one month before potential exposure to measles, mumps, rubella, or varicella viruses, passive immunization with IgG would be recommended.

## Adverse reactions to IVIG and SCIG

### Immediate reactions

Mild reactions to IVIG infusions are common, occurring in 15–20% of infusions and in 50% of patients at one time or another.<sup>35,36</sup> These AEs are mostly uncomfortable and/or unpleasant but rarely serious. Symptoms may include headache, nausea, musculoskeletal pain, and flushing and tachycardia. When severe, IVIG infusion reactions may resemble anaphylaxis, but they usually do not involve IgE and should be termed *anaphylactoid*.<sup>36</sup> A key difference between these anaphylactoid reactions that accompany IVIG infusions and true IgE-mediated anaphylaxis is that the former are usually associated with hypertension rather than hypotension. Furthermore, anaphylactoid IVIG reactions are often less severe with subsequent infusions rather than more severe as would be expected with true allergy. In most cases, symptoms during IVIG infusions can easily be managed by slowing or temporarily stopping the infusion until the symptoms subside and/or by prophylaxis or treatment with acetaminophen, nonsteroidal anti-inflammatory drugs (NSAIDs), and/or antihistamines.<sup>35,36</sup> Some patients may require corticosteroids; and many patients are given NSAIDs or steroids prophylactically. Most AEs are related to the rate of infusion and can be avoided by beginning the infusion slowly (0.01 mL/kg/minute) and gradually increasing the rate stepwise as tolerated.

Patients who are naive to IVIG replacement have had interruptions in their therapy, and/or who are actively or chronically infected have an increased risk of infusion-related AEs.<sup>51</sup> This may be related, in part, to the formation of antigen–antibody complexes as the IgG is being given, and/or the rapid release of lipopolysaccharide or other components of pathogens already present in the recipient. The risk of these reactions can be reduced by making sure that patients are afebrile and that those with active infections are on antibiotics before giving an IVIG dose. The incidence of reactions may increase when patients already on therapy are given a different brand of IVIG,<sup>35,36</sup> so whenever this occurs, it is prudent to begin the infusion slowly and/or to premedicate the patient.<sup>51</sup>

True anaphylaxis may occur in patients receiving IVIG, particularly in those who are deficient in IgA but still have the capacity to produce IgE. This occurs very rarely, but may be life threatening, and can be avoided by slow administration of low-IgA products<sup>35,36</sup> and/or by administering the IgG subcutaneously.<sup>52</sup>

Systemic reactions to SCIG infusions are rare. Gardulf *et al.* reported only 30 systemic reactions in 25 immunodeficient patients given 3232 infusions (0.93%).<sup>53</sup> A subsequent review, which included additional studies totaling over 40,000 infusions, showed that only one study reported a rate of systemic AEs >1%.<sup>27</sup> Although nearly 75% of patients may have some local discomfort associated with swelling and redness at the site of the infusions, the local symptoms usually subside within 24–48 hours and do not usually deter patients from continuing with their SCIG regimen. Because of the infrequency of systemic side effects with SCIG, premedication is rarely necessary, and close monitoring is not required during the infusion. SCIG has thus emerged as an ideal route for home use in many patients.<sup>38,53,54</sup>

The increasing use of IVIG, particularly with high doses for inflammatory and autoimmune diseases, has resulted in other serious adverse events, including thromboembolism, renal dysfunction/failure, aseptic meningitis, and hemolytic anemia.<sup>35,36,55–57</sup> This has led to the requirement (in the United States) that all IVIG products contain a “Black Box” warning about the risks of thromboembolic events (TEEs) and acute renal dysfunction/failure, as well as

“Warnings and Precautions” about hemolytic anemia, aseptic meningitis syndrome, and transfusion-associated acute lung injury (TRALI).<sup>56</sup> Most reports of acute renal failure or dysfunction were due to osmotic nephrosis related to the former use of sucrose as a stabilizer in certain products.<sup>57</sup> The risk of aseptic meningitis seems to be highest in patients receiving high-dose IVIG for neurologic diseases, and TRALI seems very rare.<sup>55,56</sup> The only “Black Box” warning on subcutaneous IgG products marketed in the United States is about the risk of thrombosis. Other potentially severe adverse effects are described in “Warnings and Precautions.” Especially in patients with other risks for thromboembolic events or hyperviscosity, adequate hydration should be ensured before administering IgG by any route, and the minimum dose and infusion rate practicable should be used.

### Thromboembolic events (TEEs)

Determining the true rates of TEEs and hemolytic incidents are difficult because the FDA and manufacturers’ pharmacovigilance efforts rely on voluntary efforts of patients/providers and because there are little data with which to calculate a denominator such as the numbers of doses given or patients treated. Hyperviscosity due to high-dose IVIG and slow blood flow in critical vascular beds may contribute to TEEs in some patients; and endothelial cell and/or platelet activation also likely play a role. There are multiple reports of myocardial infarction, transient cerebral ischemic attacks, and strokes related to high-dose IVIG therapy.<sup>56</sup> Best estimates suggest a baseline incidence of 0.16–0.6 TEEs for every million grams of IVIG sold.<sup>56,58,59</sup> Considering 50 g as a median adult dose, these rates represent roughly 0.8–3 cases per 100,000 doses. In a particularly well-investigated episode, TEEs were reported in nine patients involving seven different lots of a single product.<sup>58</sup>

Subtle changes in the procedure used to fractionate the plasma and produce that product, including the use of affinity resins to isolate certain clotting system proteins, apparently increased contact activation of factor XI, which had copurified with the IgG during the initial ethanol precipitation.<sup>55</sup> Because the total factor XI plus XIa was still within acceptable limits by the assays in use at that time, the affected product was within specifications. Only newer thrombin-generation assays have sufficient sensitivity to detect activated FXIa. Factor XI and kallikrein are difficult to separate from IgG because their isoelectric points are similar to IgG’s, and they coprecipitate during ethanol precipitation.<sup>10</sup> On the one hand, the results suggest that contamination of one or two individual lots was not responsible for the increase in factor XIa because many lots were affected. On the other hand, even with the affected lots, TEEs were extremely rare, suggesting that multiple risk factors in the individual affected patients also contributed.

Better chromatographic purification methods, and the use of specific immunoadsorbents to remove FXI/FXIa from products that are made by multiple precipitations without ion-exchange chromatography, should decrease the risk of factor-XIa-related AEs. Furthermore, the use of thrombin-generation assays should ensure the absence of procoagulant activity in current and future IgG products.

### Hemolytic reactions

An analogous problem is the presence of antibodies to erythrocytes (isoagglutinins), resulting in positive Coombs’ tests, occasional cases of clinically significant hemolytic anemia, and extremely rare

episodes of acute severe intravascular hemolysis.<sup>59,60</sup> In the original Cohn-Oncley fractionation scheme, isoagglutinins, which have higher isoelectric points than other IgGs, were greatly reduced by removing fraction III and continuing with fraction II alone.<sup>61</sup> Several current production schemes combine the fraction II and III precipitates as the starting material for IgG purification, and many manufacturers have substituted caprylic acid or fatty alcohols for the ethanol steps that removed more of the isoagglutinins, resulting in 2–4-fold increases in isoagglutinin titers in the final products.

As with TEEs, true rates of Coombs' positivity, hemolytic anemia, and acute severe hemolysis in relation to IVIG therapy are difficult to estimate. However, risk factors for clinically significant hemolytic episodes include non-O blood group, underlying associated inflammatory states, and high cumulative doses of IVIG over several days. Some studies of high-dose IVIG therapy report that as many as 20% of patients may convert to Coombs' positivity shortly after infusions, but the incidence of clinically significant anemia or acute severe hemolysis is significantly lower than that.<sup>36,55,56</sup> Analysis of US and Canadian series reported clinically significant hemolysis after IVIG in a combined total of 37 patients, including 23 patients with blood type A, 9 with type B, 4 with type AB, and only 1 with type O.<sup>59,60</sup>

Prescreening of donors to avoid using plasma units with high isoagglutinin titers in the pools from which the IVIG is prepared,<sup>62</sup> and/or the use of specific immunoabsorbents<sup>63</sup> to lower the titers of Anti-A and Anti-B, is used by some manufacturers to decrease this problem.

### Mechanisms of action of IVIG

IgG has both Fc-dependent and Fc-independent mechanisms that contribute to defense against infection<sup>64–66</sup> (Table 31.1). Fc-independent functions include neutralization of toxins, which are important virulence factors in several types of bacterial infection, agglutination and/or precipitation of infectious organisms, and neutralization of adhesion and attachment molecules. Similarly, neutralization of superantigens or toxins may explain the effects of IVIG in Kawasaki Disease and toxic shock syndromes. Fc-dependent functions include activating complement, facilitating phagocytosis (both locally at sites of tissue invasion and in bloodstream clearance by macrophages in the reticuloendothelial organs), and enhancing direct cell-mediated cytotoxicity. In general, all of these mechanisms involve stoichiometric interactions between the IgG and antigens and/or effector molecules such as C1q and Fcγ receptors. Therefore, it is not surprising that most studies comparing the efficacy of different doses of IgG replacement therapy in immune-deficient patients show fewer infections in groups that received higher doses. Pooled data from licensing studies of IVIG and SCIG clearly show that the incidence of pneumonia and other infections decreases as serum IgG levels increase.<sup>43,44</sup>

A broad array of mechanisms of action of IVIG of potential relevance to the use of high-dose therapy have been demonstrated in vitro and in animal models.<sup>64–66</sup> However, it is rarely clear which effects are most important in any given disease *in vivo*, partly because the pathogeneses of most autoimmune or inflammatory diseases are incompletely understood. Therefore, it is difficult to know whether any given effect demonstrated in vitro or in a model is really relevant in the diseased patient *per se*. One useful way to categorize mechanisms of action in these diseases is by considering whether they are likely to involve direct competition between normal molecules in therapeutic IgG and pathogenic autoantibodies.<sup>66</sup>

Clues suggesting that competitive effects are likely include strong dependency on the dose of therapeutic IgG or the amount of therapeutic IgG in the circulation at any point in time, “wearing off” of the IVIG effect as its concentration wanes, and efficacy of plasmapheresis or immunoadsorption as an effective alternative way of removing pathologic antibodies. Several mechanisms of this type are detailed below. In contrast, true “immunomodulatory effects” on the underlying disease process may be indirect, with more complex pharmacodynamics and dose-response characteristics. The effect of IVIG on the autoantibody concentration without modifying the underlying autoimmune diathesis is illustrated by a study in myasthenia gravis patients which showed that high-dose IVIG temporarily reduced the titer and ameliorated the effects of antiacetyl choline receptor antibodies but did not decrease their production and did not exert a “disease modifying effect” on the underlying immunopathogenesis.<sup>67</sup>

### Anti-idiotypic binding

Because each individual's immune repertoire arises randomly, it is readily understandable that some individuals respond to a given pathogen with antibodies that crossreact with self-antigens (e.g., *Campylobacter jejuni* and myelin gangliosides in Guillain–Barré syndrome [GBS]), whereas other individuals recognize different epitopes. Furthermore, it is easy to speculate that individuals who mount rigorous anti-id responses will rapidly bring a self-reactive response under control, whereas those whose anti-id response is weak or ineffective may continue to produce clinically significant amounts of autoantibodies.<sup>68</sup> Because IVIG contains the antibodies from tens of thousands of healthy donors, it follows that it likely contains many different anti-ids.

Shortly after its introduction, IVIG was found to neutralize “inhibitors” of the clotting protein factor VIII in hemophilia patients receiving replacement therapy.<sup>69</sup> These “inhibitors” are in fact antibodies against factor VIII (FVIII). Furthermore, F(ab') fragments of the IVIG neutralized the inhibitors, suggesting anti-id binding. Subsequent studies have shown that IVIG contains a wide variety of anti-ids,<sup>66</sup> consistent with Jerne's network theory of anti-id suppression of potential autoimmunity,<sup>8</sup> and suggest that this is actually quite common in normal physiology. The most important support for an anti-id mechanism of IVIG *in vivo* would be the observations that F(ab') or F(ab')<sub>2</sub> fragments from IVIG neutralize the autoantibody *in vitro* and/or can remove it by affinity chromatography, that the removal of the autoantibodies by plasmapheresis has a similar effect, and that autoantibodies recovered from patients reproduce the disease physiology *in vitro* or in animals and can be blocked by the therapeutic IgG preparation.

### FcRn saturation increases catabolism of endogenous antibodies

As noted above, FcRn on endothelial cells maintains the relatively high normal concentration and long half-life of IgG in the circulation.<sup>22–24,70</sup> In FcRn knockout mice and in patients with FcRn mutations, the half-life of IgG is very short, and its plasma concentration is quite low.<sup>71,72</sup> Furthermore, it is difficult to passively transfer IgG-mediated pathology into FcRn knockout mice because the half-life of the pathologic IgG is so short.<sup>72</sup> By analogy, if FcRn is saturated by high doses of *exogenous* IVIG, the catabolism of *endogenous* pathologic IgG is greatly increased. In wild-type mice, high-dose IgG dramatically increases the catabolism of pathogenic IgG, whereas, in FcRn knockout mice, high-dose IgG does not further enhance the already rapid catabolism of the exogenous pathologic IgG.<sup>24,72</sup>

Thus, by saturating FcRn with normal antibodies, IVIG increases the degradation of pathogenic IgG. This therapeutic effect of IVIG therefore requires that the total IgG concentration exceeds the binding capacity of FcRn and depends on the ratio of the serum concentration of normal IgG to the concentration of pathogenic autoantibodies. Targeting FcRn with engineered Fc fragments of IgG, monoclonal antibodies against its binding site, and other biologicals has been successful in clinical trials in myasthenia gravis, ITP, and other IgG-mediated autoimmune diseases.<sup>73,74</sup> Continued development of these agents should further define the role of FcRn in the mechanism of action of IgG therapy in autoimmune/inflammatory diseases and may actually reduce the use of plasma-derived IgG for this purpose.

### Complement scavenging

Complement components C4 and C3 contain a unique internal thioester bond that can transfer to the target during complement activation, forming a covalent bond.<sup>75</sup> Covalent binding of C4b and/or C3b increases the stability of convertases and the likelihood that the initial activation will be amplified. The C<sub>H</sub>1 domain in IgG has particularly good acceptors for this reaction, and high concentrations of soluble IgG can compete with surface-bound IgG for newly activated C3b. Inhibition of complement deposition by IVIG at concentrations readily achieved during high-dose therapy has been demonstrated in vitro and in animal models, and in humans being treated for dermatomyositis.<sup>76</sup> Clinical improvement following IVIG treatment in that condition is accompanied by decreased activation of C3 and decreased deposition of C3b and C5–9 on endomysial capillaries.<sup>76</sup> Basta *et al.* also showed that IgG could bind C3a and C5a noncovalently, thereby diminishing proinflammatory effects of complement activation.<sup>77</sup>

### Indirect actions of IVIG that do not involve competition per se

Many reviews of “the” mechanism of action of IVIG focus on laboratory data showing putative immunomodulatory effects involving networks of T cells, B cells, and cytokines.<sup>78–80</sup> However, consensus reports on uses of IgG suggest efficacy mainly in antibody-mediated diseases rather than in T-cell-mediated conditions like atopic dermatitis, graft vs. host disease, or cell-mediated allograft rejection.<sup>1</sup> Furthermore, many of these “immunomodulatory” effects of IVIG have prolonged time courses and are therefore not likely to be responsible for therapeutic effects that “wear off” before a dose of IVIG has reached its half-life.<sup>64–66,78</sup> Analysis of the pharmacodynamics of IgG therapy in any given disease may thus shed light on the mechanism by which the therapeutic IgG is acting and also on the immunopathogenesis of the disease.<sup>66</sup>

IVIG can interfere with maturation of dendritic cells in vitro and can inhibit expression of HLA-antigen complexes and the costimulatory molecules CD80 and CD86.<sup>64,79,80</sup> It certainly seems possible that decreasing or altering dendritic cell activity would decrease antigen presentation and alter the pattern of cytokine production, modulating the stimulation of different T-cell activities by the dendritic cells. IVIG can decrease the production of proinflammatory cytokines such as interleukin-1 (IL1), IL12, and interferon- $\gamma$ , and increase the production of regulatory molecules such as IL10 and IL1 RA.<sup>64,81</sup> In this way, IVIG could alter the balance between regulatory (CD25+) and effector (CD4+ or CD8+) T cells<sup>81</sup> and could also decrease the inflammatory activity of macrophages. However, when the pathology involves direct effects of antibodies and/or complement on target tissues, it is not clear that modulating T-cells

or macrophages results in significant beneficial effects. One report showed that IVIG decreased a B-cell-activating cytokine that was elevated in sera from chronic idiopathic demyelinating polyneuropathy (CIDP) patients, but the clinical correlation was poor, the time course of changes in cytokine levels was not reported, and the role of that cytokine (and autoantibodies, for that matter) in CIDP was not clear.<sup>82</sup> Reports that IVIG decreases the number of circulating “natural killer” (NK) cells; their expression of the low affinity Fc receptor (CD16) and their cytotoxic activity more likely represent in vitro blockade of CD16 than a genuine physiologic downregulation;<sup>83</sup> and the role(s) played by NK or other cells that can mediate antibody-dependent cellular cytotoxicity has not been established in the diverse autoimmune diseases for which IVIG is used.

Putative effects of IVIG in ameliorating autoimmune disease by modulating programmed cell death have been proposed, but remain controversial. Some studies suggest that anti-Fas antibodies in IVIG induce apoptosis in B cells.<sup>84</sup> However, other results suggesting that IVIG has limited or only transient effects on autoantibody production (or alloantibody, in the case of transplant rejection) would not be consistent with induction of apoptosis as a major mechanism by which IVIG acts in autoimmune disease. Because the level of Fas expression and the sensitivity of cells to proapoptotic signals vary with the degree of activation and physiologic state of the cells in question, it is not difficult to see how different results could be obtained in different experimental systems. Further compounding the issues are observations suggesting not only that IVIG preparations may contain antibodies against Fas and another important family of proteins that regulate cell death called *Siglecs*, but also that dimers in IVIG preparations may contain anti-idiotypic antibodies that can complex with and neutralize these autoantibodies.<sup>85</sup> Thus, a number of factors such as low-pH treatment during preparation and storage (which tends to dissociate dimers), stabilizers, and the percentage of dimers in any IgG preparation may also influence the results of laboratory studies, and perhaps the results with different preparations *in vivo*.

Research in animal models has focused on increased expression of the inhibitory receptor FcRIIB as a mechanism of action of a small subset of molecules in IVIG that have fully sialylated carbohydrate side chains.<sup>86,87</sup> These heavily sialylated IgG molecules putatively bind to a distinct receptor termed DC-SIGN, inducing the cytokine IL32, which increases FcRIIB expression and inhibits the activity of inflammatory macrophages.<sup>86</sup> This mechanism could potentially explain why high doses of IVIG are necessary for anti-inflammatory effects because only a small percentage of the IgG molecules are fully sialylated. Although a mouse model showed that merely 33 mg/kg of a highly sialylated Fc analog could replicate the effects of 1 g/kg of standard IVIG,<sup>87</sup> these effects appear to depend on the mouse strain and model used.<sup>88,89</sup> Thus, their relevance to therapy of human diseases is not clear.

### Dosing and scheduling IgG treatment regimens

#### Primary immune deficiencies

Antibody replacement therapy in immune deficiencies is typically initiated at doses of 400–600 mg/kg of IVIG every 3–4 weeks or 100–200 mg/kg/week subcutaneously. Doses in this range usually yield trough (in the case of IVIG) or steady-state (in the case of SCIG) serum IgG levels toward the low end of the normal range and

are quite effective in preventing sepsis or other serious bacterial infections.<sup>43,44</sup> However, based on results of clinical observations and a few controlled studies,<sup>90,91</sup> patients with chronic lung and/or sinus disease, and those suffering frequent breakthrough infections, are usually given higher doses. Several lines of evidence suggest that the resistance to acute infections is directly related to the serum (and tissue) IgG level at any point in time.<sup>43,44</sup> Analyses of large pooled data sets suggest that patients receiving IVIG every four weeks are more likely to experience infections and hospitalizations in the last week of their dosing cycle,<sup>92,93</sup> before their next dose is due. In a survey performed by the US Immune Deficiency Foundation before the use of SCIG was common, two-thirds of respondents indicated that they could feel their IVIG “wearing off” before the next dose was due.<sup>94</sup> Smoothing out of the “peaks” and “troughs” of every 3–4 weekly IVIG regimens is likely one reason why many patients prefer subcutaneous regimens in which frequent dosing achieves steady-state IgG levels. Combined studies of 88 Italian patients showed that the steady-state serum IgG level was proportional to the total monthly dose of SCIG per kg body weight regardless of the BMI.<sup>95</sup> A small case series illustrated that different patients require different “biologic IgG trough levels” to remain infection free,<sup>96</sup> and results from a cohort of 90 patients with primary antibody deficiency followed at a single center in the UK for over 20 years showed that the range of IgG doses needed to keep individual patients free from breakthrough infections varied widely: from 0.2 to 1.2 g/kg/month, resulting in trough serum IgG levels varying from 500 to 1700 mg/dL (5–17 g/L).<sup>97</sup> Thus, although guidelines can be used to recommend starting doses in previously untreated patients, dose, dosing interval, and route of administration should be individualized based on the clinical results for each patient.<sup>37,96,97</sup>

### **Secondary immune deficiencies (SID)**

A growing armamentarium of immunotherapies and other agents, such as kinase inhibitors, proteosome inhibitors, and antimetabolites that target B-cells, plasma cells, and malignancies derived from these lineages, is being used not only for hematologic malignancies but also for autoimmune diseases and antibody-mediated transplant rejection.<sup>98–100</sup> In addition to rituximab (anti-CD-20), newer antibodies against CD-20, and antibodies against CD-19, CD-22, B-cell maturation antigen, and CD-38, have completed late-stage clinical trials and/or have recently been approved. These antibodies, as well as alemtuzumab and some antilymphocyte globulins, and anti-CD19 chimeric antigen receptor (CAR-) T-cells deplete B-cells and/or plasma cells and may lead directly to secondary antibody deficiency (SAD) and/or frank hypogammaglobulinemia.<sup>101,102</sup> Other agents, such as cytokine and costimulation blockers, antimetabolites (especially fludarabine), and inhibitors of Bruton’s tyrosine kinase (the enzyme whose mutations cause X-linked agammaglobulinemia), other intracellular signaling inhibitors, and proteosome inhibitors such as bortezomib, alone or in combination, may also contribute to clinically significant antibody deficiency.<sup>99–101</sup> With increased efficacy of targeted therapies, and advances in supportive care, the survival of patients with hematologic malignancies and those with solid organ transplants is improving. Infection is a major cause of morbidity and mortality in these patients, and SIDs are likely major contributors.

Because of the heterogeneity of underlying conditions and complications of therapy for the primary diseases, there have been few large randomized controlled trials of IgG replacement in patients

with SID. Nevertheless, reviews in the United States,<sup>101,102</sup> a consensus study in the EU,<sup>103</sup> and the EMA core SmPC for IVIG<sup>2</sup> all suggest similar algorithms and criteria for considering IgG replacement in these patients for SID: 1). occurrence of severe or recurrent infections, especially if unresponsive to antimicrobial therapy, and 2). failure to mount at least a twofold increase in specific antibody titer in response to immunization, or frank hypogammaglobulinemia (serum IgG < 400 mg/dL [4 g/L]).

As with primary immune deficiencies, starting doses should be in the range of 400–600 mg/kg every 3–4 weeks for IVIG and 100–200 mg/kg/week for SCIG. Monitoring of the patient’s IgG infection status and IgG levels should actually begin at the time of diagnosis of the primary disease since many patients with chronic lymphocytic leukemia, other B-lineage malignancies, and/or autoimmune disease may actually have hypogammaglobulinemia or antibody deficiency even before beginning treatment. IgG dose adjustments and duration of therapy should be based on the individual patient’s clinical response.

### **Acute autoimmune or inflammatory diseases**

In contrast to immune deficiencies, in which the frequency of infection can be used to monitor and individualize IgG treatment, there are few such metrics available for guiding therapy in most acute inflammatory or autoimmune diseases treated with IVIG, so the regimen of 2 g of IVIG per kg is usually used. The core SmPC for IVIG in the EU includes class labeling for ITP, Guillain–Barré syndrome (GBS), multifocal motor neuropathy (MMN), chronic inflammatory demyelinating polyradiculoneuropathy (CIDP), secondary immunodeficiencies, and Kawasaki disease.<sup>2</sup> In contrast, GBS, and secondary immune deficiencies are not FDA-approved indications for IVIg in the United States, even though IVIG is considered “standard of care” for these conditions. In the United States, labeling for CIDP and MMN has been granted only to those individual products for which efficacy has been demonstrated. One brand each is labelled for Kawasaki disease and Dermatomyositis in adults.

*Kawasaki disease* is an acute, severe, and potentially fatal inflammatory vasculitis syndrome of uncertain etiology that is most common in young children.<sup>104</sup> IVIG is the major treatment used, and a meta-analysis of studies in which different doses of IVIG were coadministered with aspirin showed a correlation between the dose of IVIG and its efficacy in preventing coronary artery aneurysms, which occurred in only 3.8% of patients who received the highest dose studied: 2 g/kg.<sup>105</sup> Furthermore, a randomized trial in 549 subjects<sup>106</sup> and the meta-analysis cited above both reported that administering the IVIG as a single infusion over 8–12 hours led to more rapid resolution of symptoms and was more effective than giving it as five consecutive daily doses of 400 mg each. Thus, the commonly used first-line treatment for Kawasaki disease is 2 g/kg of IVIG given as a single large bolus over 8–12 hours. The observation that more rapid administration of the large infusion increases its efficacy suggests that the peak IgG level achieved—rather than the total dose, AUC, or trough level—may be the most important determinant of efficacy in this particular situation. This conclusion, in turn, is consistent with the hypothesis that the target of IVIG in Kawasaki syndrome is intravascular per se, perhaps blocking the effects of a superantigen or toxin on endothelial cells and/or circulating leukocytes.

*Guillain–Barré syndrome* (GBS) is an acute autoimmune polyneuropathy causing flaccid paralysis that frequently follows resolution of an infectious disease, most notably gastroenteritis due to *C. jejuni*.<sup>107</sup> The leading hypothesis for the pathogenesis of GBS is

that antibodies produced by the host's immune system in response to the lipo-oligosaccharide of the infectious agent crossreact with ganglioside antigens on the patient's myelin, activating complement and causing dysfunction and demyelination of peripheral nerves.<sup>107,108</sup> As such, GBS is frequently cited as a leading example of the *molecular mimicry* theory of autoimmune disease. Although GBS is generally considered an acute, monophasic disease, as many as 5% of patients succumb during the acute episode, and up to 20% may have slow recovery and/or prolonged disability.<sup>107-110</sup> First-line treatments for GBS include high-dose IVIG and/or plasma exchange. Laboratory evidence supports hypotheses that IVIG acts predominantly by blocking the binding of autoantibodies to the nerves, presumably due to the presence of anti-idiotypes, and/or by blocking complement deposition.<sup>66,109,111,112</sup> It is likely that plasmapheresis also acts primarily by removing autoantibodies. However, different antibodies targeting different epitopes may be involved in different patients and different clinical variants, and neither measurements of autoantibodies nor in vitro determinations of the ability of IVIG to neutralize them are used to select or adjust the dose or schedule of IVIG treatment. Practice parameters of the American Academy of Neurology consider plasma exchange and IVIG equally effective first-line treatments.<sup>113</sup> A *Cochrane Review* reached the same conclusion but noted that the full course of IVIG treatments was more likely to be completed than the series of plasma exchanges, and that there was little additional benefit of combining the two modalities.<sup>114</sup> The most common dose of IVIG is 2 g/kg, usually given over 2–5 days. One study of 39 subjects suggested that six days of 0.4 g/kg (total dose = 2.4 g/kg) was preferable to three days of 0.4 g/kg (total dose = 1.2 g/kg) in terms of the number of days until subjects could walk with assistance, but the difference did not reach significance except in a smaller subgroup.<sup>115</sup> Although serum IgG levels achieved by patients receiving 0.4 g/kg/day for five days were quite variable, patients with the highest increments in IgG level from baseline to day 14 showed the best response in terms of the percentage able to walk unaided six months after the acute episode and vice versa.<sup>116</sup>

These results suggest that caution should be taken when the maximum dose of IVIG is limited to a preset total number of grams, or in which dosing is based on lean or ideal body mass rather than actual body mass. Such rules may result in underdosing some GBS patients, and the efficacy of lower doses in this acute situation has not been established. The International GBS Outcomes Study (IGOS) is being carried out to determine how to identify patients at high risk of death or long-term disability and who might benefit from more rigorous treatment.

*Immune thrombocytopenic purpura* (ITP), as noted in this chapter, was the first condition in which beneficial effects of IVIG in an autoimmune disease were reported (in ITP, autoantibodies mediate destruction of the patient's own platelets). ITP patients may suffer or be at risk for clinically important bleeding episodes, including acute severe blood loss and intracerebral hemorrhage. Corticosteroid therapy is frequently sufficient to increase the platelet count enough to control symptoms such as nosebleeds and menorrhagia. However, when more severe and/or acute bleeding requires a faster increase in the platelet count, IVIG is often used at a dose of 1–2 g/kg given over several consecutive days.<sup>117,118</sup> This increases the platelet count to >50,000 within seven days of beginning a course of therapy in >80% of patients. IVIG may also be used preoperatively to raise the platelet count and decrease the risk of bleeding in ITP patients who require urgent surgery. In patients with Rh<sup>+</sup> red cells, anti-Rh(D) immune globulin (Rhophylac<sup>®</sup> and WinRho<sup>®</sup>SDF) may be used as

an alternative to IVIG, obviating many of the potential adverse effects of high-dose IVIG.<sup>119</sup> The Rh(D) immune globulin is believed to act by creating immune complexes with the recipient's red cells, which then saturate phagocytic receptors in the reticuloendothelial organs, sparing the platelets. This, in turn, is associated with some destruction of the antibody-coated red cells, but the resulting hemolysis is rarely clinically significant. IVIG is repeated at monthly intervals in some patients with chronic ITP, but many such individuals are managed with corticosteroids, splenectomy, rituximab, and/or thrombopoietin receptor agonists.

IVIG may be expected to have effects in autoimmune neutropenia, autoimmune hemolytic anemia, and alloimmune thrombocytopenia (sensitization of baby's platelets with maternal IgG transferred across the placenta) similar to those in ITP, but these disorders are less common than ITP. Furthermore, if IVIG is used, it would frequently be given together with corticosteroids, with which it should have synergistic effects. High-dose IVIG is used as a treatment for pure red cell aplasia secondary to parvovirus B19 infection.<sup>120</sup> In that situation, the IVIG likely helps the host control the infection.

### Chronic autoimmune or inflammatory diseases

*Chronic inflammatory demyelinating polyneuropathy*: In many ways, CIDP resembles a chronic form of GBS.<sup>121,122</sup> CIDP differs in that few patients recall preceding infections or other triggering events. CIDP is considered by many to be an autoantibody-mediated disease, and plasma exchange is very effective. However, unlike the putative role of antiganglioside antibodies in GBS, no single major target antigen(s) has been identified in CIDP.<sup>66,120-122</sup> Based upon results of a randomized, placebo-controlled trial of IVIG in 117 CIDP patients (the ICE study), the FDA approved IVIG for CIDP using a loading dose of 2 g/kg followed by maintenance dosing of 1 g/kg every three weeks.<sup>123</sup> In many CIDP patients, the effects of IVIG are transient, suggesting a mechanism of action involving competition with autoantibodies rather than inhibition of their production. End of dosing-interval "wear-off" effects are common with every 3–4 week IV treatment regimens,<sup>66</sup> and doses and intervals other than those used in the ICE trial are prevalent.<sup>124-126</sup> A prospective study reported that 60% of CIDP patients required IVIG more often than once every two weeks to maintain optimal strength.<sup>126</sup> A large, randomized, controlled multicenter study showed that SCIG was also effective in CIDP at doses of 0.2 g/kg/week and 0.4 g/kg/week,<sup>127</sup> and this efficacy was confirmed in a 48-week open-label follow-up study.<sup>128</sup> Hizentra<sup>®</sup>, the 20% SCIG which was used in that study, is labeled for use in CIDP in the United States. Anecdotal reports and small series suggest that continuously maintaining high serum IgG levels by the use of SCIG may result in more stable maintenance of strength, and guidelines and study results suggest that long-term dosing should be individualized for each patient.<sup>125,129</sup>

*Multifocal motor neuropathy (MMN)*: MMN is characterized by multiple motor nerve conduction blocks with sparing of sensory nerves.<sup>130,131</sup> The electrophysiology is consistent with segmental demyelination, but several studies suggest immunologic target(s) on axons rather than, or in addition to, Schwann cells or myelin. Unlike GBS and CIDP, corticosteroids and plasma exchange are usually not effective in MMN,<sup>132,133</sup> raising questions about its immunopathogenesis. About half of MMN patients have IgM antibodies against the ganglioside GM1, and these seropositive patients tend to have more severe weakness, disability, and eventual axon loss than seronegative patients.<sup>134</sup> Efficacy of IVIG was demonstrated

in multiple anecdotal and case series reports in the mid-1990s. Small controlled studies soon followed, and the results of a 44-subject, randomized, double-blinded, placebo-controlled crossover trial were reported by Hahn *et al.* in 2013, and led to labeling of Gammagard® in the United States for improving muscle strength and decreasing disability.<sup>135</sup> The mean maximal grip strength declined 31% during placebo treatment and increased 3.75% during IVIG treatment ( $p = 0.005$ ).<sup>121</sup> IVIG has also been recommended as first-line treatment by a European Federation of Neurological Sciences/Peripheral Nerve Society task force,<sup>136,137</sup> and it is usually given in doses of 1–2 g/kg every 3–4 weeks. Interestingly, even early reports noted that improvement in strength and conduction began within a few days after the IVIG, but lasted only 1–2 months, at best.<sup>138–140</sup> As with CIDP, many patients complain that their strength deteriorates in the third or fourth week after an IVIG dose. Small case series suggest that SCIG may help to ameliorate these “wear-off effects” and promote more constant activity, but long-term follow-up studies of SCIG are needed to determine if this will prevent the eventual deterioration that now characterizes most MMN patients.

**Autoimmune mucocutaneous bullous (blistering) diseases:** Autoimmune mucocutaneous bullous (blistering) diseases are a family of conditions including multiple subtypes of pemphigus and pemphigoid in which the separation of intraepidermal or subepidermal layers of skin occurs because of antibodies against intercellular adhesion molecules such as desmoglein.<sup>141</sup> Most of these disorders have relapsing-remitting courses, and subtypes are characterized by differences in the locations and depth of the blisters, which in turn are probably related to differences in the specific targets of the autoantibodies. In general, corticosteroids are first-line therapies, and most patients are also given cytotoxic drugs such as cyclophosphamide or azathioprine. “High-dose” IVIG is often added as an alternative or addition to steroids in patients who develop complications of steroid treatment and is regarded as a “steroid-sparing” therapy.<sup>141–143</sup> IVIG may also be preferentially used in patients with poor tolerance of cytotoxic agents. A major effect of IVIG seems to be reduction of autoantibody titer, although this has been reported to take several months,<sup>142</sup> so it is not clear whether the mechanism is competitive or mediated by other pathways. In cases of severe mucosal and/or ocular involvement, high-dose IVIG may be used to achieve a more rapid remission of acute attacks or exacerbations.

### IgG in transplantation

IgG therapy has three main uses in solid organ transplantation. High-dose IVIG is employed along with plasmapheresis and rituximab in regimens designed to “desensitize” or remove preformed anti-HLA antibodies (“panel reactive antibodies” [PRA]) before transplantation in patients who have developed these antibodies in response to exposure to alloantigens in a previous transplant and/or in the fetuses of multiparous women.<sup>144</sup> In this situation, the IVIG is likely acting by anti-idiotype blocking, exchanging with endogenous IgG in the extravascular spaces, so the latter can be removed by plasmapheresis, and/or saturating FcRn to increase the catabolism of the endogenous anti-HLA antibodies. Particularly with kidney transplant patients, caution must be taken in selecting the IVIG product and scheduling the infusions to avoid adverse effects due to isoagglutinins or sucrose. The newer IVIG preparations with non-sugar stabilizers and those with reduced isoagglutinin content should ameliorate these concerns. The second major use also involves high-dose IVIG, which is used to treat acute antibody-

mediated rejection.<sup>145</sup> In this situation, part of the effect of the IVIG is undoubtedly to neutralize and/or accelerate the catabolism of the antibodies against the graft that are causing the rejection. However, immunomodulatory and inflammatory activities, including inhibiting complement, blocking Fc receptors, and altering the cytokine milieu, are also likely important. Many recipients of solid organ transplants become at least transiently hypogammaglobulinemic as a result of their immunosuppressive regimen, because of GI losses in visceral transplants<sup>146</sup> and/or because of preexisting hypogammaglobulinemia or specific antibody deficiency that may have been undiagnosed before the transplant (particularly in lung transplant recipients).<sup>147</sup> Such patients may benefit from antibody replacement, which may be given by the IV or subcutaneous routes, using regimens like those used for patients with primary antibody deficiency. Formerly, cytomegalovirus (CMV) hyperimmune globulin was used prophylactically after transplants, especially in antibody-negative recipients of organs from CMV+ donors (so-called D+/R– transplants). This has become much less common in the current era in which antiviral chemotherapy with gancyclovir and valgancyclovir is available, but it may still be used in patients who do not tolerate those agents and/or who develop active CMV disease.

In recipients of hematopoietic stem cell transplantations (HSCTs) for severe combined immune deficiency (SCID), particularly the X-linked form due to mutations in the cytokine receptor common  $\gamma$  chain, B-cell reconstitution frequently occurs much slower than hematopoietic and T-cell reconstitution.<sup>148</sup> Most SCID patients are given IgG replacement for at least a year after transplantation, and some require it for life.

Based on the results of a large, randomized, double-blind, placebo controlled study in France,<sup>149</sup> an independent meta-analysis in Canada,<sup>150</sup> and a *Cochrane Database Review*,<sup>151</sup> IVIG is not recommended for routine prophylaxis of infection after allogeneic HSCT, and has not been shown to be effective for prophylaxis or the treatment of graft vs. host disease.<sup>101</sup> However, in some malignant lymphoproliferative diseases, especially when rituximab is used in combination with conventional chemotherapy (e.g., cyclophosphamide, hydroxydaunorubicin, oncovin, and prednisone or prednisolone [CHOP]) or fludarabine, and in some cases in which rituximab is used (with or without cytotoxic agents) for nonmalignant antibody-mediated disorders, prolonged depletion of circulating B cells and significant hypogammaglobulinemia may occur, resulting in severe and/or recurrent infections. This is also likely in patients receiving CAR-T cells targeting CD19 or other B-cell lineage antigens. Such patients should be evaluated for IgG and/or specific antibody deficiency, and IgG replacement therapy using regimens similar to those used for primary immune deficiency should be considered.<sup>101,103,151,152</sup>

### Hyperimmune globulins

Hyperimmune globulins are made from plasma obtained from normal subjects with high titers of antibodies to the desired microbe or antigen detected by screening or because they are convalescing or have been repeatedly immunized.<sup>142,143</sup> Plasma is processed as for regular (IM) ISG or IVIG, and the final product is tested to ensure an adequate antibody titer to the microbe or other antigen. In general, hyperimmunes contain about fivefold higher titers of specific antibodies to the microbe or antigen for which they are labeled than standard IVIG preparations (on the basis of a unit of specific antibody per gram of IgG).<sup>153,154</sup> Thus, to get the same amount of specific antibody, fivefold higher doses of standard IVIG would have to

be given, constituting an extremely high dose of the latter. For example, to get the recommended amount of specific IgG for protection against respiratory syncytial virus required 750 mg/kg of the hyperimmune RespiGam® but would require 3750 mg/kg of “standard” IVIG. To put this in a current perspective, the same amount of specific IgG is contained in 15 mg/kg of the monoclonal anti-RSV antibody palivizumab (Synagis®), which can be given as a simple IM injection.<sup>143</sup> Other hyperimmunes target the Rh(D) red cell antigen (RhoGAM®, Rhophylac®, etc.) to prevent sensitization of Rh(D)-negative women to Rh(D)+ fetal erythrocytes and also for ITP in Rh(D)+ individuals. Hyperimmune globulins are also available for CMV (CytoGam® and Cytotect®), botulism toxins (Human Botulism Immune Globulin-IV® and Baby-BIG®), hepatitis B virus (BayHep B® and HyperHepB S/D®), rabies virus (HyperRAB® and Imogam-Rabies HT), tetanus (HyperTet-SD®), and varicella-zoster virus (VariZIG®). The same adverse effects may occur with IV hyperimmunes as with standard IgG preparations. However, the doses of IV hyperimmunes are generally lower than the high doses of IVIG used for immunomodulatory effects, and so are less likely to produce serious adverse effects unless they are administered too rapidly. Antivenoms used for spider and snake bites<sup>155</sup> and antithymocyte globulin used for immunosuppression (i.e., in transplant recipients) are usually animal antisera and may be associated with anaphylaxis and/or serum sickness.

## Summary

Given the diversity of the immune repertoire and the multiple interactions of its Fc domains with receptors and other effector systems, it is not really surprising that polyclonal IgG, pooled from tens of thousands of individuals, has a broad multiplicity of uses. Modern preparations are highly purified and contain stabilizers designed to prevent aggregate formation, and thus can be given intravenously or subcutaneously with relative freedom from

severe systemic adverse reactions. Caution and monitoring are still necessary as occasional serious and/or life-threatening AEs still occur, especially in individuals with identifiable risk factors. Doses in the range of 600–1000 mg/kg/month are generally used for replacement therapy for immune-deficient patients. This may be given in IV boluses every 3–4 weeks, or fractionated into smaller increments given by the subcutaneous route weekly or even more often. Although the former require venous access and are frequently monitored by trained medical personnel, the latter are frequently self-administered in the home, greatly facilitating the convenience of long-term IgG therapy and the quality of life for patients and their families. High-dose IVIG therapy (1–2 g/kg, usually every 2–4 weeks) is used for a growing number of autoimmune/inflammatory diseases, generally in clinical settings, and accounts for a continuously increasing global demand for IgG products.

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## **SECTION V**

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# **Apheresis**

## CHAPTER 24

# Basic principles of apheresis and the collection of blood components by apheresis

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### Terminology and definitions

Apheresis is derived from a Greek word that means *separate* or *remove*, and it was first used in 1914 by Able, Rowntree, and Turner to describe a procedure where blood was withdrawn from dogs and separated into cells and plasma, with the plasma being discarded and the cells returned with a replacement fluid.<sup>1</sup> Apheresis procedures can be divided into two categories, *cytapheresis*, where the cellular elements are removed, and *plasmapheresis*, where plasma is removed.

### Basic concepts common to all apheresis procedures

#### Blood component separation

Separation of blood components is performed by centrifugation, filtration, or a combination.<sup>2,3</sup> Centrifugation uses centrifugal force and blood-component-specific gravity to separate the elements in the blood. The elements are then removed by siphoning off the desired component or spilling the lighter components over a barrier that retains the heavier components. In some systems, elutriation is also used. Two opposing forces—centrifugal force that pushes heavier elements away from the centrifuge center and pump withdrawal force that pulls the elements to the centrifuge center—separate based on size rather than specific gravity. Effective separation by centrifugation is dependent on the centrifuge *g*-force, determined by the rotations per minute and rotor radius, and the time that blood is exposed to the centrifugal force.<sup>3</sup> Filtration separates elements based upon size.<sup>3</sup> The filter can be a bundle of hollow fibers with pores in their walls or a membrane surface with pores.<sup>3</sup> The pore size determines what passes through the filter and what is retained. Cascade or double-membrane filtration systems can remove substances from the plasma by using two filters, a primary membrane plasma separator to separate the plasma from the cells and a plasma fractionator with smaller pores to remove proteins, such as immunoglobulins.<sup>4</sup>

The Autopheresis C and the Aurora (Fresenius-Kabi, Lake Zurich, IL, USA) combines centrifugation and filtration to increase

plasma collection efficiency. A grooved cylinder covered with a filter membrane is encased within a larger cylinder. Whole blood enters the larger cylinder and surrounds the rotating grooved cylinder. The rotation moves the cells away from the filter toward the inner wall of the larger cylinder while the plasma moves toward the rotating filter. Pressure causes the plasma to flow through the filter to a collection bag while cells exit the bottom of the larger cylinder and are returned. The rotation improves efficiency by keeping platelets and cells from plugging the membrane pores.<sup>3</sup>

#### Anticoagulation

Contact between the plastic disposables and the blood activates the coagulation cascade and clots the circuit in the absence of anticoagulation. To prevent this, blood is anticoagulated prior to extensive contact with the tubing. In the United States, citrate or heparin are the primary anticoagulants with additional medications available in other countries.<sup>5,6,7</sup> Citrate anticoagulants contain citric acid, which binds calcium so that it cannot participate in the coagulation cascade.<sup>5,7</sup> It is necessary to lower the ionized calcium concentration to 0.2–0.3 mmol/L to inhibit coagulation. This would be incompatible with life, yet citrate anticoagulation is safely used due to compensatory mechanisms that increase calcium through release from albumin, mobilization from bone stores, and resorption by the kidneys.<sup>6,7</sup> Citrate is also rapidly metabolized by the liver, kidneys, and skeletal muscle releasing the chelated calcium.<sup>6,7</sup> Citrate anticoagulation produces regional anticoagulation, anticoagulation of the blood within the apheresis device but not in the patient donor.

Heparin anticoagulates blood by increasing antithrombin activity.<sup>6</sup> It is not used as widely as citrate due to systemic anticoagulation and the risk of heparin-induced thrombocytopenia.<sup>6</sup> Heparin is used in procedures where citrate interferes with the mechanism of action of the apheresis device, and it is frequently used in therapeutic plasma exchange (TPE) performed with filtration-based devices. The use in filtration TPE is related to plasma removal efficiency. In a centrifugal device, the plasma extraction ratio is higher (80%), resulting in the removal of most of the administered anticoagulant. The lower plasma extraction ratio (30%) of filtration

devices means a large percentage of the anticoagulant is returned to the patient, causing a greater risk of toxicity.<sup>5</sup> Heparin is not used as sole anticoagulant for the collection of most blood components.

Heparin and citrate may be used in combination to minimize the side effects of both by reducing the amount of citrate needed and its electrolyte disturbances and reducing heparin dose to minimize the length of systemic anticoagulation.<sup>8</sup> This dual anticoagulation is used in procedures on low-weight patients, such as children, where the total volume of anticoagulant administered during the procedure may be large relative to the individual's blood volume.<sup>8</sup> This combination of anticoagulants is commonly used for hematopoietic progenitor cell collections in pediatric patients and may be used for hematopoietic progenitor cell collection in adults by some centers as well.

### Vascular access

In donor apheresis, vascular access is obtained through peripheral venipuncture. A large-bore steel needle (17 gauge or greater) is inserted into an antecubital vein as the draw while an intravenous catheter (18 gauge or larger) is inserted into a peripheral vein of the opposite arm to serve as the return.<sup>9</sup> In blood donors, this is effective and safe.

Underlying disease, previous medical treatment, previous IV insertion, hypovolemia, and multiple apheresis procedures may make peripheral access difficult in patients, and it may be necessary to insert a central venous catheter. Central venous catheters capable of supporting apheresis must have a large diameter and short length to minimize resistance to flow and have rigid walls to prevent collapse under negative pressure in order to allow adequate flow rates.<sup>9</sup> Such catheters are almost always dual lumen catheters to allow a single line to be utilized for both the draw and return. The use of triple lumen catheters is uncommon as the presence of the third lumen frequently results in too small of a diameter of the draw and return causing increased resistance with pressure alarms from the apheresis device. Catheters used for dialysis are acceptable for apheresis. The size of the catheters used varies according to the size of the patient undergoing therapy with 8 French dual lumen catheters utilized for patients 10–20 kg, 7–10 French catheters for patients 20–40 kg, and 10–12.5 French diameter twin or dual lumen catheters for those >40 kg.<sup>10</sup>

Central venous catheter placement represents the greatest risk for apheresis complications,<sup>11</sup> with complications including pneumothorax, bleeding, thrombosis, and infection.<sup>5</sup> Deaths due to these complications have been reported.<sup>12</sup> Studies have found that adequate access can be obtained in patients using peripheral veins. One TPE study involving multiple sclerosis patients found inadequate peripheral access in only 4.5% of patients.<sup>13</sup> An additional, more recent, single center study found that peripheral venous access was successful in 72% of therapeutic procedures but only 29% of TPE.<sup>14</sup> More recent studies examining vascular access for apheresis procedures globally have demonstrated a variation in practice with Europe demonstrating predominantly peripheral access (63% in 2003 and 71% in the period from 2003 to 2007) while North America, South America, and Asia demonstrated predominantly the use of central venous catheters.<sup>15,16</sup>

To minimize risks, each patient should have their peripheral access evaluated and used, if possible. Transitioning to such a practice, however, may be difficult. Recent studies have demonstrated that utilizing ultrasound guidance to obtain peripheral access can successfully reduce central venous catheter use in both adult and pediatric patients.<sup>17,18</sup> In adults, Salazar *et al.* demonstrated a

reduction in the need for such catheters in 20% of their patients where peripheral access could not initially be obtained.<sup>17</sup> Mahon and colleagues found that the use of ultrasound guidance in difficult peripheral venous access pediatric patients resulted in a “small amount of time gained” but a significant improvement in patient and parent satisfaction.<sup>18</sup>

Beyond peripheral venous access and central venous catheters, other forms of access can include peripheral arterial access in the form of arterial lines, use of subcutaneous implantable ports, use of AV fistulae, and the use of AV grafts. These last three forms of access are predominantly utilized for long-term chronic apheresis treatment as they may require weeks to months to “mature” following surgical placement. Peripheral arterial access is used acutely and short term but is relatively uncommon and used predominantly in very small pediatric patients.<sup>19</sup>

### Blood donor apheresis

The 2017 National Blood Collection and Utilization Survey demonstrated a continued shift from whole blood to apheresis donation.<sup>20</sup> This preference in the use of apheresis-derived blood products has been driven by the ability to optimize the products collected based upon the donor's characteristics and inventory needs. For example, although AB plasma is in high demand as the universal plasma product, AB red blood cells are only compatible with 4% of the population and are therefore of limited use. Collecting only plasma from an AB donor allows that donor to donate plasma more frequently and does not generate a low-demand product likely to outdated. Similarly, by not collecting red blood cells from donors donating COVID-19 convalescent plasma, the deferral between donations is shorter allowing for the collection of more product. Multicomponent collections (e.g., a platelet collection with a concurrent collection of plasma) can also be performed, allowing even greater optimization. Table 24.1 lists the various blood components and their combinations that can be collected. Table 24.2 lists advantages of collecting blood components by apheresis.

Compared to 2015, in 2017 there was a decrease in red blood cell (RBC) units produced from whole blood donation by 3.2% and units collected by apheresis of 0.6%. There was a decline in the number of RBC units transfused by 6.1%. While there was an overall decrease in both RBC sources and an overall decrease in RBC transfusions, the greater decline in RBCs derived from whole blood demonstrates a preference for apheresis-derived RBCs, driven by the ability to optimize product collections.

The total number of apheresis platelets collected in 2017 compared to 2015 increased by 4.6%. There was also an increase in whole-blood-derived platelets distributed by 10.3%. However, there was a substantial decline in the number of whole-blood-derived

**Table 24.1** Blood Components Collected by Apheresis

Granulocytes
Hematopoietic progenitor cells
Plasma: multiple doses may be collected depending upon donor characteristics
Platelet: single, double, or triple
Double platelet and single plasma
Platelet and single or double plasma
Red blood cell: single or double
Red blood cell and plasma
Red blood cell and platelet
Red blood cell, platelet, and plasma

platelets transfused by 52.3% with an accompanying increase in apheresis platelets transfused of 2.3%. These numbers indicate that there was the preferential use of apheresis platelets compared to whole-blood-derived platelets, as has been seen in previous National Blood Collection Utilization Surveys. In 2017, 91.3% of platelets distributed were collected by apheresis. This trend could be influenced by the availability of pathogen-reduced platelet products, reduced manipulation of the platelet product by the transfusing facility (no need for pooling of whole-blood-derived platelets), decreased donor exposure, and other factors. Given pending deadline for implementation FDA requirements concerning bacterial detection for platelet products, this trend may continue in the future.

### Donor apheresis instrumentation

Devices cleared by the US Food and Drug Administration (FDA) and currently available in the United States will be briefly described. Table 24.3 lists the different products that each of these devices can collect.

#### Alyx (Fresenius-Kabi, Lake Zurich, IL, USA)

The Alyx is a single-needle instrument designed for double red cell and multicomponent collection (Table 24.3). Blood is pumped by the donor pump into an "in-process" bag. Blood is then pumped by the in-process pump into the centrifuge. Because the in-process pump is slower than the donor pump, unprocessed blood accumulates in the in-process bag. Plasma is drawn from the centrifuge by the plasma pump and delivered to the plasma bag to be returned to the donor or saved for plasma collection. The packed RBCs are pumped into the RBC bag for collection (or returned during a plasma collection). In RBC collection, when a predetermined

amount of blood has been processed, the plasma and saline are returned to the donor by the donor pump. Simultaneously, the in-process pump delivers unprocessed blood from the in-process bag, so the centrifuge is continuously processing blood. During RBC collection, when the appropriate amount of RBCs is collected, the extra blood components are returned to the donor. A preservative solution is added to the RBCs, which are pumped through a leukoreduction filter into final storage bags. The extracorporeal volume (ECV) is 110 mL plus the volume of the products. The Alyx is a compact device that can be utilized on mobile blood drives.<sup>3</sup>

#### Fenwal Amicus (Fresenius-Kabi, Lake Zurich, IL, USA)

The Amicus can perform single- and multicomponent collections (Table 24.3). Blood is pumped into a double chamber belt wrapped around a spool. Prior to entering the centrifuge, plasma is added to the whole blood to adjust the hematocrit (HCT). When blood enters the centrifuge, the lighter platelets quickly migrate toward the center of the centrifuge and are withdrawn by the platelet-rich pump (PRP) from the inlet side of the separation chamber. As blood flows through the separation chamber, the HCT increases, and mononuclear cells flow to the center of the centrifuge and are drawn by the PRP pump toward the platelet outlet. However, when they encounter the lower HCT of the entering diluted blood, they fall to the outside of the chamber. They continue to circulate in the center of the separation chamber until they are returned to the donor during reinfusion. The packed RBC and granulocytes flow to the far end of the separation chamber and are returned to the donor. The PRP is pumped into the second chamber (collection chamber), where the platelets concentrate and the platelet-poor plasma is directed into a collection bag or returned to the donor.<sup>3</sup> When the procedure is complete, the platelets are manually resuspended and then pumped into the platelet storage bags.

The interface between the blood components in the separation chamber is monitored by an interface detector and maintained by a microprocessor. The Amicus can perform double- or single-needle procedures, with the later used for multicomponent collections (Table 24.3). RBC leukoreduction is done off-line by manually filtering the product. The ECV without products is 205 mL for double-needle procedures and up to 329 mL for single-needle procedures.<sup>3</sup>

#### Autopheresis-C and Aurora (Fresenius-Kabi, Lake Zurich, IL, USA)

The Autopheresis-C and Aurora perform plasma collection (Table 24.3). The centrifugal filtration system described under blood component separation is used by both. The devices are single-needle systems using citrate anticoagulation. Both have ECVs of 200 mL, not including the volume of the products being collected.<sup>3</sup>

**Table 24.2** Advantages of Apheresis Collected Blood Components

- Reduced donor exposure: full transfusion dose collected from one donor
- Frequent repeat donors: "pedigreed" donors with repeated screening and testing
- Higher quality products: more quality control per component collected
- Consistent and standardized product volumes and yields
- Ability to match donors to patients: HLA matching for platelet transfusions
- Ability to collect only the product of interest from donor: collect AB plasma or COVID convalescent plasma without collecting RBCs leading to longer donor deferral and unneeded product
- Double, triple, or multiple full-dose blood component collections
- Safety enhancement for the patient with reduced frequency of certain transfusion reactions
- Reduced frequency of donor reactions
- Ability to perform pathogen reduction

**Table 24.3** Products Collected by Donor Apheresis Devices Available in the United States

Device	Platelet	cRBC	Double RBC	Plasma	cPlasma	Granulocytes	HPC
Alyx		X	X	X	X		
Amicus	X	X			X		X
Autopheresis-C/Aurora				X			
MCS+ 8150			X		X		
MCS+ 9000	X				X		
PCS-2				X			
Spectra Optia		X					
Trima Accel	X	X	X	X	X	X	X

RBC: red blood cell; cRBC: concurrent RBC; cPlasma: concurrent plasma; HPC: hematopoietic progenitor cell.

### **Trima Accel (Terumo Blood and Cell Technologies, Lakewood, CO, USA)**

The Trima Accel is capable of single- and multicomponent collections (Table 24.3). Blood is pumped into a rotating single-stage circular channel. The collect line from the centrifuge is attached to a conical leukocyte reduction chamber. The “collect pump” pumps the product into collection bags. The plasma is collected in the plasma collection bags or is returned to the donor, depending upon the procedure being performed. The packed cells go to the return reservoir or RBC collection bag, again depending upon the procedure being performed. A microprocessor directs component flow from the centrifuge through a valve system to the proper collection bags. The plasma-cell interface is maintained by algorithms that utilize donor data entered at the procedure beginning. Because of this, there is no interface detector, but there is an RBC detector that will monitor RBC spillover during the collection. The Trima Accel is a single-needle system. Double RBC units can be collected, filtered, and additive solution added, all on-line. The ECV, without products, is 196 mL for the platelet kit and 182 mL for the RBC-plasma kit.<sup>3</sup>

### **Spectra Optia (Terumo Blood and Cell Technologies, Lakewood, CO, USA)**

The Spectra Optia, while predominantly used as for therapeutic apheresis (see Chapter 24), can be used for granulocyte collection. It uses a circular separation channel with ports positioned at different distances from the outside wall. The Spectra Optia is automated with an interface detector that monitors the position of the plasma/cell interface, at the port of the collect line. The operator can fine tune the product content by monitoring the collect line color and adjusting the plasma pump. The Spectra Optia typical ECV during granulocyte collection is 253 mL while the maximum is 297 mL, not including the product volume.<sup>3</sup>

### **Haemonetics MCS+ 9000 and 8150 (Haemonetics Corporation, Braintree, MA, USA)**

MCS stands for *mobile collection system*. The MCS+ 9000 is used for platelet collection and concurrent plasma, and the MCS+ 8150 is used for double RBC collection and RBC with concurrent plasma (Table 24.3). The MCS+ 9000 uses a conical-shaped Latham bowl, and the MCS+ 8150 uses a bowl with vertical walls.<sup>3</sup>

With the MCS+ 9000, blood fills the spinning conical bowl from the bottom, with the RBCs migrating to the outside wall and plasma staying on the inside wall with the buffy coat (white blood cells [WBCs] and platelets) lying between. The cell/plasma interface is monitored by a light beam reflected off the inside of the top of the bowl. Plasma exits the bowl by first passing through an optical-based line sensor. When the plasma-buffy coat interface is detected in the bowl, plasma is rapidly pumped into the bowl from the bottom, flushing the lighter platelets from the bowl. When the platelets are detected by the line sensor, a valve opens to the collect bag, which then closes when WBCs are detected. At that point, the bowl contains only RBCs, which are returned to the donor along with the plasma. The cycle is then repeated. WBC removal occurs through an on-line leukoreduction filter. When concurrent plasma is collected, the desired volume of plasma is retained during the last reinfusion.<sup>3</sup>

In the MCS+ 8150, the vertical-wall centrifuge bowl (blow-molded bowl) also fills from the bottom. Instead of a light-beam-based sensor at the top of the bowl, the line sensor detects the presence of RBCs in the bowl effluent line and stops the cycle. A

small amount of saline is pumped to the donor, and then a predetermined amount of RBCs is transferred to the RBC reservoir bag. The remaining RBCs, plasma, and additional saline are returned to the donor. The cycle is repeated until the programmed RBC volume is collected. The donor is disconnected when all remaining plasma and RBCs have been returned. Additive solution is pumped to the reservoir bag, and the set is disconnected. Leukoreduction is performed by gravity through preattached filters as blood drains into two RBC product bags.<sup>3</sup>

The MCS+ 9000 and MCS+ 8150 ECV are dependent on the size of bowl used, donor HCT, and phase of the cycle. The range is 542 mL (38% HCT) to 391 mL (54% HCT). The instruments are designed to be portable and can be used for mobile blood drives.<sup>3</sup>

### **Haemonetics PCS-2 (Haemonetics Corporation, Braintree, MA, USA)**

The Haemonetics PCS-2 is used for plasma collection (Table 32.4). The MCS+ 8150 vertical wall bowl is used with plasma diverted to a collection bag until the bowl fills with RBCs. The bowl contents are then returned to the donor. Cycles are repeated until the target plasma volume is collected. The ECV depends upon the donor hematocrit ranging from 480 mL (38% HCT) to 359 mL (52% HCT). The device is portable.<sup>3</sup>

## **Product and procedure requirements**

### **Granulocytes**

Chapter 19, “Granulocyte collection and transfusion,” discusses granulocyte collection and clinical use, and the reader is referred to this chapter for procedure and product requirements.

### **Hematopoietic progenitor cells**

Chapter 53, “Hematopoietic stem cells and transplantation,” discusses the collection and processing of hematopoietic progenitor cells, including procedure and product requirements. The reader is referred to this chapter.

### **Plasma**

In 2017, 564,000 plasma units were collected by apheresis in the United States. These apheresis units, however, represent only 8.7% of the plasma collected for transfusion with the majority collected from whole blood donation.<sup>21</sup> Between 2015 and 2017, there was a decline in plasma units transfused by 13.6% with an accompanying decline in the production of plasma units from both whole blood (13%) and apheresis (46.3%). The increased cost and complexity of apheresis plasma collections in the presence of shrinking demand resulted in this change. Plasma is also collected as *source plasma*, plasma to be fractionated and manufactured into products such as albumin, intravenous immune globulin, and coagulation factor concentrates (see Chapter 22, The purification of plasma proteins for therapeutic use).

The regulatory and accreditation requirements for plasma collection depend upon donation frequency. Donors may donate more frequently than every four weeks (*frequent*) or less frequently than every four weeks (*infrequent*). In general, frequent donors donate “source plasma” and infrequent donors donate plasma for transfusion. Infrequent plasma donors must fulfill whole blood donor requirements.<sup>22</sup> AABB Standards for Blood Banks and Transfusion Services also require that donors taking warfarin be deferred for one week following their last dose to allow for vitamin-K-dependent

coagulation factor regeneration.<sup>23</sup> Frequent plasma donors must fulfill criteria outlined in the US Code of Federal Regulations (CFR).<sup>23,24</sup> These require that total serum protein is greater than 6.0 g/dL prior to each donation, a serum protein electrophoresis or quantitative immunodiffusion be performed every four months, and a physical examination performed by a physician be performed annually.<sup>24</sup> This ensures that “frequent” plasma donation does not deplete plasma proteins that could harm the donor and affect the potency and efficacy of the products.

Both frequent and infrequent plasma donors are limited to the total plasma volume that can be donated in 12 months. Donors weighing 110–175 lbs. can donate up to 12 L, and those weighing >175 lbs. can donate up to 14.4 L.<sup>22</sup> Frequent plasma donors are limited to two donations within a seven-day period, with at least two days between the donations.<sup>24</sup>

The SARS-CoV-2 pandemic has resulted in a development in renewed interest of utilizing convalescent plasma from individuals who have recovered from infection.<sup>25</sup> Donors donating such plasma may be either frequent or infrequent plasma donors. Donor criteria for the collection of COVID-19 Convalescent Plasma (CCP) have been issued by the FDA with numerous updates.<sup>26</sup> At the time of writing of this chapter, donors donating CCP must fulfill the criteria outlined in Table 24.4. Indications for the appropriate use of CCP are discussed in Chapter 3, Responding to the regulatory challenges during emergencies and epidemics, and Chapter 21, Plasma and cryoprecipitate for transfusion.

## Platelets

Donor requirements for apheresis platelet donation are the same as whole blood donation.<sup>23,27</sup> Both the AABB and FDA have requirements that address a donor’s exposure to antiplatelet medications.

The FDA requires deferral of apheresis platelet donors who have ingested aspirin or piroxicam within two days prior to collection.<sup>27</sup> Apheresis platelet donors who have taken clopidogrel or ticlopidine, drugs whose metabolites inhibit platelet function and have a longer antiplatelet effect, are deferred for 14 days.<sup>27</sup> AABB Standards require that apheresis platelet donors taking “medications known to irreversibly inhibit platelet function” be evaluated prior to donation.<sup>23</sup> These criteria do not apply to whole blood donors whose donations are used to manufacture platelets unless they represent the single source of platelets to the patient (e.g., transfusing an aliquot from a single whole-blood-derived platelet to a pediatric patient).

**Table 24.4 Donor Criteria for the Collection of COVID-19 Convalescent Plasma.** Source: Based on Food and Drug Administration.<sup>26</sup>

- Evidence of COVID-19 infection as defined by either 1 or 2
- 1. Symptoms of COVID-19 and positive results on a diagnostic test approved, cleared, or authorized by the FDA
- 2. No prior symptoms or positive diagnostic test but reactive results on two different FDA approved, cleared, or authorized tests for SARS-CoV-2 antibodies
- Resolution of symptoms at least 14 days before donating
- Male donors or female donors who have never been pregnant or for female donors who have been pregnant with a negative test for HLA antibodies since the most recent pregnancy
- Donors who have received a COVID-19 vaccine may donate if they had symptoms of COVID-19 infection and a positive diagnostic test, received the vaccination after diagnosis, and are within six months of resolution of COVID-19 symptoms
- Donors who have received monoclonal antibodies may donate three months after the administration of the monoclonal antibody

Platelet donors must have a minimum 150,000/ $\mu$ L platelet count. A predonation count must be used to program the apheresis instrument and can be a post collection count from the donor’s previous donation or a count on a sample drawn immediately prior to the current donation. If the count is not available prior to the start of collection, the procedure can begin but the count must be entered when available. If a preprocedure count cannot be obtained, an average of the donor’s previous platelet counts, a default count recommended by the instrument manufacturer, or a default count determined by the collection center may be used.<sup>27</sup> When a preprocedure platelet count cannot be determined on a first-time donor, a triple-platelet product cannot be collected.<sup>23</sup> If capable, the instrument must be programmed to ensure that the donor’s platelet count does not fall below 100,000/ $\mu$ L.<sup>27</sup>

Platelet donors have restrictions on donation frequency and the total number of donations that can occur within a 12-month period.<sup>23,27</sup>

Donors cannot donate more than 24 times in a rolling 12-month period.<sup>27</sup> This limit arose from the observation that donors collected on early apheresis devices experienced declines in total lymphocyte numbers, T-lymphocyte numbers, and immunoglobulin G (IgG) levels for up to eight months.<sup>28</sup> It had been suggested that with newer devices, this was no longer a problem.<sup>28</sup> However, a recent study examining donors donating using the Trima Accel demonstrated statistically significant declines in CD4 and CD8+ T-cells with a dose response to the number of donations in two donor groups (3–19 donations per year or 20–24 donations per year) compared to a control group of 1–2 donations per year.<sup>29</sup> The authors of this study could not demonstrate any effect lymphopenia on donor health.<sup>29</sup> An additional study by the same authors examined 30 platelet donors who had undergone 20–24 platelet donations in the prior year with the Amicus.<sup>30</sup> This group of donors did not show the same reduction in CD4 and CD8+ T-cells. The leukocyte reduction method of the Trima Accel and Amicus is substantially different and therefore the authors concluded that lymphopenia due to platelet donation is likely device dependent.<sup>30</sup> A nationwide cohort study from Sweden examining 74,408 apheresis platelet donors and comparing the risk of infections in donors donating on devices using the leukocyte reduction chamber used in the Trima Accel to those who had donated platelets at a time when such chambers were not in use has been published.<sup>31</sup> The authors found an increased risk of immune-suppression-related infections, mostly reactivation of varicella-zoster, and an increased risk of bacterial infections in donors donating on devices with leukocyte reduction chambers when matched for the same donation frequency.<sup>31</sup> These findings support continued limitations on the number of apheresis platelet donations if certain leukocyte reduction methods are used.

In addition to lymphopenia, a study has suggested that long-term platelet donation could result in baseline platelet count decline and a delay in platelet recovery.<sup>32</sup> This study resulted in the addition of the limitations on the number of times a donor can donate in a week and the requirement for a longer period of deferral between donations when double or triple products are donated.<sup>27</sup> Donors can donate twice per week but must have two days between donations if they are donating a single dose of platelets. If donating a double or triple product, they are deferred for one week.<sup>23,27</sup> It should be noted, however, that declines in baseline platelet count and delayed recovery with increasing donation frequency have not been seen in other studies.<sup>33</sup>

The FDA has also placed limits on the total amount of plasma removed during a platelet donation and the total amount of RBCs lost within eight weeks.<sup>27</sup> Donors weighing <175 lbs. can have no

**Table 24.5** Impact of Red Cell Loss on Donor Eligibility. Source: Modified from Food and Drug Administration.<sup>27</sup>

First Red Cell Loss (mL)	Total of First and Subsequent Red Cell Loss in Preceding 8 Weeks (mL)	Eligibility
<200	<200	No deferral
<200	>200 but ≤300	Defer for 8 weeks from second loss
>200 but ≤300	NA	Defer for 8 weeks from first loss
<200	>300	Defer for 16 weeks from second loss
≥300	NA	Defer for 16 weeks from loss

NA: not applicable.

more than 500 mL of plasma removed, whereas those weighing ≥175 lbs. can have 600 mL removed.<sup>27</sup> RBC loss, from whole blood donation or loss during the apheresis donation, can result in prolonged donor deferral.<sup>21,23</sup> The loss of red blood cells during apheresis platelet collection, however, is usually minimal with significant red cell loss occurring only in the presence of instrument malfunctions that prevent the reinfusion of blood from the apheresis device. Even then, the extracorporeal red cell volume of most devices is less than 100 mL. These deferrals are listed in Table 24.5.

The FDA requires that 75% of the apheresis platelet products contain at least  $3.0 \times 10^{11}$  platelets,<sup>24</sup> whereas AABB requires that 90% contain  $3.0 \times 10^{11}$  platelets.<sup>23</sup> The products must also have a pH >6.2 at issue or the end of storage.<sup>23</sup> If products are leukocyte reduced, then 95% of the units sampled must have  $\leq 5 \times 10^6$  residual leukocytes.<sup>23</sup>

### Red blood cells

RBCs can be collected as a single or a double unit. Single units are usually collected as part of a multicomponent collection due to the costs associated with the collection. Advantages of apheresis RBC collections include fewer donor reactions, better inventory management, standardized RBC doses, and decreased donor exposure if both units of a double RBC are given to the same patient.<sup>34</sup> Disadvantages include stricter donor criteria that are dependent upon the apheresis device used, longer donation time, and potential apheresis-related complications.<sup>34</sup>

Apheresis RBC donors must meet whole blood donation criteria<sup>23,35</sup> and must also meet criteria defined by the device manufacturer. These criteria define height, weight, and hematocrit requirements based upon the donor's sex.<sup>35</sup> Of note, the hematocrit requirements of the manufacturer may differ from those defined by the FDA for whole blood donation (females Hgb 12.5 g/dL, Hct 38%; males Hgb 13 g/dL, Hct 39%). The FDA requires that the hemoglobin/hematocrit method used to qualify the donor be a quantitative method.<sup>35</sup>

The maximum allowed RBC volume removed during the collection is defined in the device operator's manual with no specific requirements from the FDA.<sup>35</sup> AABB standards require that the amount of RBC removed not reduce the donor's hemoglobin below 10 gm/dL or the hematocrit below 30%.<sup>23</sup>

Donation frequency is determined by the type of RBC product donated. Single-unit RBC donors are deferred for 8 weeks, whereas those donating two units are deferred for 16 weeks.<sup>35</sup> The deferral length may vary based upon the absolute RBC loss in cases where there is an incomplete procedure or there has been additional RBC

loss due to other donations (Table 24.5).<sup>27</sup> As with whole blood donation, iron deficiency among apheresis donors is a significant concern with the potential for significant health effects. See Chapter 7, Chronic effects of blood and plasma donation, for further discussion concerning these issues.

The FDA requires that 95% of the products collected meet the "expected or target RBC volume" and "any other target parameters" defined in the device operator's manual. The FDA defines how the testing to validate and monitor the apheresis RBC collections is performed.<sup>35</sup> For validation, the FDA requires testing 100 consecutively collected units. For monitoring, at least 50 units, consisting of single and double RBCs, must be monitored each month. When a center collects fewer than 50 units, all of the units must be tested.<sup>35</sup> AABB standards require that the method used results in a mean hemoglobin of ≥60 g or 180 mL RBCs. At least 95% of the units must have >50 g of hemoglobin or 150 mL RBCs. If the RBC unit is leukocyte reduced, the method must have a mean hemoglobin ≥51 g or a 153 mL RBC volume and a residual leukocyte count ≤5 × 10<sup>6</sup>. Ninety-five percent of units must have >42.5 g of hemoglobin or 128 mL RBCs.<sup>23</sup>

### Multicomponent collections

Multiple different components can be collected by apheresis from a single donor (Table 24.2). With multicomponent collections, the donor and product requirements that apply to the individual products apply to the procedure. Donation frequency requirements seen with plasma donation apply when plasma is collected concurrently with a platelet or RBC product. Red cell loss deferrals given in Table 24.5 and the plasma volume limits described with plasma and platelet donation also apply.

### Apheresis complications

#### Donor reaction overview

Among American Red Cross apheresis donors, the reaction frequencies were 577.5/10,000 for platelet donation and 538.3/10,000 for double red cell donation, compared to 348.9/10,000 for whole blood donation.<sup>36</sup> Severe reactions, requiring outside medical management, occurred in 3.2/10,000 whole blood, 2.9/10,000 platelet, and 2.9/10,000 double red cell donations. The differences were not statistically significant, although the reaction types with severe reactions to whole blood donation were predominantly vasovagal reactions, whereas those due to apheresis donation were phlebotomy related.<sup>36</sup> One study found severe reactions in 0.01% of apheresis platelet donations,<sup>37</sup> a rate 20 times that seen with whole blood donation (0.0005%).<sup>38</sup> Reaction types and frequencies in apheresis and whole blood donors are listed in Table 24.6. Venipuncture-related complications are most frequent with apheresis donation, with first-time donation being the most common risk factor.<sup>36,39</sup>

**Table 24.6** Frequency of Reactions among Apheresis and Whole Blood Donors

Reaction	Apheresis (%) <sup>39</sup>	Whole Blood (%) <sup>40</sup>
Hematoma	1.15	9–16
Citrate toxicity	0.4	Not applicable
Vasovagal	0.05	2–5
Vasovagal with loss of consciousness	0.08	0.1–0.3

## Therapeutic apheresis reaction overview

Reaction frequencies among patients undergoing therapeutic apheresis vary among studies. McLeod observed reactions in 4.75% of procedures, and Couriel and Weinstein observed reactions in 17%.<sup>12,41</sup> A difference between these studies was that Couriel and Weinstein considered central line complications as an apheresis complication, whereas McLeod did not. In the study by Couriel and Weinstein, all severe reactions, including one patient death, were associated with central line placement.<sup>12</sup> Shemin *et al.* observed reactions in 36% of TPE procedures.<sup>42</sup> The reactions observed by McLeod were transfusion reactions related to replacement fluids (1.6%), citrate toxicity (1.2%), hypotension (1.0%), vasovagal reactions (1.0%), tachycardia (0.4%), respiratory distress (0.3%), tetany and seizure (0.2%), and chills and rigors (0.2%).<sup>41</sup>

## Common apheresis complications

### Citrate toxicity

Citrate chelates calcium, making it unavailable for the coagulation cascade. Ionized calcium levels necessary for anticoagulation occur within the apheresis device, not in the patient donor, due to compensatory mechanisms. Despite this, levels of ionized calcium can reach a point whereby spontaneous neuron depolarization occurs, resulting in the signs and symptoms listed in Table 24.7.<sup>43</sup> The table

lists these in the order of appearance, so it is possible to avoid more severe reactions by identifying the early, mild symptoms.<sup>43</sup> Hypocalcemia can also suppress myocardial function, induce arrhythmias, and prolong the QT interval.<sup>36</sup> Treatments of hypocalcemia are listed in Table 24.8. Prevention of hypocalcemia includes continuous intravenous calcium administration during the procedure and correction of low levels prior to the start of the procedure.<sup>43</sup>

Although hypocalcemia is commonly thought of as citrate toxicity, citrate anticoagulation results in a variety of electrolyte and acid–base disturbances. The second most common divalent cation in the blood is magnesium, which is also chelated by citric acid. Significant magnesium decline has been seen during apheresis procedures. Signs and symptoms of hypomagnesemia are similar to those of hypocalcemia, including muscle spasms, muscle weakness, decreased vascular tone, and impaired cardiac contractility, and therefore may present as hypocalcemia that fails to respond to calcium supplementation.<sup>43</sup> Treatment consists of procedure discontinuation with magnesium supplementation.

Citrate metabolism generates bicarbonate, producing a metabolic alkalosis.<sup>43</sup> In most circumstances, this is not significant but in the presence of renal impairment and/or increased citrate load when plasma is used as the replacement fluid, severe metabolic alkalosis may occur. Symptoms are nonspecific and present as

worsening hypocalcemia, although suppressed respiratory drive can occur as the body tries to compensate. Metabolic alkalosis resolves with time, although in severe cases, dialysis may be necessary.<sup>44</sup>

Metabolic alkalosis can also produce hypokalemia. To buffer the generated bicarbonate, protons leave cells with a resulting net potassium influx to maintain a neutral charge. Significant potassium decline, up to 11% in a large-volume stem cell collection study, can occur.<sup>45</sup> Most patients are asymptomatic, but symptoms can include weakness, hypotonia, and cardiac arrhythmia. Treatment consists of oral or intravenous replacement.<sup>45</sup>

### Hypotension

Hypotension is a common reaction during apheresis and can result from numerous mechanisms. As indicated in Table 24.6, citrate-induced hypocalcemia can cause hypotension. Other causes include hypovolemia, vasovagal reactions, and the presence of angiotensin-converting enzyme inhibitors (ACEIs). In hypovolemia, the amount of blood within the extracorporeal circuit is greater than can be tolerated by the patient donor. It is characterized by hypotension and tachycardia.<sup>43</sup> Hypovolemia is relatively uncommon among healthy apheresis donors because the total amount of blood withdrawn into the extracorporeal circuit and the collected products is limited to 10.5 mL/kg.<sup>23</sup> Hypovolemia is common among patients as they may be volume depleted due to underlying disease and poor oral intake. Vasovagal reactions are characterized by low blood pressure with an inappropriately low pulse. Increased parasympathetic output in response to sympathetic drive leads to vasodilation and bradycardia.<sup>46</sup> Both hypovolemia and vasovagal reactions are treated by pausing the procedure, placing the patient donor in the Trendelenburg position, and infusing fluids.<sup>46</sup>

An uncommon but important cause of hypotension, in patients undergoing TPE with albumin replacement fluid and lipid apheresis with the Kaneka MA-03 Liposorber system, is the use of ACEIs. This reaction is characterized by flushing, hypotension, bradycardia, and dyspnea occurring after a few milliliters of blood is returned to the patient due to bradykinin generation in the apheresis device. Bradykinin may be generated by prekallikrein activating factor that is present in some albumin lots.<sup>47</sup> In the Liposorber system, bradykinin is generated when blood comes into contact with the negatively charged dextran sulfate beads within the Liposorber columns.<sup>48</sup> Normally, bradykinin has a short half-life, metabolized by kinase I and kinase II. ACEIs inhibit these, resulting in unopposed bradykinin effects. Treatment options are limited, and when severe reactions occur, procedures must be terminated. Avoiding ACEI administration for 24–48 hours before the procedures can prevent these reactions. ACEIs can be safely administered immediately following the procedures.

**Table 24.7** Signs and Symptoms of Hypocalcemia due to Citrate Toxicity.  
Source: Based on Winters (2006).<sup>43</sup>

- Perioral and acral paresthesias
- Shivering
- Lightheadedness
- Twitching
- Tremors
- Nausea and vomiting
- Hypotension
- Carpopedal spasm
- Tetany
- Seizure

**Table 24.8** Treatment of Hypocalcemia due to Citrate

- Slow reinfusion rate to allow metabolism of citrate
- Increase the blood-to-citrate ratio so that less citrate is given
- Administer oral calcium carbonate
- Administer intravenous calcium gluconate or calcium chloride

Source: Based on Winters (2006).<sup>43</sup>

### Allergic and anaphylactic reactions

In therapeutic apheresis, allergic and anaphylactic reactions are most often triggered by the replacement fluid. Allergic and anaphylactic reactions can occur when either plasma-containing blood products or albumin is used. They can also occur in donor apheresis.<sup>43</sup> In donors, substances present in the disposables or substances used in the procedure trigger histamine release. For example, there have been reports of apheresis platelet donors developing allergic and anaphylactic reactions to residual ethylene oxide present from sterilizing the disposables.<sup>49</sup> In granulocyte collections, hydroxyethyl starch (HES) is used as a sedimenting agent to enhance the product yield. HES can activate the alternate complement pathway generating C3a and C5a, which can trigger mast cell release.<sup>50</sup> Simple allergic reactions can be treated with oral or intravenous antihistamines. In donors, procedures would be discontinued, whereas in patients, they could be resumed after ensuring that the symptoms are not progressing. In severe reactions, procedures should be discontinued but vascular access maintained. Treatment of severe reactions would include administration of epinephrine, fluids, and potentially aminophylline for bronchoconstriction. These patients may also require intubation to maintain their airway. At the end of an apheresis procedure, the contents of the centrifuge are returned to the patient donor. In the setting of severe allergic or anaphylactic reactions where procedures are stopped early, this should be avoided in order to prevent allergen infusion.

### Air embolism

Air embolism is a rare, but potentially fatal complication, of apheresis. This complication occurs when a volume of air greater than 3–8 mL/kg enters the venous circulation. It is rare as all modern apheresis instruments include air detectors that immediately stop the return of blood to the donor or patient when air is detected in the system. Air embolism is most likely to occur, therefore, in patients with central venous access. It occurs in this setting when connecting or disconnecting the apheresis device from the catheter without closing the clamp on the catheter and the patient taking a deep inspiration resulting in a negative intrathoracic pressure. Air embolism results in bronchoconstriction, pulmonary artery vasoconstriction, and right ventricular outflow obstruction producing

wheezing, hypotension, cyanosis, and cardiovascular collapse. Treatment consists of immediately placing the patient into Trendelenburg position on their left side so that the air moves from the right ventricular outflow track. With time the air will dissolve or if a large amount is present, it may be necessary to insert a catheter into the right ventricle and aspirate the air.<sup>43</sup>

### Summary

The term *apheresis* has been used to describe a family of medical procedures for more than 100 years. Both donor collections and therapeutic procedures have in common the basic mechanisms of blood separation, anticoagulation, and vascular access, and as a result, share some of the same complications. The use of apheresis to collect blood products for transfusion continues to expand despite an overall reduction in the total number of transfusions in the United States. The percentage of red blood cells and platelets collected by apheresis has grown between 2015 and 2017. While the collection of plasma products for transfusion decreased during that time period, the collection of COVID-19 convalescent plasma has recently become an important priority for blood collectors. Apheresis blood collections represents an important and effective way to tailor collections to maximize donations and provide products to meet the special needs of patients.

### Key references

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## CHAPTER 25

# Therapeutic apheresis: plasma processing

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## Introduction

The pathophysiology or symptoms of some diseases are due to the excessive accumulation of blood cells or plasma constituents. When medical options to address the core pathophysiology in these conditions is limited or ineffective, blood cell separators can be used therapeutically.<sup>1</sup> Therapeutic apheresis includes plasma exchange, red blood cell exchange, therapeutic cytoreduction, photopheresis, and specific selective adsorption. In the United States, in 2006 approximately 112,109 therapeutic apheresis procedures were performed. Subsequent summary data are not available, but therapeutic apheresis is projected to be a \$120,000,000 business as of 2015. From an estimated \$178 million industry in 2020, the therapeutic plasma exchange market is projected to increase to \$243 million by 2025.<sup>2</sup>

## Therapeutic plasma exchange (TPE)

John J. Abel of the Johns Hopkins University used the term “plasmapheresis” to describe a method for removing large quantities of plasma from experimental animals.<sup>3</sup> Therapeutic possibilities in humans have been driven by advances in centrifugal blood separation technologies, starting with collection chambers filled by intermittent blood flow, such as the Cohn and Latham bowls in the 1950s, through challenges of rotating seals to modern, fully automated continuous flow systems. Commercially available apheresis devices range from tabletop platforms optimized for component collection to portable yet highly customizable systems that can interface with other extracorporeal technologies at an ICU bedside.<sup>4</sup>

## Techniques of plasma exchange

### Instruments

Collection of plasma for the purposes of blood product manufacture or further processing into plasma derivatives is discussed in Chapter 22. Medical devices require review by different regulatory agencies around the world. We focus on the procedure as it occurs in the United States, which is regulated by the Food and Drug Administration (FDA); therefore, it is required to consult local authorities for instruments that may be approved in other jurisdictions. For therapeutic applications, plasma is separated either by

centrifugation or membrane separation, using distinct instruments. Centrifuge-based methods are commonplace in the United States, whereas membrane-based methods have gained popularity in Japan and Germany. In plasma exchange using centrifugal blood cell separators, the whole blood enters the instrument, where an anticoagulant is added to allow for smooth flow through the tubing and most of the red cells, leukocytes, and platelets are separated from the cell-poor plasma. Separated plasma from the patient is diverted into a waste bag and is replaced with one or more of several available fluids according to the indication for the exchange and patient-specific factors. Membrane filtration plasma exchange is similar to ultrafiltration performed on dialysis machines and employs a filter with pores between 0.3 and 0.5 mm which allow even large plasma proteins to pass through, while all cellular components remain.<sup>5</sup> Since centrifuge-based instruments allow the flexibility to remove (or collect) blood's cellular components as well as plasma, transfusion medicine practitioners and services primarily employ this technology, and we restrict this chapter to centrifugal therapeutic apheresis.

Several instruments can be used for TPE, including the Terumo Optia (which replaced the Spectra instrument and is the most common device), Fresenius Kabi Amicus, Fresenius COM.TEC (including AS104 as a predicate device), and Haemonetics MCS. Due to the risks associated with apheresis, the FDA regulates new devices under the 510(k) pathway and maintains a list of approved devices online.<sup>6</sup> Membrane-based separation systems are regulated by the FDA under a distinct classification from centrifuge-based systems used for therapeutic purposes (“MDP” vs. “LKN” product codes). The Terumo and Fresenius instruments are continuous-flow systems that make it easier to control the patient's fluid volume, and the Haemonetics instrument uses repeated cycles of filling and emptying the blood cell separator. In the United States, the Spectra has been the most widely used, with most centers moving to the Optia when the Spectra became obsolete. The extent of use of Amicus instruments for therapeutic apheresis is not clear, although they are widely used in collections. Details of operating these instruments are not included here. The manufacturer's operating manuals must be used because of the complexity of these procedures. Thorough quality control programs are essential to be sure staff are properly trained, fluids monitored, alarms tested, lines and

fluid attachments secured, and medications and replacement solutions used correctly. Particularly important clinical parameters include understanding the extracorporeal blood volume, inlet:anticoagulant ratio, and pump pressures, which can help diagnose midprocedure clinical changes as well as overall suitability for a patient to undergo a plasma exchange. The plasma removed must be discarded as biohazardous waste.

### Vascular access

The majority of therapeutic plasma exchanges (TPEs) will have a withdrawal rate between 60 and 150 mL/minute using centrifugal systems, allowing most procedures to process one plasma volume in approximately one and a half to three hours. Vascular access for TPE is most commonly via peripheral veins or central venous catheters (CVCs) that can be tunneled or nontunneled, although arteriovenous fistulas, grafts, or power-injectable (a.k.a. "vortex") ports are possible options for long-term use as well. Membrane-based systems require higher flow rates that prohibit peripheral venous access. Factors such as the desired flow rate; the vascular anatomy; patients' mobility and ability to care for a catheter; the urgency, frequency, and anticipated duration of TPE; or whether other treatments that require venous access are needed all help determine the selection of vascular access.<sup>7-9</sup>

Peripheral venous access is desirable because it can be done immediately and is without the potentially serious risks associated with the use of CVCs. Steel dialysis-type needles, typically inserted under aseptic conditions in the antecubital veins, are required to maintain adequate negative pressure on the draw line for continuous flow procedures. For adults, 17 gauge needles can accommodate 80 mL/minute and greater flow rates.<sup>10</sup> Forced-air warming blankets, warm compresses, and arm exercises can increase venous blood flow, making venipuncture more successful in the cooperative patient. A vein assessment by an apheresis nurse is an important step in the planning of appropriate vascular access for elective procedures. A small study of neurological patients receiving TPE showed only 50% of patients were able to complete a planned course of therapy with only peripheral access.<sup>7</sup> According to the 2007 International Apheresis Registry, peripheral access is more common in Europe and Australia (66–70%) than in Asia and the Americas (2–15%).<sup>11</sup> Ultrasound-assisted peripheral venous access has increased, thanks to small devices with intuitive interfaces optimized for vascular access, such as the Sonosite SII, which can be used by apheresis clinic staff.<sup>12</sup> CVC use was reduced to only 1–3% at an academic apheresis clinic in Toronto after they initiated an ultrasound-guided program for both therapeutic apheresis and cellular therapy collections.<sup>13,14</sup> The use of single-arm needle attachments has improved the ability to complete an apheresis course peripherally if a patient can tolerate the longer time required to perform a discontinuous flow procedure. Unfortunately, CVCs are often required due to small vein caliber, poor vascular tone, altered mental status, or the need for several procedures.<sup>15</sup> Patients with seizure disorders may also be better suited to CVC access over peripheral to protect against serious antecubital fossa trauma from the steel needles in the event of a seizure during the procedure. In children, peripheral venous access is not recommended given their small venous caliber and inability to tolerate immobility as required to complete a procedure.<sup>9,16</sup>

If CVC placement is necessary, the apheresis personnel should be involved to ensure appropriate catheter selection. Poiseuille's equation (in which  $R$  is proportional to viscosity  $\times$  length/radius) states that there are primarily three determinants of resistance to flow in a

catheter: radius, length, and viscosity.<sup>17</sup> Shorter catheters allow for less resistance to flow with the added benefit of allowing the anticoagulant to reach the blood sooner. A larger diameter offers less resistance and also ensures that a small clot or fibrous sheath will not block the lumen.<sup>17,18</sup>

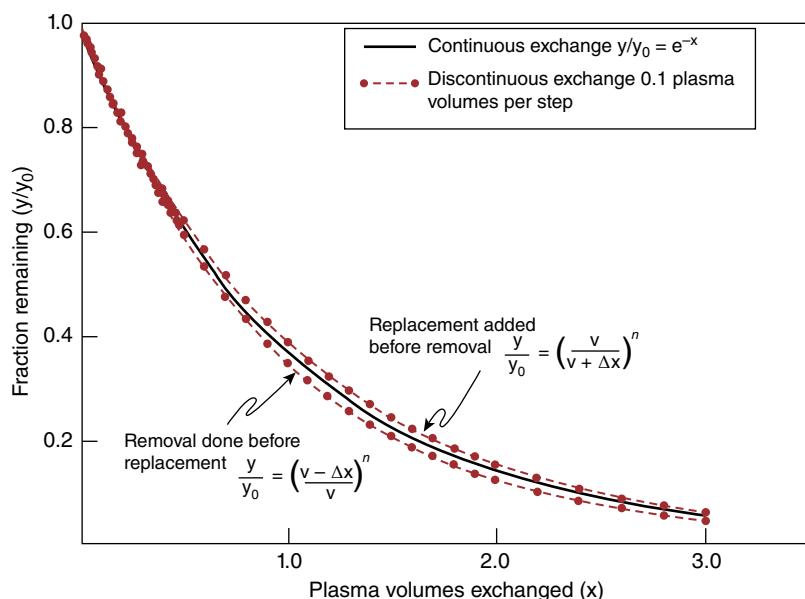
Shorter catheters allow for less resistance to flow with the added benefit of allowing the anticoagulant to reach the blood sooner. A larger diameter offers less resistance and also ensures that a small clot or fibrous sheath will not block the lumen.<sup>17</sup> The catheters must have sufficient rigidity so as not to collapse under negative pressure during blood removal.<sup>17,19</sup> In general, if a CVC can support hemodialysis, it will be sufficient for TPE. Double-lumen catheters are most commonly color coded with red for the draw line and blue as the return line. There are potential risks with all catheter tip positions, but it is generally accepted that the tip should be preferentially in the superior vena cava (SVC) or inferior vena cava (IVC), outside the pericardial sac, and should not touch the vein or heart wall at a sharp angle.<sup>20</sup> Radiographic confirmation of the catheter position is typically necessary prior to use. Recent advances in vascular access technology, including devices such as the Arrow vascular positioning system (VPS), which combine Doppler ultrasound, electrocardiography, and algorithmic logic, allow catheter use immediately after placement.<sup>19,21</sup> The majority of evidence-based guidelines on CVC care have been inferred from hemodialysis literature. In order to prevent fibrin sheaths and thrombosis, CVCs must be flushed with saline and locked with an anticoagulant after each use and at regular intervals when not in use. Catheter-locking solutions include heparin and sodium citrate (4%), with use of tissue plasminogen activator (tPA) instillation if a catheter used for regular procedures becomes problematic.<sup>10</sup> To reduce the risk of bleeding, the solution should be aspirated if the heparin dose is 1000 units per mL or greater.<sup>10</sup>

The average plasmapheresis treatment course for TTP is 13 procedures, but can extend up to several weeks.<sup>22</sup> Most other indications require 5–7 procedures for an urgent course of treatments, but chronic schedules may be preferred in some circumstances. For patients with renal disease who also require hemodialysis, the use of a pre-existing arteriovenous fistulae or grafts may be convenient, but these approaches are not typically taken if the patient requires access for apheresis alone. Rather, the use of subcutaneous port-CVC systems has gained in popularity due to their low rates of infection, long-term use, and patient satisfaction. Cost-savings and convenience have also been reported.<sup>10,23</sup>

### Exchange volume

The volume of plasma to be exchanged is usually based on the estimated plasma volume of the patient. The fraction of the unwanted compound that remains in patient's plasma volume decreases as an exponential decay due to the continuous mixing of the replacement solution with the patient's plasma during the plasmapheresis (Figure 25.1). After an exchange equal to the patient's plasma volume, the unwanted component will be reduced to approximately 35% of the initial value. Exchanging two times the patient's plasma volume further reduces the unwanted component only to approximately 15% of the initial value. A plasma exchange is therefore the most efficient at the beginning, and due to its diminishing effectiveness and increasing likelihood of possible adverse effects from the anticoagulant, the procedure is usually limited to between two and four hours.

The molecule targeted for removal by TPE is often an immunoglobulin G (IgG) antibody, which is approximately 50% extravascular.



**Figure 25.1** Calculated fraction of intravascular substance remaining during a plasma exchange, assuming no equilibration with extravascular material. Source: Chopek and McCullough (1980).<sup>31</sup> Reproduced with permission of American Association of Blood Banks.

Since the removal of accessible (i.e., intravascular) IgG becomes progressively less efficient during a TPE procedure, most practitioners limit an exchange to 1–1.5 times the patient's estimated plasma volume. An exchange of this magnitude will remove 60–75% of intravascular material while limiting side effects from depletion of normal plasma components. The intravascular IgG level will rise during the ensuing 1–2 days by equilibration with extravascular sources, and further removal by a subsequent exchange can then be undertaken more efficiently. It does not appear that a rebound overshoot in antibody levels occurs after plasma exchange, but rapid re-equilibration of IgG occurs because 50% of IgG is extravascular.<sup>24,25</sup> Because IgG removal is often the goal of TPE, it is worthwhile to consider certain aspects of IgG metabolism. The subclasses IgG1, IgG2, and IgG4 together constitute about 90% of total IgG. Their catabolic rates are proportional to total IgG level, and their half-lives are therefore inversely proportional to concentration. Animal studies concerning levels of specific antibody were interpreted to show that the synthetic rate for IgG exhibits negative feedback, increasing when IgG or specific antibody levels or both are lower.<sup>26</sup> However, Junghans<sup>27</sup> has shown that "knockout" mice genetically deficient for the FcRn receptor catabolize IgG quite rapidly and maintain very low IgG levels, but they have the same IgG synthetic rate as normal mice. This argues against negative feedback regulation of IgG synthesis and suggests that a reduction in antibody levels induced by TPE would not produce a meaningful "rebound" increase in IgG synthesis. Moreover, serial monitoring of pathologic as well as post-vaccination antibodies in myasthenic patients failed to demonstrate any rebound phenomenon.<sup>28</sup>

A reduction of 80–90% can be obtained with four to six exchanges in 14 days. A typical treatment series for IgG removal consists of five procedures approximately every other day. The effects of a series of TPEs on extravascular, intravascular, and total IgG are shown schematically in Figure 25.2. Treatment of some patients such as those with TTP or acute Guillain–Barré disease might involve plasma exchange daily for several days. There appears to be a limit of the linear removal of antibodies in

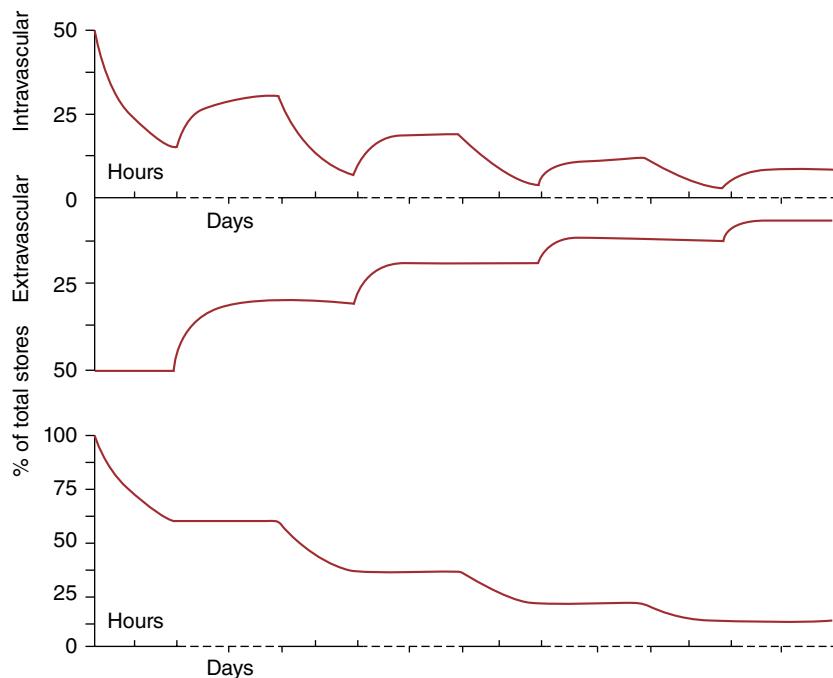
patients undergoing peri-transplant desensitization with TPE and IVIg,<sup>29</sup> likely attributable to a component of maintained antibody synthesis.

A model that takes into account the size of the exchange relative to the patient's blood volume, the amount of material available to exchange, the amount of material in both the intravascular and extravascular compartments, the mobility of the material between the pools, and the production and catabolic rate of the material showed good agreement with in vivo observations in patients with hyperbilirubinemia and hypercholesterolemia.<sup>25</sup> Many of these parameters cannot be measured in vivo. Efforts to estimate them do not fit clinical observations, which is likely due to the dynamic clinical state of patients experiencing disease processes that require therapeutic apheresis.<sup>30</sup>

### Replacement solutions

The goal of plasma exchange is to remove a pathologic material in an euvolemic manner, with minimal perturbation of homeostatic volume status. Saline replacement alone can suffice when only 500–1000 mL of plasma is removed in a manual plasmapheresis, but in order to maintain intravascular volume, a replacement volume of colloid solutions (Table 25.1) equivalent to the removed plasma volume is given during the dynamic fluid exchange. If a patient is hemodynamically unstable, the management of replacement fluids can be adjusted to compensate. This can be done by infusing the replacement solutions at either (1) less than 100% fluid balance (volume replaced is less than volume removed) if the patient is fluid overloaded, or (2) greater than 100% fluid balance (volume replaced is greater than volume removed) if the patient is in need of extra fluids.

For the majority of indications, the standard replacement medium is 5% human serum albumin in normal saline;<sup>32</sup> however, substitution of 25–50% of the total with saline has been shown to be well tolerated in certain patient groups.<sup>33</sup> Although it is a pooled product, 5% albumin is preferred over plasma as a source of replacement colloid because (1) it is treated to inactivate bloodborne



**Figure 25.2** Computer-generated curve estimating amounts of intravascular and extravascular immunoglobulin (IgG; upper curves), and total IgG (lower curve) during a course of four one-plasma volume therapeutic plasma exchanges with an IgG-free replacement medium. Published formulas were used for rates of removal during exchanges and re-equilibration after exchanges. No correction was made for continuing synthesis.

**Table 25.1** Colloid Replacement Fluids for TPE

Fluid	Advantages	Disadvantages
5% Albumin	Virus inactivation Ease of use Reactions rare	High cost Most proteins not replaced
Single-donor plasma*	All proteins replaced	High cost Inconvenient <sup>b</sup> Citrate reactions Transfusion reactions possible Viral infection risk Depletes precious supply
Pooled solvent/detergent-treated plasma	All proteins partially replaced <sup>c</sup> Lipid-coated viruses inactivated Pooled plasma dilutes single donor allergens	Very high cost Inconvenient <sup>b</sup> Citrate reactions Transfusion reactions possible Depletes precious supply
6% Hetastarch	Low cost Viral safety Ease of use Slow catabolism	No proteins replaced Hypotensive reactions Dosage limit

\* Fresh-frozen plasma, plasma frozen in 24 hours, or plasma cryoprecipitate reduced.

<sup>b</sup>Must be thawed before use and must be ABO compatible.

<sup>c</sup>Coagulation factors  $\geq 80\%$  of normal levels.

TPE: therapeutic plasma exchange; US: United States.

pathogens, (2) it can be given without regard to blood type, and (3) it does not require thawing or other preparation before use. The transient depletion of coagulation factors and other plasma proteins that occurs with albumin solution replacement is mild enough that fresh-frozen plasma (FFP) replacement is not typically warranted in patients without known coagulation disorders. An exception to this might be during a series of several daily procedures when it may be prudent to monitor the fibrinogen level and platelet count to assess the need for platelets, FFP, or cryoprecipitate. When there is a need to replace essential plasma constituents, such as with TTP

or depleted coagulation factors, FFP should be used. The advantages of plasma include supplementation of immunoglobulins, antithrombin, and fibrinogen, along with other proteins. The disadvantages of plasma include a viral transmission risk, an increased citrate load, ABO incompatibility risk, allergic reactions, and the potential for sensitization. Partial replacement with saline (crystalloid) can be used; however, this should not exceed 25–30% of the replacement volume.<sup>18</sup>

There are a few circumstances in which the replacement of patient plasma with donor plasma is used. Patients with ITP

customarily receive exchanges with FFP in light of abundant evidence that patients with TTP respond better to plasma than to albumin. Cryoprecipitate-reduced plasma is also effective for this purpose.<sup>19</sup> Some plasma may also be given toward the end of an exchange to patients with preexisting thrombocytopenia or humoral coagulopathy, who are considered to be at increased risk for bleeding complications when the dilutional coagulopathy of an albumin exchange is superimposed, or to patients with ongoing blood loss regardless of pretreatment coagulation status.

One group reported infusing a solution of hydroxyethyl starch (HES), a less costly volume expander, in the early part of an exchange.<sup>20</sup> They reason that recommended dosage limitations for HES will not actually be exceeded because much of the infused HES will be removed in exchange for albumin infused later. This group has seen successes, albeit with a higher incidence of side effects. HES is not recommended for patients with renal impairment, underlying coagulopathy, or a history of hypersensitivity to HES.

The apheresis physician should determine whether any combination of replacement fluids is used and, if so, the relative proportions of each fluid. This should be based upon the patient's cardiovascular stability, underlying disease, frequency of procedures, coagulation status, and cost.

### Complex circuits

Critically ill patients who may be candidates for therapeutic apheresis may have comorbidities that require other extracorporeal interventions. Extracorporeal membrane oxygenation (ECMO) and continuous renal replacement therapy (CRRT) are two common therapies that affect the circuit volume and access placements.<sup>34</sup> The total circuit volume must be added to the patient's total blood volume when performing procedure calculations. For concomitant ECMO procedures, anticoagulation is monitored and adjusted by the perfusionist and so the TPE protocol usually does not require supplemental anticoagulation. Apheresis machines can be directly connected to ECMO devices in various configurations so additional vascular access is not required.<sup>35,36</sup> When CRRT cannot be paused to allow TPE through the existing vascular access, membrane-based TPE or a second catheter may be considered.<sup>5</sup>

### Biochemical changes resulting from plasma exchange

Removal of such a large volume of plasma has several biochemical effects (Table 25.2).<sup>31,37,38</sup> Because some platelets are in the plasma being removed, there is about a 30% decrease in the platelet count, which takes about three days to return to baseline. The changes in the proteins IgG, IgM, IgA, factor V, ferritin, transferrin, lactic dehydrogenase, serum glutamic oxaloacetic transaminase, and alkaline phosphatase closely follow the decrease expected based on the volume of plasma removed. When no FFP is used for replacement, coagulation test results are abnormal at the end of the plasma exchange.<sup>39</sup> For instance, the prothrombin time is usually 20 seconds or more, the partial thromboplastin time is more than 180 seconds, and the fibrinogen is decreased by about 70%.<sup>31,38</sup> These test values return to baseline in about 24 hours, except fibrinogen, which normalizes in 72 hours (Figure 25.3).<sup>31</sup>

Complement components C3, C4, and CH50 can be depleted when albumin is used as the replacement fluid; however, this is unlikely unless plasma exchange is performed daily for several days due to its rapid rate of synthesis.<sup>37</sup>

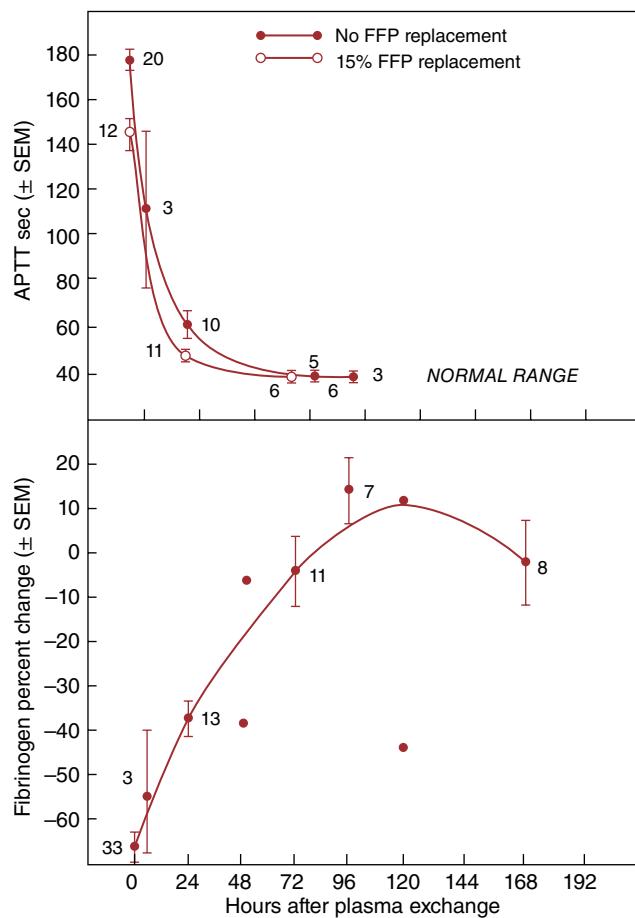
There are no clinically important changes in electrolytes as a result of plasma exchange (Table 25.2). The ionized calcium level is particularly important because citrate is often used as the procedural anticoagulant, exerting this effect by binding calcium. Citrate toxicity thus manifests through hypocalcemia, causing symptoms of paresthesia, muscle cramping, tremors, shivering, lightheadedness, and anxiety; when more severe, it can cause grand mal seizures, tetany, and, most dangerous of all, electrocardiographic abnormalities (Figure 25.4). These serious physiological disturbances occur far before potential bleeding due to a theoretical citrate-induced coagulopathy. If FFP is used for replacement, this provides additional citrate. Studies of citrate and calcium metabolism during normal-donor plateletpheresis have established that symptoms only begin to occur when the rate of citrate infusion exceeds 60 mg/kg/hour.<sup>40-43</sup> Although there is some reduction in ionized calcium levels even when albumin is used as the replacement solution,<sup>44</sup> the citrate infusion rates are below 60 mg/kg/hour and only approach this rate when FFP is used and the flow rates are substantial. Thus, supplementation with calcium during plasma exchange should be based on each patient's situation.

**Table 25.2** Comparison of Changes Induced by Plasma Exchange of 1.0–1.5 Plasma Volumes with Equal Volume Replacement

	Albumin Replacement	FFP Replacement
Hematology	↓ Platelets (30–50%) ↑ Granulocytes (2000–3000/mL) ↓ Hemoglobin (10–15%)	↓ Platelets (30–50%) ↑ Granulocytes (2000–3000/mL) No change in hemoglobin
Proteins	↓ Pathological antibodies (60–75%) (60–75%) ↓ All other proteins (60–75%) Long-term effects depend upon TER, FCR, and S (see text)	↓ Pathological antibodies All other proteins change to approximate levels present in FFP
Coagulation	↓ Individual factors (60–75%) Transient coagulopathy (24–48 hours)	All factors approximate levels in FFP
Electrolytes	Slight ↓ potassium Albumin: Ø Bicarbonate (6 mEq/L) ↑ Chloride (4 mEq/L) PPF: ↑ Bicarbonate ↓ Chloride	↓ Potassium (0.7 mEq/L) ↑ Bicarbonate (3 mEq/L) ↓ Chloride (6 mEq/L)
Citrate and calcium	Slight ↑ citrate (0.2 mM/L) ↓ Total calcium (1.4 mg/dL) ↓ Ionized calcium (0.5 mEq/L)	↑ Citrate (1.1 mM/L) Slight ↓ total calcium (0.3 mg/dL) ↓ Ionized calcium (0.6 mEq/L)

FCC: fractional catabolic rate; FFP: fresh-frozen plasma; PPF: plasma protein fraction; S: synthesis; TER: transcapillary escape rate.

Source: Chopek and McCullough (1980).<sup>31</sup> Reproduced with permission of American Association of Blood Banks.

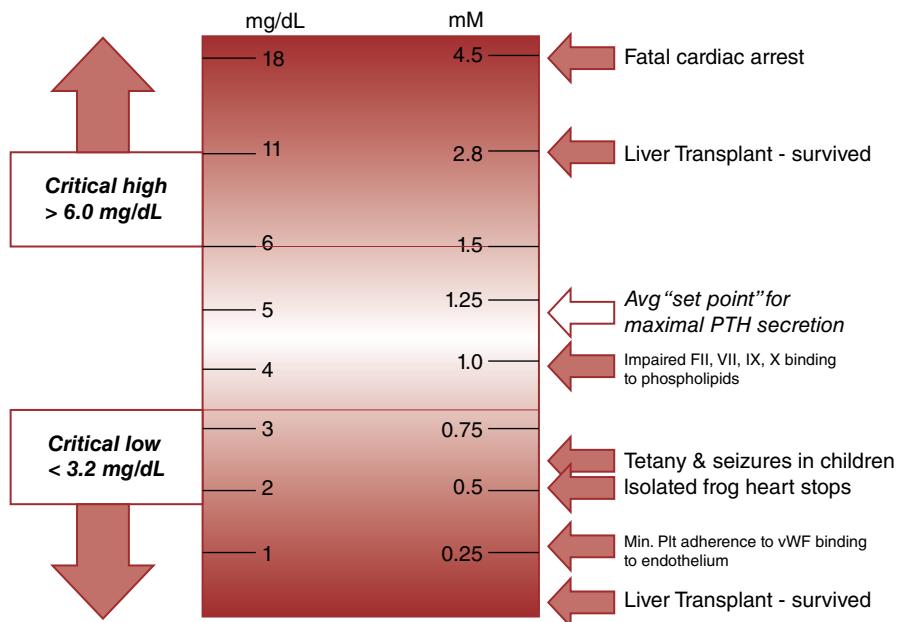


**Figure 25.3** Normalization of fibrinogen level and activated partial thromboplastin time after plasma exchange using albumin for replacement.<sup>31</sup>

The differences between albumin and FFP as replacement solutions are summarized in Table 25.2.

### Complications of plasma exchange

Apheresis of normal donors for the collection of blood components is well tolerated with few side effects and only very rare serious complications. However, therapeutic apheresis is carried out in ill patients who should not be expected to react the same as a healthy donor.<sup>45</sup> Deaths in patients undergoing TPE may be attributable to progression of their serious underlying medical condition.<sup>46,47</sup> The mortality rate is estimated to be three per 10,000 procedures.<sup>48</sup> Most deaths have resulted from cardiac or respiratory arrest, but deaths due to anaphylaxis, pulmonary embolus, and vascular perforation have also been reported.<sup>49</sup> The FDA receives an average of three medical device reports a year related to therapeutic apheresis devices, and in a review in 2015, none of the reports from the prior decade was attributable to the device, verifying the overall safety of this class of instruments.<sup>50,51</sup> The nature and incidence of complications will depend somewhat on the condition of the patient prior to plasma exchange. In one comprehensive report of complications of plasma exchange, side effects occurred during 12% of procedures and involved 40% of patients.<sup>52</sup> The incidence of severe complications was 0.5% of procedures. A national registry of therapeutic apheresis procedures in the former East Germany reported complications in 22% of 1945 procedures in 419 patients.<sup>53</sup> There were severe complications in 2%, including cardiac arrhythmia, bronchospasm, adult respiratory distress syndrome (ARDS), and thromboembolic problems. Of the 419 patients, 87 died; of these, 64 died of their underlying disease and 19 of related causes not thought to be due to the plasma exchange. Twelve patients died during or immediately after the plasma exchange, and four of these fatalities were thought to be due to the apheresis procedure. Two of these fatalities resulted from ARDS, one from myocardial infarction, and one from pulmonary embolus. The complication rate was almost twice as great when FFP was used as the replacement solution



**Figure 25.4** Relationship of clinical signs and symptoms to ionized calcium concentration.

compared to albumin, although the nature of the complications was not described. The Canadian Apheresis Study Group reported that adverse reactions occurred in about 9% of 58,000 procedures.<sup>54</sup> About two-thirds of these were mild, and only 8% were severe, resulting in a severe reaction rate of 7 per 1000 procedures. In a recent four-year series of pediatric ICU patients undergoing over 600 TPE procedures, adverse events were reported in 16.3% of procedures. The majority of complications were related to circuit clotting or vascular access malfunction (69%) without a significant impact to the patient.<sup>55</sup>

The complications of plasma exchange are due to vascular access, replacement solutions, or the procedure itself.

### Vascular access

Placement of vascular access devices is often required, although peripheral venous access should be used whenever possible. There are numerous complications associated with catheters, some of which can be fatal. Vascular access devices may cause either immediate or delayed adverse effects. Immediate adverse effects include bleeding, arterial puncture, arrhythmia, air embolism, thoracic duct injury, catheter malposition, pneumothorax, and hemothorax.<sup>20,56–58</sup> Delayed complications include infection, venous thrombosis, venous sclerosis, catheter migration, catheter embolization, myocardial perforation, and nerve injury.<sup>9,56,57,59–61</sup> Complications with CVCs differ with insertion sites and physician experience, with more than 15% of patients experiencing some type of complication.<sup>62–64</sup> In one study of 385 CVC procedures, there was an overall mechanical complications rate of 33%.<sup>56</sup> Another study showed that 11% (3 of 28) of CVCs placed in patients undergoing TPE procedures experienced life-threatening complications associated with catheter use. These complications were pneumothorax, air embolus upon removal, and catheter-related bloodstream infection.<sup>7</sup> Infectious complications range from 5% to 26%; however, the implementation of procedures such as checklists and care bundles has been shown to reduce catheter-related bloodstream infections.<sup>65–67</sup> The reported rate of vascular access complications associated with TPE procedures is lower when compared to hemodialysis procedures; however, this is likely explained by the longer duration of catheter use along with higher comorbidities in hemodialysis patients.

### Replacement solutions

Complications related to replacement solutions include citrate-induced hypocalcemia, coagulation factor depletion, depletion of other functional proteins, electrolyte abnormalities, and transfusion reactions or disease transmission when plasma is used. Adverse reactions to albumin are rare.<sup>68</sup> Exchanges of plasma for albumin produce temporary deficiencies of other plasma proteins, such as coagulation factors; however, these are usually subclinical, and levels are rapidly restored by ongoing synthesis and reequilibration.<sup>69</sup> Hypotonic replacement solutions should not be infused during the procedure as this can cause hemolysis.

Allergic reactions such as urticaria or mild fevers are rather common even when albumin is the replacement solution. Plasma replacement carries a higher risk of urticarial and hypocalcemic reactions.<sup>70</sup> Progenic reactions can occur to specific lots of albumin.<sup>68</sup> An unusual case of hemolysis due to infusion of hypotonic replacement solution has been reported.<sup>71</sup> The 25% albumin was diluted to 5% in sterile water rather than saline, resulting in the hypotonic solution. Depletion of coagulation factors leading to a bleeding diathesis should not occur because this can usually be

prevented by spacing out TPE procedures or judiciously using an FFP as part of the replacement solutions. The same is true for other functional proteins and electrolytes. If FFP is used, febrile or allergic reactions are more common because of the proteins in the plasma. Antibodies in the plasma may cause transfusion-related acute lung injury (TRALI),<sup>72</sup> and ABO-incompatible plasma can cause hemolysis. When reactions occur during plasma replacement, it may be difficult to identify the offending unit because of the timing or the reaction in relation to the infusion rate.

Inclusion of some proportion of saline in replacement fluids is particularly desirable for the treatment of hyperviscosity syndromes to promote rapid correction of either plasma or whole blood viscosity. There is an economic incentive to substitute some saline for albumin as well. In a study of 3642 procedures, 67% of which used 20% saline and with 105% fluid replacement, the incidence of hypotension increased from 1.5% to 3% in partial-saline procedures compared to all-albumin procedures.<sup>73</sup> Increasing the fluid replacement to 110% can be well tolerated even with up to 50% saline in appropriate patients without vasomotor, renal, or cardiopulmonary comorbidities.

### Apheresis procedure

Mild reactions are rather common during plasma exchange; they are usually chills (possibly due to infusion of room-temperature replacement solutions) or lightheadedness (possibly due to a vagal reaction). These can also be symptoms of hypocalcemia due to citrate infusion, and so when the symptoms begin the operator often slows the blood flow rate and the symptoms subside. Fluid shifts can also contribute to procedural hypotension or hypertension that are usually well tolerated but may be problematic for patients with baseline fluid overload, congestive heart failure, or arrhythmias. Transient hypotension can be caused by hypovolemia resulting from the blood required to fill the extracorporeal circuit. Thus, the volume of the circuit in relation to the patient's blood volume must be considered, and for smaller patients it may be desirable to prime the circuit with albumin. If this is done, the dilutional effect of the priming solution must also be considered. Hypertension can occur if the volume of fluid returned exceeds that removed. Thus, it is important that the operator closely monitor the fluid balance during the procedure.

Anaphylactoid reactions consisting of flushing, hypotension, bradycardia, and dyspnea have occurred in patients taking angiotensin-converting enzyme (ACE) inhibitors for hypertension.<sup>74–76</sup> Bradykinin (BK) causes vasodilation and smooth muscle contraction in some tissues. ACE is the major peptidase that inactivates BK.<sup>76</sup> Patients receiving ACE inhibitor drugs have less ability to inactivate BK and can experience serious hypotension and may not be responsive to fluids.<sup>77</sup> Thus, situations that promote BK release may lead to hypotensive reactions in patients taking ACE inhibitors. This is thought to occur during therapeutic apheresis, possibly due to contact between the patient's blood and the foreign surfaces of plastic bags, tubing, centrifuge systems, and blood filters. Discontinuation of the ACE inhibitor for 24–48 hours before therapeutic apheresis prevents these reactions.<sup>76</sup>

Anticoagulation must be carefully managed during any therapeutic apheresis procedure. Fortunately, clinically serious thromboembolic complications are rare, and the most common clotting issues relate to catheter function or machine flow rates, not patient morbidity. Bleeding complications are mitigated by thoughtful replacement fluid selection and routine use of anticoagulants in the circuit. Citrate is a popular anticoagulant because of its short

half-life and low cost. Complications related to citrate are more likely when a blood product is the replacement fluid (discussed above) since they provide an additional source of citrate and usually begin with perioral and distal paresthesias heralding citrate-induced hypocalcemia, but metabolic alkalosis can also occur. Patients with liver dysfunction, altered mental status, or pediatric patients are at particular risk, but all patients are at risk due to the prolonged exposure throughout the procedure. Patients may also experience gastrointestinal issues, such as nausea or vomiting, and muscle cramps after the early sensory disturbances. In extreme cases, tetany, prolongation of the QT interval, arrhythmias, or hypotension may occur if the deficiency is not treated (Figure 25.4). It may be prudent to evaluate pre-, intra-, and post-procedural ionized calcium levels and perform electrocardiogram monitoring in patients who are unable to reliably communicate symptoms of hypocalcemia (e.g., pediatric and altered mental status patients) in addition to providing empiric calcium repletion during the procedure. Treatment of citrate-related complications consists of either slowing the rate of the procedure or administration of supplemental calcium. If resolution of symptoms does not occur, the evaluation of a magnesium level may be appropriate.

Iatrogenic removal of protein-bound medications or therapeutic antibodies can also be an adverse effect of plasma exchange since it is a nonspecific removal of plasma proteins and their bound analytes. Careful review of the patient's medications beyond ACI inhibitors and therapeutic anticoagulants along with consideration of the volume of distribution, dosing, and pharmacokinetics is important prior to the first procedure.<sup>78,79</sup> Most pharmacologic agents should be administered after, rather than before, a plasma exchange if possible to reduce iatrogenic removal. Agents on a continuous infusion may require intraprocedural monitoring or titration.<sup>80</sup>

### **Therapeutic apheresis in children**

TPE or cytapheresis can be performed on even very small children.<sup>81,82</sup> The major consideration is that the instruments are designed for adults, and thus the extracorporeal volume may be too large for small patients. This can be overcome by priming the instrument with red cells, albumin, or other combinations of fluids. Vascular access can also present additional concerns since insertion and maintenance can be more complicated in children.<sup>83</sup> The blood flow rates through the instrument are not high for adults but may represent a large portion of a small child's blood volume. Therefore, problems can arise quickly if there are difficulties with the lines or blood flow. Also, the rate of return of blood and solutions can be much greater in relation to the total blood volume of a small child, and thus citrate or other complications can occur more frequently than in adults if adjustments in blood flow are not made to reflect the small patient's blood volume.<sup>84</sup> Critically ill pediatric patients are more frequently supported by additional extracorporeal therapies than adults, making the impact of circuit volume an even more important factor in already complex and fragile pathophysiologic states.<sup>85</sup> Nearly a quarter of >1000 apheresis procedures reported from a quaternary pediatric hospital were for indications considered to be controversial or of uncertain benefit, reflecting the additional challenges of consultations for apheresis in children.<sup>86</sup>

### **Diseases treated by plasma exchange**

Apheresis allows for the separation of the different blood elements and nonselective removal of pathogenic cells, antibodies, toxic metabolites, and other plasma molecules. The therapeutic effect is based on different mechanisms, including (1) removing antibodies

directly involved in the pathogenesis of the disease (e.g., autoantibodies against the acetylcholine receptor in myasthenia gravis or alloantibodies causing solid organ transplant rejection), (2) removing toxic metabolites (e.g., acute liver failure), and (3) removing other plasma molecules and proteins (e.g., removal of lipid molecules in familial hypercholesterolemia). Not all diseases in which antibodies play a role in the pathophysiologic mechanism demonstrate a positive clinical response to apheresis treatment. This is the case of dermatomyositis and rheumatoid arthritis among others.

One of the main challenges in apheresis medicine, as in medicine in general, is obtaining high-quality evidence to support therapeutic decisions. There is a limited number of randomized controlled trials evaluating the efficacy of therapeutic apheresis, resulting in decisions made based on low-quality evidence. Some of the factors contributing to the scarcity of clinical studies include the low frequency of the disease, which results in small sample size, the challenges of recruiting large institutions with large apheresis capacity to enroll patients, defining clinical equipoise (i.e., reluctant providers to randomize patients based on their own beliefs), designing a control group (i.e., considering what would be an appropriate placebo), and funding.

The American Society for Apheresis (ASFA) took the lead in appraising the literature and developing guidelines with an indication category system, and evaluating the strength of the evidence using the GRADE system.<sup>87</sup> The guidelines are typically revised every three years, being the eight and last edition published in 2019. Each disease has a fact sheet describing the most relevant aspects of the pathophysiology and the category indication for the use of apheresis. There are currently 84 fact sheets included in the last edition. The indications are classified based on categories: Category I: diseases for which apheresis therapy is a standard first-line therapy; Category II: diseases in which apheresis is a valuable second-line therapy when first-line measures fail or are poorly tolerated; Category III: diseases in which there is uncertainty due to inadequate data or controversy due to conflicting reports; and Category IV: diseases in which there are negative data from controlled trials or anecdotal reports.

In this section, we group diseases classified as categories I and II based on the system involved and provide a brief description of the disease and the evidence to support TPE.

### **Therapeutic plasma exchange for neurologic disorders**

Immune-mediated processes, especially the formation of circulating antibodies to structures in the nervous system, have been implicated in several neurologic diseases, and TPE has become an important therapy for some of them. See Table 25.3 with a list of neurologic conditions classified as categories I or II. Neurologic disease with categories III and IV are not included in this table and can be found in the original publication.

### **Guillain–Barré syndrome**

Guillain–Barré syndrome (GBS) affects the peripheral nervous system. A typical clinical course begins with symmetric distal paresthesias, followed by leg and arm weakness. Symptoms spread proximally and reach a peak of severity by 14–30 days after onset. About one-fourth of patients with GBS have mild illness and remain ambulatory throughout. A nerve conduction defect resulting from demyelination is found in most cases, but there are variants in which axonal damage is evident in motor fibers or both motor and sensory fibers.<sup>88,89</sup>

**Table 25.3** Therapeutic Plasma Exchange for Neurologic Disorders

Disease Name	Indication	Category	Grade
Acute inflammatory demyelinating polyneuropathy (Guillain–Barré syndrome)		I	1A
Chronic inflammatory demyelinating polyradiculoneuropathy (CIDP)		I	1B
Myasthenia gravis		I	1B
	Acute, short-term treatment	I	1B
	Long-term treatment	II	2B
<i>N</i> -Methyl-D-aspartate receptor antibody encephalitis		I	1C
Paraproteinemic demyelinating polyneuropathies	IgG/IgA/IgM	I	1B
Acute disseminated encephalomyelitis (ADEM)		II	2C
Lambert–Eaton myasthenic syndrome		II	2C
Multiple sclerosis	Acute attack/relapse	II	1A
Neuromyelitis optica spectrum disorder (NMOSD)	Acute	II	1B
PANDAS	PANDAS exacerbation	II	1B
Phytanic acid storage disease (Refsum's disease)		II	2C
Steroid-responsive encephalopathy associated with autoimmune thyroiditis (Hashimoto's encephalopathy)		II	2C
Voltage-gated potassium channel antibodies		II	1C

PANDAS: pediatric autoimmune neuropsychiatric disorders associated with streptococcal infections.

Source: Based on Padmanabhan *et al.* (2019).<sup>87</sup>

GBS is often associated with a history of recent infection with *Campylobacter jejuni*, cytomegalovirus, Epstein–Barr virus, *Mycoplasma pneumoniae*, or other organisms. It has been postulated that antibodies are formed in response to a strain-specific lipopolysaccharide that is antigenically similar to one of the myelin gangliosides (e.g., GM1 after *C. jejuni* infection).<sup>88,90</sup> Spontaneous recovery is the usual outcome of GBS and may be associated with the decline in antibody levels. Neither oral nor pulse intravenous steroids are helpful in GBS; however, large randomized controlled trials have documented that TPE can shorten recovery time and reduce disability.<sup>91,92</sup>

Later studies have shown that intravenous immunoglobulin (IVIG) is also beneficial in GBS. A large multicenter trial compared IVIG, TPE, and TPE followed by IVIG, with 121–130 patients in each treatment group.<sup>93</sup> The trends favored TPE plus IVIG, although none of the differences was statistically significant. Some authorities now favor IVIG for initial treatment, citing its relative simplicity, wider availability, and lower incidence of adverse effects.<sup>88</sup> Meta-analyses have supported this approach.<sup>94,95</sup>

### Chronic inflammatory demyelinating polyneuropathy

Chronic inflammatory demyelinating polyneuropathy (CIDP) is an acquired neuropathy that may follow either a continuously progressive or an intermittent, relapsing course. Both weakness and sensory loss are usually present, and both distal and proximal sites may be affected. Nerve conduction studies suggest demyelination, which may be apparent in nerve biopsy tissue if this is obtained. Patchy inflammatory infiltrates may be seen in nerve root biopsies. The cerebrospinal fluid usually has a moderately elevated protein concentration and a cell count less than 10/ $\mu$ L.<sup>96</sup> Although it is usually idiopathic, CIDP may also occur in the context of an associated condition, such as inflammatory bowel disease, chronic active hepatitis, connective tissue disease, Hodgkin's disease, human immunodeficiency virus (HIV) infection, or monoclonal gammopathy.<sup>96</sup>

The precise cause of CIDP remains unknown; however, the disease associations, the clinical similarities to GBS, and histopathology suggest an immune process. The presence of a monoclonal protein in some cases points to an antibody mediated disorder, as does the finding that an animal model of CIDP (experimental allergic neuritis) can be passively transferred with serum.

Treatment options include corticosteroids, IVIG or TPE, or a combination of treatments. TPE has been shown to be effective

in improving symptoms in randomized clinical trials.<sup>97–99</sup> Trials comparing TPE vs. IVIG demonstrated similar positive outcomes.<sup>100,101</sup>

### Myasthenia gravis

About 85% of patients with myasthenia have circulating antibodies to a portion of the  $\alpha$ -subunit of the acetylcholine (ACh) receptor molecule (AChR) on the motor end plate of muscle cells. Approximately 10% of the patients will demonstrate antibodies against muscle-specific kinase (MuSK), usually associated with more severe disease; a minority of patients will demonstrate antibodies against lipoprotein receptor-related protein 4 (LRP4) usually associated with less severe presentation.

TPE is a widely accepted therapy for myasthenia; however, it is not recommended for all patients. It is instead reserved for those with severe disease and those who are intolerant or unresponsive to other therapies. Patients whose breathing, swallowing, or walking is inadequate are good candidates for the rapid improvement from TPE, even as an initial treatment.<sup>102</sup> Numerous trials have suggested that TPE can lead to rapid symptomatic improvement in concert with lower levels of circulating AChR antibody and in patients that are refractory to other therapies.<sup>103</sup> TPE has also been effective in patients who test negative for antibody, suggesting that some pathogenic antibodies may not be detected by current assays.<sup>104–106</sup> Myasthenia may respond to IVIG, and it is considered equally effective with TPE.<sup>107</sup> Immunomodulators are also indicated to decrease ongoing antibody production.<sup>108</sup>

### *N*-Methyl-D-aspartate receptor antibody encephalitis

*N*-Methyl-D-aspartate receptor (NMDAR) encephalitis is the most frequent form of autoimmune encephalitis characterized by psychiatric (hallucinations, catatonia, and altered behavior) and autonomic (changes in blood pressure and heart rate, and excess salivation) symptoms. It is most commonly seen in children and young adults with female predominance. Some patients present with an occult tumor, in particular females with ovarian teratoma. Pathophysiology of the disease involves the presence of IgG antibodies directed against NMDAR. First-line treatment options include the treatment of the tumor if present, steroids, TPE, and IVIG. There are small case series evaluating the efficacy of TPE with positive results.<sup>109,110</sup> Second-line therapy includes immunomodulators such as rituximab, mycophenolate mofetil, and cyclophosphamide.

### Paraproteinemic demyelinating polyneuropathies

Patients with monoclonal gammopathies often present with some sort of polyneuropathy. Polyneuropathy classification can be rather complex considering their presentation (acute, subacute, and chronic), nerves involvement (sensory and/or motor fibers), and type of immunoglobulin involved (IgG, IgA, and IgM).

Most clinical features of neuropathies associated with monoclonal gammopathy are similar to CIDP, and although progression may be slower overall, spontaneous improvement is uncommon. The prevalence of neuropathy is higher in patients with IgM paraproteins than in those with IgG or IgA except in osteosclerotic myeloma, where the prevalence of neuropathy with IgG or IgA proteins is quite high, sometimes as a part of the POEMS syndrome (polyneuropathy, organomegaly, endocrinopathy, monoclonal protein, and skin changes). Patients with POEMS do not benefit from TPE. Patients with multiple myeloma or Waldenström's macroglobulinemia should be treated with appropriate chemotherapy and may experience improvement in the neuropathy as a result. Patients with IgM-associated neuropathy can present antibodies against myelin-associated glycoprotein (MAG). These patients typically present with distal sensory involvement. Current available data using immunotherapies, including the use of TPE, demonstrated very limited benefit on these patients.<sup>111</sup>

TPE can be helpful in MGUS-associated neuropathy. In a sham-controlled trial in 39 patients, twice-weekly exchange led to improvement in disability scores, weakness scores, and electrodiagnostic parameters in the blinded portion of the trial. Scores also improved in sham-treated patients who received true TPE in an open follow-up study.<sup>112</sup> In this and other studies, a response was noted more frequently in patients with IgG and IgA paraproteins than in those with IgM.

### Acute disseminated encephalomyelitis

Acute disseminated encephalomyelitis (ADEM) affects the central nervous system, in particular the white matter of the brain and the spinal cord. Pathophysiology of the disease involves demyelinating lesions associated with an autoimmune destruction of oligodendrocytes. The onset of neurologic symptoms usually follows a viral or bacterial infection and includes acute encephalopathy and multifocal neurologic deficits such as weakness, ataxia, seizures, and others. It is more frequent in children and has good prognosis. The diagnosis is made based on clinical symptoms and MRI findings demonstrating demyelinating lesions. Treatment involves immunosuppressive medication, including high-dose corticosteroids. Although there are no clinical trials to support its use, TPE has demonstrated positive outcomes and it is considered second-line therapy for patients who do not respond to steroids.<sup>113</sup>

### Lambert-Eaton myasthenic syndrome

Lambert-Eaton myasthenic syndrome (LEMS) is characterized by muscular weakness and fatigue. The syndrome is most often seen in patients with autoimmune diseases or cancer, with almost half of cases being in patients with small cell lung cancer. The pathophysiology of LEMS involves the neuromuscular junction affecting the nerve cell ending. Autoantibodies directed at the P/Q type voltage-gated calcium channel (VGCC) are found in the majority of patients. These antibodies reduce the amount of ACh released during depolarization events, causing weakness in affected skeletal muscles as well as dysfunction in autonomic nerves.<sup>114,115</sup>

Curiously, cholinesterase inhibitors are not as effective in LEMS as they are in myasthenia gravis.<sup>116,117</sup> More useful are agents that

prolong nerve action potentials by blocking voltage-gated potassium channels (VGKCs), such as aminopyridines, which are the first choice for symptomatic control in LEMS. Immunosuppressive drugs such as prednisone and azathioprine may also be beneficial in LEMS, and paraneoplastic cases may respond to specific antitumor therapy.<sup>114,116</sup>

TPE has demonstrated positive results in LEMS in the context of case series and case reports. Responses are usually less dramatic than those seen in myasthenia gravis. IVIG has also been reported to be active in both the short and long terms.<sup>116,118</sup>

### Multiple sclerosis

Multiple sclerosis (MS) is a disease characterized by localized neurologic dysfunction that is caused by demyelinated "plaques" in the CNS. The clinical presentation can be in the form of acute attacks that resolve fully or partially (relapsing-remitting), seen in approximately 70% of patients, or the form of a slow, continual progression of disease (chronic progressive). The frequency of attacks in relapsing-remitting MS tends to decrease with disease duration, and over time some patients evolve to a chronic progressive pattern.<sup>119</sup>

The radiologic hallmark of MS is the presence of discrete areas or plaques of demyelination in white matter, which are easily visualized with magnetic resonance imaging (MRI). The mechanism of the disease remains unexplained; it is suspected a combination of humoral and cellular immune system dysregulation.<sup>105,120</sup> These demyelinated plaques have four distinct histopathologic features, with the type II characterized by the deposition of complement and antibodies.<sup>111</sup> Plasma exchange is most effective in patients with type II lesions.<sup>121</sup>

Autoantibodies to myelin basic protein (MBP) or myelin oligodendrocyte glycoprotein (MOG) can be found both in CNS lesions<sup>122</sup> and in serum<sup>123</sup> in some MS patients. Although they are predictive of progression to MS in patients with a clinically isolated demyelinating event,<sup>124</sup> such antibodies do not produce MS in experimental animals.<sup>125</sup> Also, anti-MOG does not correlate with histopathologic classification or apparent responsiveness to TPE.<sup>126</sup>

The study of treatments for MS is complicated by the natural history of the disease, particularly the tendencies for acute attacks to subside and for attack frequency to decline, but also by other spontaneous fluctuations in disease activity. Immunosuppressive and immunomodulatory agents have been the mainstays of drug therapy in MS. Resolution of acute attacks is thought to be hastened by brief courses of either glucocorticoids or adrenocorticotropic hormone. The relentless progression of disability is probably not halted by these measures, although aggressive treatment of optic neuritis with intravenous steroids may delay the onset of frank MS that often follows. Immunomodulatory monoclonal antibodies, such as alemtuzumab, daclizumab, natalizumab, and rituximab, were also investigated with mixed results.<sup>127-129</sup>

The rationale for TPE in MS is uncertain, given the paucity of evidence that any circulating factor has a role in the etiology of acute attacks or chronic progression. TPE has nevertheless been used, and encouraging results have been reported from uncontrolled studies.<sup>130,131</sup> TPE has better outcomes when used in acute attacks and little or no value in chronic/progressive MS.

### Neuromyelitis optica spectrum disorder (NMOSD)

Neuromyelitis optica, also known as Devic's disease, is a demyelinating autoimmune inflammatory process affecting, in particular, the optic nerve and spinal cord. The spinal cord is typically affected in the form of longitudinal extensive transverse myelitis. Other areas of the brain that can be affected and are used as diagnostic criteria.<sup>132</sup>

The disease can present as an isolated acute attack (monophasic, minority of patients) or as a relapsing disease. It affects all age groups, and affects disproportionately more females and individuals of African descent.<sup>133</sup> NMOSD is associated with the presence of serum aquaporin-4 immunoglobulin G antibodies (AQP4-IgG) in approximately 70% of the patients; autoantibodies to myelin oligodendrocyte glycoprotein or anti-MOG have also been found in these patients. Treatment of acute attacks includes high-dose steroids. TPE is reserved for patients with severe manifestations or not responsive to steroids.<sup>134,135</sup>

### Pediatric autoimmune neuropsychiatric disorders associated with streptococcal infections (PANDAS)

PANDAS is a pediatric syndrome characterized by the sudden onset of a neuropsychiatric symptoms after a group A streptococcus infection. These symptoms include obsessive compulsive disorder, attention disorders, tics, and abnormal movements such as choreoathetosis and catatonia. It typically affects males more than females between 7 and 9 years of age. A crossreactive antibody response to streptococcal antigens could mediate symptoms in some of these patients. The first line of therapy includes antibiotic treatment against *Staphylococcus*, combined with psychiatric medications such as diazepam, valproic acid, carbamazepine, and haloperidol. For more severe or refractory cases, IVIG and TPE are considered second-line therapy.<sup>136</sup> Sydenham chorea is a related movement disorder that may follow a group A streptococcal infection in children. Patients with Sydenham chorea may also exhibit obsessive-compulsive symptoms, and it is considered a neuropsychiatric manifestation of acute rheumatic fever. TPE is not considered second-line treatment, and its use is not routinely recommended.<sup>137</sup>

### Phytanic acid storage disease (Refsum's disease)

Refsum's disease results from deficiency of the peroxisomal enzyme phytanoyl-CoA hydroxylase, which participates in the degradation of phytanic acid by  $\alpha$ -oxidation. Accumulation of diet-derived phytanic acid in plasma lipoproteins and in tissue lipid stores leads to symptoms, which may include peripheral neuropathy, cerebellar ataxia, retinitis pigmentosa, anosmia, deafness, ichthyosis, renal failure, and arrhythmias. Slow progression is the usual course, but rapid deterioration and even sudden death may follow a marked increase in plasma phytanic acid.<sup>138,139</sup>

Primary treatment is based on the restriction of dietary intake of phytanic acid via dairy products, meats, and fats. It leads to gradual clearing of phytanate stores by slow  $\omega$ -oxidation and gradual symptomatic improvement in most patients. Appropriate caloric intake must be maintained; however, because extreme dietary restrictions may cause the mobilization of calories from endogenous fat, increasing plasma phytanic acid levels acutely and resulting in clinical exacerbations. TPE will remove large quantities of phytanic acid incorporated into plasma lipids.<sup>140</sup> Apheresis therapy is most appropriate for patients who have very high plasma phytanate levels and an associated exacerbation of symptoms. Skin disease, neuropathic symptoms, and ataxia usually improve as plasma levels drop. Cranial nerve defects usually do not.<sup>140</sup>

### Steroid responsive encephalopathy associated with autoimmune thyroiditis (Hashimoto's encephalopathy)

Hashimoto's encephalopathy is characterized by acute onset of encephalopathy, high serum concentration of antithyroid antibodies, and responsiveness to glucocorticoids. Most patients present with seizures, psychosis, stroke-like symptoms, and subclinical

hypothyroidism, but normal thyroid function or hyperthyroidism can be present.<sup>141</sup> Antithyroid peroxidase (anti-TPO) and antithyroglobulin can be present, but their role in the pathophysiology of the disease is controversial. Psychiatric medication and high-dose steroids are the first-line treatment with excellent response. TPE and IVIG are considered second-line therapy for patients who do not respond to steroids.<sup>142</sup>

### Voltage-gated potassium channel antibody (VGKC) related diseases

Voltage-gated potassium channels modulate neuronal excitability by returning the polarized cells to a resting state. Several antigens are targeted for autoantibody formation, including leucine-rich glioma inactivated 1 (LGI1), contactin-associated protein-2 (CASPR2), and contactin-2. LGI 1 has been associated with limbic encephalitis, characterized by amnesia, seizures, disorientation, psychiatric disturbances, and neuromyotonia.<sup>143</sup> CASPR2 has been associated with Morvan syndrome, characterized by limbic encephalitis, neuromyotonia, pain, and autonomic dysfunction.<sup>144</sup> First-line therapy includes treatment for underlying neuropsychiatric symptoms with antiepileptic medication and immunotherapy including steroids and IVIG. TPE is considered second-line therapy, and its efficacy has been described in small case series and case reports.<sup>143,145</sup>

### Therapeutic plasma exchange in hematologic disorders

There are several hematological conditions for which TPE is used as a first- or second-line therapy (Table 25.4). TPE has been attempted as a therapeutic tool in many other hematological conditions with varied therapeutic results. These conditions are classified as Category III, implying that TPE treatment has been attempted but its efficacy is not yet determined. These diseases include but are not limited to the removal of coagulation factor inhibitors; hemolysis, elevated liver enzymes, and low platelet count (HELLP); hemophagocytic lymphohistiocytosis (HLH); heparin-induced thrombocytopenia and thrombosis (HIT/HITT); and refractory immune thrombocytopenia purpura (ITP) and post-transfusion purpura (PTP). In this section, we describe conditions for which TPE is considered first- or second-line therapy.

### TPE for thrombotic microangiopathies (TMAs)

Frequent indications for TPE include diseases presenting with thrombotic microangiopathy (TMA), including TTP. TMA is characterized by occlusive microvascular disease and intraluminal

**Table 25.4** Hematological Conditions for Which TPE is Considered First- or Second-Line Therapy.

Disease Name	Indication	Category	Grade
Thrombotic thrombocytopenic purpura		I	1A
Complement-mediated thrombotic microangiopathy	Factor H autoantibody	I	2C
Drug-associated thrombotic microangiopathy	Ticlopidine	I	1B
Hyperviscosity in hyperimmunoglobulinemia	Symptomatic Prophylaxis for rituximab	I	1B 1C
Catastrophic antiphospholipid syndrome (CAPS)		I	2C
Autoimmune hemolytic anemia: severe	Severe cold agglutinin disease	II	2C
Cryoglobulinemia	Symptomatic/severe	II	2A

thrombus formation, and manifests clinically as microangiopathic hemolytic anemia and thrombocytopenia. Diseases presenting with TMA can present with overlapping symptoms that pose a challenge when making a diagnosis and deciding treatment. TMAs have different pathophysiologic mechanisms that will determine the type of treatment indicated. Some examples of TMA include congenital TTP (cTTP), immune TTP (iTTP), infection-associated hemolytic-uremic syndrome (IA-HUS), complement-mediated hemolytic-uremic syndrome (CM-HUS) solid organ and bone marrow transplant associated TMA, disseminated intravascular coagulation (DIC), *Streptococcus pneumoniae*, Cobalamin C deficiency, connective tissue diseases, pregnancy-associated TMAs (HELLP syndrome and pre-eclampsia), drugs (most relevant ticlopidine, gemcitabine, bleomycin, mitomycin, and others), pancreatitis, and malignancies.<sup>146</sup> In this section, we discuss diseases presenting with TMA for which TPE is indicated as a first- or second-line therapy.

### **Thrombotic thrombocytopenic purpura**

TTP is characterized by microangiopathic hemolytic anemia and thrombocytopenia. Central nervous system changes, fever, and renal abnormalities may be seen in advanced cases, although frank renal failure is unusual. Idiopathic TTP was formerly assigned a mortality rate of 90%, but empiric studies in the 1970s and 1980s and a clinical trial in the 1990s demonstrated much improved survival in patients receiving daily TPE with plasma replacement.<sup>147</sup> The pathogenesis of TTP involves the severe deficiency (<10%) of the metalloproteinase ADAMTS13 that cleaves ultralarge von Willebrand factor (vWF) multimers secreted by endothelial cells, yielding the smaller vWF polymers found in normal plasma.<sup>148,149</sup> Severe ADAMTS13 deficiency can be acquired (immune TTP [iTTP]) or genetic (Upshaw-Schulman syndrome). A genetic deficiency should be suspected in children and pregnant women presenting with TTP, which can be confirmed with genetic testing.<sup>150</sup> iTTP is most common in adults and it is confirmed by the presence of an autoantibody with ADAMTS13 inhibitor effect.<sup>148,149,151,152</sup>

Immune and congenital forms have similar clinical presentation; persistence of ultrahigh-weight vWF in the circulation promotes inappropriate adherence of platelets to endothelial cells and to each other, leading to consumptive thrombocytopenia and to microvascular obstructions that cause mechanical trauma to red cells and varying degrees of end-organ ischemia. The clinical presentation and severity are quite variable, with a range that goes from patients who present with asymptomatic thrombocytopenia to patients who present with minor symptoms and sudden death.<sup>153</sup> Relapses after the initial episode are common.

ADAMTS13 activity during remission is variable and unpredictable.<sup>153</sup> Response to treatment is also variable, with patients who recover clinically with increased ADAMTS13 activity after a series of daily TPE, and no relapse. There are other patients with refractory disease requiring prolonged TPE treatment and intensive immunosuppression presenting with both decreased or normal ADAMTS13 activity and no relapse, and other patients with refractory disease and relapses.<sup>153</sup>

First-line therapy includes a combination of TTP and steroids. Plasma infusion can be started if TPE is not readily available. Periodic plasma infusion may prevent attacks in congenitally deficient patients by supplying active enzyme.<sup>152</sup> Idiopathic cases respond better to TPE (78% response rate vs. 63% for plasma infusion),<sup>147</sup> presumably because exchanges remove inhibitory antibody

as well as replace the deficient enzyme.<sup>147</sup> TPE is performed daily until the platelet count has normalized.

Rituximab has been used in different situations: in combination with TPE as first-line therapy<sup>154,155</sup> or for patients who are refractory or fail TPE and steroids, and to prevent relapses without TPE.<sup>156</sup>

Caplacizumab, an anti-von Willebrand factor humanized, bivalent variable-domain-only immunoglobulin fragment, has recently been evaluated in two clinical trials for the treatment of TTP. Caplacizumab inhibits interaction between von Willebrand factor multimers and platelets. The TITAN trial included 75 patients who were randomized to Caplacizumab or placebo. Patients in the intervention group recovered faster but had an increased risk of bleeding.<sup>157</sup> The HERCULES trial included 145 patients with a diagnosis of TTP undergoing TPE. The addition of caplacizumab resulted in faster recovery of platelet counts and decreased number of exacerbations (defined as disease recurrence during therapy or within 30 days of stopping TPE) but increased number of relapses (defined as disease recurrence between 30 days and 12 months of stopping TPE). Mucocutaneous bleeding was more frequent in the Caplacizumab group.<sup>158</sup>

The clinical manifestation and response to the treatment of patients with TTP is quite heterogeneous. The best treatment approach should be evaluated in the context of clinical presentation, previous events, and level of evidence supporting the efficacy of the interventions being considered.<sup>159</sup>

### **Complement-mediated thrombotic microangiopathy**

Complement-mediated thrombotic microangiopathy (CM-TMA), formerly known as atypical hemolytic uremic syndrome (aHUS), is a rare, life-threatening disorder associated with dysregulation of the alternative pathway of the complement system.<sup>160</sup> It typically presents in children and young adults with TMA, diarrhea, and acute kidney failure. Multiple mutations affecting complement regulatory proteins have been identified. Mutations that result in loss of function of complement regulatory proteins include complement factor H (CFH), complement factor I (CFI), thrombomodulin (THBD), or CD46/membrane cofactor protein (MCP). Mutations in complement activating proteins including complement factor B (CFB) and C3 can result in gain of function with subsequent dysregulation.<sup>161</sup> The diagnosis is challenging and other causes of TMA need to be ruled out. It is common to start treatment with TPE until the diagnosis of CM-TMA is confirmed through genetic analysis. Eculizumab, a monoclonal antibody directed against C5 is considered first-line therapy. The benefit from TPE is controversial, but some patients do benefit from it, most likely due to the removal of mutated complement proteins and substitution with normal proteins present in donor plasma.<sup>162,163</sup>

**Thrombotic microangiopathy, drug associated, ticlopidine**  
TMA has been observed in some patients taking the antiplatelet drugs ticlopidine<sup>164</sup> and clopidogrel (although this is an ASFA Category III indication).<sup>165</sup> Antibody to ADAMTS13 has been detected in such case,<sup>166</sup> and TPE has been reported to improve outcome (76% survival vs. 50% for unexchanged patients in a retrospective study of ticlopidine recipients).<sup>164</sup>

### **Hyperviscosity in hypergammaglobulinemia**

Hyperviscosity was the first condition to be treated successfully with manual plasmapheresis, the precursor to TPE. The full-blown syndrome consists of neurologic symptoms, a bleeding diathesis, a peculiar retinopathy marked by alternating dilated and constricted

segments in retinal veins, and hypervolemia caused by expansion of plasma volume. Symptoms are uncommon if the relative serum viscosity is below four and become more likely when it exceeds six. The hyperviscosity syndrome is most often seen in patients with Waldenström's macroglobulinemia, who have IgM paraproteins, but it may also occur in multiple myeloma.<sup>167,168</sup>

At higher paraprotein levels, a relatively large change in viscosity may follow a relatively small change in concentration. It is this non-linear relationship that allowed the two-unit manual plasmapheresis technique available in the 1950s to lower viscosity enough to relieve symptoms. Because most IgM paraproteins are roughly 80% intravascular, the same relationship also predicts that a one plasma-volume automated exchange will provide a wide margin of safety and can therefore be repeated less frequently for hyperviscosity than is necessary for many other conditions. Viscosity measurements should guide therapy, but treatment every 1–2 weeks may be adequate.<sup>167</sup>

Paraproteins may interfere in platelet and clotting factor interactions in the absence of hyperviscosity. Such coagulopathies are found in 60% of patients with macroglobulinemia, 40% of patients with IgA myeloma, and 15% of patients with IgG myeloma.<sup>169</sup> In instances that are clinically significant, TPE therapy can help restore adequate hemostasis.

TPE is considered first-line therapy for patients with symptomatic hyperviscosity and as a prophylactic treatment for patients receiving treatment with rituximab. Note that TPE provides symptomatic relief but has no effect on underlying disease process. Treatment with rituximab can result in transient increase of circulating IgM and subsequent hyperviscosity in patients at risk, including those with a baseline elevated serum viscosity.<sup>170</sup>

### Catastrophic antiphospholipid syndrome (CAPS)

Catastrophic antiphospholipid syndrome (CAPS) is a life-threatening autoimmune disease characterized by microvascular venous or arterial thrombosis resulting in multiorgan failure. The disease is characterized by the persistent presence of autoantibodies against phospholipids present in a variety of cells. These antibodies have a prothrombotic effect inducing endothelial, platelet, and coagulation cascade activation, affecting the microvasculature and resulting in acute thrombotic microangiopathy. Virtually any organ can be affected, but life-threatening presentations usually involve the brain, kidneys, lungs, and heart. Patients can present with stroke, acute kidney failure, thrombocytopenia, and anemia. These signs and symptoms can overlap with TTP, which should be considered in the differential diagnosis.<sup>171</sup> CAPS can be precipitated by an acute event such as trauma, infection, or inflammatory process.<sup>172</sup> Laboratory diagnostic criteria include the presence of lupus anticoagulant, moderate-to-high titer anticardiolipin antibodies, and/or moderate-to-high titer anti-b2-glycoprotein I antibodies.<sup>173</sup> The first line of therapy for patients with CAPS is immediate anticoagulation to halt the progression of thrombosis. TPE and immunomodulatory agents, such as IVIG, prednisone, and cyclophosphamide, are also part of the first-line treatment to remove and decrease antibody levels, respectively.<sup>173</sup>

### Autoimmune hemolytic anemia (AIHA)

AIHA is caused by autoantibodies to red cells antigens. Such antibodies are classified as either "cold" or "warm" agglutinins, depending on the temperature of maximal activity. Cold agglutinins are usually IgM antibodies directed against the I/i antigens; they bind most strongly at low temperatures and may produce a syndrome of complement-mediated intravascular hemolysis (cold agglutinin

disease [CAD]). Warm agglutinins are usually IgG, bind stronger at body temperature, and produce a predominantly extravascular hemolytic syndrome (warm autoimmune hemolytic anemia [WAIHA]). Autoimmune hemolytic anemia can be idiopathic but can also be associated with infections, lymphoproliferative disorders, or other autoimmune diseases. The direct antiglobulin test (DAT) is positive for 3d complement in CAD, and for IgG and sometimes complement in WAIHA.

Most patients with warm autoantibodies do not develop hemolysis, but the patients who do develop hemolysis will need treatment. Standard therapy is aimed at lowering antibody production and inhibiting destruction of sensitized cells. Combined treatment of prednisone with rituximab is considered first-line treatment, and IVIG and splenectomy are often effective.<sup>174</sup> Other immunosuppressive drugs, such as cyclophosphamide, azathioprine, dapsone, and others, have been used for refractory or recurrent disease.<sup>175</sup> TPE is not routinely used as a therapeutic strategy.<sup>176,177</sup>

Pharmacological treatment CAD is typically more challenging and steroids have demonstrated marginal benefits.<sup>178</sup> First-line therapy include rituximab and bendamustine therapy.<sup>179</sup> Complement inhibitors, such as the monoclonal antibody against C1 sutimlimab, demonstrated to be safe and efficacious in stopping hemolysis.<sup>180</sup> Other complement inhibitors directed against more downstream complement proteins, such as eculizumab that inhibits C5, also demonstrated efficacy in stopping hemolysis.<sup>181</sup> Because the IgM antibodies in CAD are predominantly intravascular and only loosely bound to cells, their removal by TPE relatively more efficient when compared to IgG antibodies. TPE is considered second-line therapy, and it is typically used in more severe or refractory cases or before surgical procedures that require hypothermia.<sup>182</sup>

### Cryoglobulinemia

Cryoglobulins are abnormal serum proteins that precipitate reversibly at 4 °C; some will precipitate at higher temperatures. Such precipitates always contain immunoglobulin, and immunoelectrophoretic or immunofixation analysis allows distinction of three types. Type I cryoglobulins consist of a single species of monoclonal immunoglobulin. These are usually found in B-cell lymphoproliferative disorders such as myeloma or Waldenström's macroglobulinemia. Cryoglobulin levels are often quite high (>500 mg/dL) and may cause Raynaud phenomenon or acral necrosis due to microvascular obstruction, as well as other symptoms. Type II cryoglobulins contain both monoclonal and polyclonal immunoglobulins. The former is usually an IgM with anti-IgG specificity, and the latter is polyclonal IgG bound to the IgM in an immune complex. Most cases occur in the context of chronic hepatitis C infection. They typically manifest a cutaneous vasculitis on the lower extremities and may have visceral manifestations of immune complex disease as well.<sup>183</sup> Type III cryoglobulins are mixed polyclonal, often with IgM anti-IgG that binds IgG in immune complexes. These may arise in acute infections, such as hepatitis B, or in chronic inflammatory states, such as severe rheumatoid arthritis. Clinical manifestations resemble serum sickness.

If there is an underlying condition, cryoglobulin levels and related symptoms may decrease with the treatment of this primary disorder, for example chemotherapy for myeloma or interferon for hepatitis C virus infection.<sup>184</sup> For idiopathic and secondary cases of mixed cryoglobulinemia, prednisone alone or combined with rituximab, azathioprine, or cyclophosphamide have been proven to be effective.<sup>185</sup> Alkylating agents may be useful in patients with severe symptoms resistant to prednisone.

TPE will reduce cryoglobulin levels and control symptoms, even in the absence of other treatments,<sup>186,187</sup> but inconvenience and expense mitigate against such use. It should be started promptly for patients who seek treatment for severe acral ischemia or visceral manifestations of vasculitis, in whom it can help achieve control of symptoms until immunomodulatory therapy takes hold.<sup>188</sup> Patients with chronic vasculitic skin ulcers may also benefit.<sup>189</sup> In all cases, replacement fluids should be warmed to body temperature before infusion.

### TPE in other immunologic and metabolic disorders

TPE has been used as a therapeutic tool in a number of rheumatic diseases and other diseases that are considered to have an immune or autoimmune etiology (see Table 25.5). Not every disease that is antibody mediated benefits from TPE. In this section, we discuss diseases for which TPE is considered first- or second-line therapy.

#### Goodpasture syndrome

Goodpasture syndrome (GPS) is characterized clinically by pulmonary hemorrhage and rapidly progressive glomerulonephritis. Light microscopy of renal biopsies shows crescent formation in many glomeruli, and immunofluorescent and electron microscopy reveal linear subendothelial immune deposits that may also be evident in a lung biopsy. In 95% of cases, there is a circulating antibody that binds to glomerular basement membrane (anti-GBM). Such antibodies are specific for a noncollagenous sequence near the carboxy terminus of the  $\alpha 3$  chain of type IV collagen, which is found in appreciable quantities only in renal and pulmonary basement membranes. Untreated GPS progresses quickly and relentlessly, and most patients die of uremia or complications of lung hemorrhage.<sup>190</sup>

The preferred treatment for GPS is high-dose prednisone and cyclophosphamide, combined with intense TPE, to quickly reduce anti-GBM levels and minimize progression of tissue damage.<sup>191</sup> Exchanges are usually carried out daily and may be continued for up to two weeks. A single controlled trial failed to show an advantage for GPS patients who received TPE; however, this study has been largely discounted because the TPE schedule (every three days) was not sufficiently aggressive and because the extent of renal damage at entry was worse in the TPE group than in controls.<sup>192</sup> Early treatment is recommended because patients who are already dialysis-dependent at the onset of TPE are unlikely to recover renal function.<sup>193</sup> Patients whose renal biopsies show irreversible lesions

are not likely to benefit from TPE unless they also have pulmonary hemorrhage. Patients affected with diffuse alveolar hemorrhage have a very good response to TPE.

#### ANCA-associated vasculitis

ANCA-associated vasculitis (AAV) is an autoimmune disorder characterized by necrotizing inflammation of small- and medium-sized vessels and the presence of antineutrophil cytoplasmic antibodies (ANCA). There are clinical diseases associated with AAV, including granulomatosis with polyangiitis (GPA), microscopic polyangiitis (MPA), and eosinophilic GPA (EGPA). The major antigens targeted by ANCA are proteinase 3 (PR3) and myeloperoxidase (MPO). Most patients present with renal disease in the form of rapidly progressive glomerulonephritis.<sup>194</sup>

First-line therapy of the disease to induce remission includes glucocorticoids in combination with either cyclophosphamide and rituximab, followed by maintenance therapy with low-dose steroids combined with azathioprine, mycophenolate mofetil, or rituximab. When compared to glucocorticoids alone, TPE was shown to be effective in increasing renal function in patients who did and did not require dialysis.<sup>195,196</sup> In contrast, a recent randomized clinical trial reported no benefit in mortality or end stage kidney disease when plasma exchange was used in patients receiving either low- or high-dose steroids.<sup>197</sup> A recent meta-analysis including five randomized clinical trial and 937 patients comparing TPE vs. no TPE and TPE vs. steroids concluded that there was no difference in clinical outcomes when TPE was used. Most importantly, TPE is not without risks and adds significant expense to the treatment. Careful consideration should be made when evaluating TPE indication.

#### Vasculitis with hepatitis B-associated polyarteritis nodosa

Polyarteritis nodosa (PAN) is characterized by necrotizing vasculitis affecting medium-sized arteries. It can affect any organ, most commonly the skin, peripheral nervous system, and visceral vessels, but typically does not affect the lungs. The underlying pathophysiology involved the immune-complex deposition with antigen excess. PAN can be idiopathic, or can present as two variants of clinical forms, including cutaneous PAN and hepatitis B infection-associated PAN (HB-PAN).<sup>198</sup> Idiopathic and cutaneous forms are typically treated with corticosteroids with or without cyclophosphamide. Treatment for HB-PAN involves antiviral therapy (vidarabine, interferon- $\alpha$ , and lamivudine) combined with TPE.<sup>199,200</sup>

#### Focal segmental glomerulosclerosis

Focal segmental glomerulosclerosis (FSGS) is one of the leading cases of end stage renal disease in the United States. It is characterized by podocyte injury of different underlying pathologic mechanisms including primary (idiopathic) and secondary forms. Secondary forms include genetic mutations (more than 40 genes affected), adaptive FSGS (excessive nephron workload or reduced nephron capacity), APOL1 FSGS (patients of sub-Saharan African descent), infection/inflammation associated FSGS (HIV, CMV, EBV, and others), and medication associated FSGS (interferon- $\alpha$ ,  $\beta$ , or  $\gamma$ ; bisphosphonates, sirolimus, and others). There are also distinct morphological patterns, including the tip lesion and the collapsing variant. The combination of clinical presentation, classification, and histological patterns needs to be considered to tailor treatment options and determine expected efficacy.<sup>201</sup> First-line therapy for primary FSGS includes prednisone with or without cyclophosphamide, and calcineurin inhibitors for patients who are

**Table 25.5** Immunologic and Metabolic Disorders

Disease Name	Indication	Category	Grade
Antiglomerular basement membrane disease (Goodpasture's syndrome)	Diffuse alveolar hemorrhage (DAH)	I	1C
Vasculitis, ANCA-associated	Dialysis independence	I	1B
	Rapid progressive glomerulonephritis	I	1A
	Diffuse alveolar hemorrhage (DAH)	I	1C
Vasculitis, hepatitis B polyarteritis nodosa		II	2C
Focal segmental glomerulosclerosis (FSGS)	Recurrent in transplanted kidney	I	1B
Wilson's disease	Fulminant	I	1C
Myeloma cast nephropathy		II	2B
Overdose, envenomation, and poisoning	Mushroom poisoning	II	2C
Systemic lupus erythematosus	Severe	II	2C
Thyroid storm		II	2B

resistant to steroids.<sup>202</sup> Other immunosuppressive medications such as azathioprine and levamisole have also been used.<sup>203</sup> Rituximab has been evaluated in observational studies with positive therapeutic effect.<sup>204</sup> The rationale to use TPE is based on the presumed existence of a circulating factor that contributes to the pathogenesis of the disease. A promising candidate is the soluble urokinase plasminogen activator receptor (suPAR),<sup>205</sup> but its role as a disease marker or in disease pathogenesis is controversial.<sup>206</sup> Plasmapheresis is typically reserved for patients with recurrence of the disease after transplant.<sup>207,208</sup>

### Wilson disease

Wilson disease is a recessive genetic disorder caused by a mutation in the Wilson disease protein (*ATP7B*) gene, which results in abnormal copper secretion with subsequent pathologic deposition in several organs including the brain, liver, and cornea. The main treatment involves chelation therapy with penicillamine and trientine. Patients with liver cirrhosis are candidates for liver transplant. Patients TPE has been helpful in acute hepatic failure in Wilson's disease in preparation for liver transplant.<sup>209,210</sup>

### Myeloma cast nephropathy

Multiple myeloma (MM) is a plasma cell proliferative disorder that commonly affects the kidneys. The most common pathophysiology of renal failure is light chain cast nephropathy. Other mechanisms include immunoglobulin light chain amyloidosis and monoclonal immunoglobulin deposition disease. Several randomized controlled trials evaluated the efficacy of TPE in patients with MM and renal disease and demonstrated benefits on the use of TPE.<sup>211,212</sup> Another randomized trial failed to demonstrate any benefit with the use of TPE, but an important limitation was the lack of biopsy as an including criterion.<sup>213</sup> The combination of bortezomib and TPE also had positive renal function recovery.<sup>214</sup>

### Overdose/mushroom poisoning

Toxic effects may occur after exposure to excessive doses of pharmacologic agents or to harmful agents in the environment. Management techniques for both types of events are similar and may include removal of toxin still in the gastrointestinal tract, enhancement of renal elimination, and direct removal from blood by hemodialysis, hemoperfusion (e.g., over charcoal columns), or TPE.<sup>215</sup> Specific antidotes may also be given if available. Serious events are usually treated with multiple measures.

TPE has been reported to be beneficial, when combined with other therapies, in cases involving substances such as methyl parathion, vincristine, and cisplatin that bind tightly to plasma proteins. TPE has been reported in poisonings due to ingestion of the *Amanita phalloides* mushroom; however, diuresis clears more *Amanita* toxin.<sup>191</sup>

Unfortunately, the literature on this topic is older and entirely anecdotal. Furthermore, TPE has always been used in combination with other therapies that are presumably effective. This complicates the formulation of firm, rational guidelines.<sup>216</sup> Nevertheless, it seems reasonable to offer TPE to a severely affected patient with an overdose or poisoning who has a high blood level of a toxic agent that binds to plasma proteins. It should also be noted that TPE has shown minimal or no beneficial effect in overdosage of drugs known to bind to tissue proteins and lipids, including barbiturates, chlordcone, aluminum, tricyclic antidepressants, benzodiazepines, quinine, phenytoin, digoxin, digitoxin, prednisone, prednisolone, tobramycin, and propranolol.<sup>191</sup>

### Systemic lupus erythematosus (SLE)

SLE has long been regarded as the prototypic autoimmune disease. The most important diagnostic criterion, circulating antibodies to DNA, especially double-stranded DNA (anti-dsDNA), identifies patients who may have a variety of other autoantibodies and a disparate array of clinical syndromes in which skin disease, joint disease, cytopenias, or nephritis may be the sole or dominant problem.

Immunosuppressive measures are the cornerstone of therapy for SLE. Most patients are given prednisone in varying doses, and those with severe disease may also receive azathioprine or cyclophosphamide. The plethora of autoantibodies that seem relevant to clinical signs made SLE an obvious target for TPE. It was one of the first illnesses to be treated with automated TPE in the early 1970s, and early case reports and uncontrolled series suggested a favorable effect.<sup>217</sup>

Lupus nephritis is a particularly devastating manifestation in which glomerular deposition of immune complexes and anti-DNA is believed to have a prominent role in pathogenesis. Thus, it seemed an attractive setting for randomized trials of TPE. A controlled trial with only eight patients suggested benefit.<sup>218</sup> However, in a multicenter randomized controlled trial comparing oral cyclophosphamide plus TPE to oral cyclophosphamide alone, there was no advantage for the patients receiving TPE.<sup>219</sup> A later international trial, which enrolled patients with a variety of severe manifestations, was structured to exploit enhanced sensitivity to a properly timed pulse dose of intravenous cyclophosphamide that was believed to follow pathogenic antibody removal by TPE.<sup>220</sup> This trial also failed to show any advantage for all patients treated with TPE<sup>221</sup> or for a subgroup with nephritis.<sup>222</sup> Thus, large controlled studies have failed to confirm any worthwhile effect of TPE in SLE.

There are some severe LES manifestations for which TPE could be considered, including CAPS, TTP, cryoglobulinemia, and DAH.<sup>223</sup>

### Thyroid storm

Thyroid storm, also known as thyrotoxic crisis, is an acute, life-threatening disorder characterized by excess of thyroid hormone. It is most frequently associated with patients presenting with hyperthyroidism, in particular Grave's disease. Other forms of thyrotoxicosis not associated with hyperthyroidism include thyroiditis (postpartum and subacute thyroiditis) and extrathyroidal source of hormone.<sup>224</sup> Patients typically present with signs and symptoms of excess thyroid hormone and increase sympathetic activity, including irritability, psychosis, tachycardia, arrhythmias, heart failure, and fever. If untreated, it can progress to stupor, coma, and death. These episodes are usually triggered by a stressor, such as infection, radiation thyroiditis, stopping antithyroid medication, or thyroidectomy. First-line therapy includes medications to stop the synthesis and release of thyroid hormones, such as propylthiouracil and iodine, respectively, and beta blockers to decrease the adrenergic effects. TPE can be added for patients not responding to first-line therapy. TPE has been shown to be effective in decreasing thyroid hormones in the acute phase but levels bounce back the following day.<sup>225,226</sup>

### TPE in transplantation

TPE has been used before transplant to remove pre-existing ABO or HLA antibodies (desensitization), or after transplant in the setting of antibody-mediated rejection (AMR). A summary is provided in Table 25.6. For solid organ transplantation, ABO compatibility between the donor and recipient is critical to ensure organ

**Table 25.6** TPE in Transplantation

Disease Name	Indication	Category	Grade
Liver transplantation, ABO-incompatible	Desensitization, living donor	I	1C
Renal transplantation, ABO compatible	Desensitization, living donor	I	1B
	Antibody-mediated rejection	I	1B
Renal transplantation, ABO incompatible	Desensitization, living donor	I	1B
	Antibody-mediated rejection	II	1B
Cardiac transplant	Desensitization	II	1C
Lung allograft rejection	Bronchiolitis obliterans syndrome	II	1C
HSCT, ABO incompatible	Major HPC, marrow	II	1B
	Major HPC, apheresis	II	2B

viability and successful engraftment. The importance of HLA compatibility is critical for stem cell transplant but varies between solid organs. In this section, we describe solid organ and stem cell transplantation for which TPE has been shown to be effective in removing antibodies. The role of photopheresis in the transplant setting is described in Chapter 26.

### Desensitization

Desensitization involves the removal of circulating antibodies in the recipient to prevent hyper acute rejection of the transplanted organ. This becomes critical when a patient is in acute need of an organ, but compatible donors are not available. Liver transplant can be a life-saving procedure for patients presenting with fulminant liver failure or other forms of severe liver dysfunction. The mortality rate of patients with kidney disease increases as the kidney disease progresses.<sup>227</sup> ABO compatibility is a major determinant of organ availability, and many patients die while waiting for a compatible transplant. Strategies to increase the donor pool include the use of living donors (for liver and kidney transplants) and the use of incompatible organs. Incompatible transplants can be performed in the setting of desensitization protocols to decrease antibody synthesis and remove circulating antibodies.

For liver transplants, the use of desensitization protocols in recipients of incompatible organs from living donors demonstrated good survival rates. Different protocols can include a combination of rituximab, TPE, IVIG, steroids, splenectomy, and prostaglandin E1 infusions.<sup>228</sup> A series of TPE before transplant effectively removes ABO antibodies preventing hyperacute rejection. ABO isoagglutinins typically bounce back after transplant requiring a series of TPE posttransplant. Rituximab has been shown to be effective as the main desensitization strategy. Biliary complications and antibody-mediated rejection are more frequent complications in incompatible transplants when compared to compatible transplants.<sup>229</sup>

In the case of kidney transplantation, TPE can be used to remove HLA and ABO antibodies for desensitization in the setting of living donors and antibody-mediated rejection (AMR). HLA-sensitized patients presenting with donor-specific antibodies (DSAs) have longer waiting time to receive an organ, which has a negative impact on their overall health and survival. These patients might be able to find a suitable living donor through kidney-paired donation or desensitization.<sup>230,231</sup> Desensitization protocols for HLA-sensitized patients include a combination of TPE and immunosuppressive drugs, including IVIG, rituximab, and bortezomib.<sup>232,233</sup> ABO-incompatible kidney transplants demonstrate positive outcomes.<sup>234</sup>

Desensitization protocols include a combination of intensive TPE to decrease isoagglutinin titers, IVIG, and immunosuppressive medication including tacrolimus and mycophenolate mofetil, daclizumab, and steroids.<sup>235</sup>

Desensitization protocols can also be used in patients waiting for a cardiac transplant. Not all patients are eligible for this treatment and the outcomes are not equivalent to compatible transplant due to higher risk of AMR. A combination of TPE, IVIG, and rituximab with or without bortezomib is typically used.<sup>236</sup>

### Rejection

Standard post-transplant management for kidney and heart transplantation consists of prophylactic immunosuppression with glucocorticoids, a calcineurin inhibitor (cyclosporine or tacrolimus), and an antimetabolite such as azathioprine or mycophenolate mofetil. High-risk recipients and those who have rejection episodes in spite of these standard measures are treated with pulse steroids, T-cell antibody preparations, or both.<sup>237,238</sup>

Case reports and uncontrolled series published in the late 1970s and early 1980s suggested TPE was beneficial in renal transplant rejection. Then, five controlled trials<sup>191</sup> were reported in the mid- and late-1980s. Four showed no significant benefit for patients receiving TPE in addition to standard drug therapy, even in the sub-groups whose transplant biopsies showed “vascular” histologic changes. In the one study suggesting benefit, the mean treatment time was 10–11 months after transplant, when antibody-mediated rejection is less likely. The last and largest study concluded that TPE therapy for renal transplant rejection could no longer be recommended.<sup>239</sup> Nevertheless, the use of TPE for this purpose continued to be reported.<sup>191</sup>

In the more recent past, a new wave of enthusiasm for TPE has focused on patients with clinical and histologic evidence of acute rejection who also have circulating donor-specific antibody and/or deposition of complement component C4d in transplant biopsy tissue, suggesting a humoral mechanism.<sup>240</sup> The antibody can be shown by a positive crossmatch with donor cells or by flow cytometric reactions with donor antigens. C4d deposition is demonstrated by immunofluorescence microscopy.<sup>241</sup>

In summary, evidence from controlled trials has not shown global efficacy for TPE in reversing established rejection episodes in renal allografts. Anecdotal experience focusing on patients with circulating antibody and immunopathologic evidence of humoral rejection seems more promising, but controlled trials in such patients have not been published. Clarification of the role of TPE in cardiac transplantation awaits controlled studies targeting patients with documented humoral rejection and circulating DSA.

### Controversial applications of therapeutic plasma exchange

Every new patient consultation is an assessment of pros and cons. There are patient- or institution-specific factors that impact the feasibility or appropriateness of embarking on a course of apheresis therapy, just as there is for performing any medical procedure. Many apheresis services utilize the ASFA guidelines to establish a framework of diagnostic settings when there is some evidence, of often variable quality, particularly for Category I or II indications, where apheresis “is accepted as a first- or second-line therapy.”<sup>287</sup> Category III diagnoses are defined as entities where the “optimum role of apheresis therapy is not established”<sup>87</sup> and have not been reviewed here based on the lack of strong evidence in favor of an important role for apheresis in disease management. Clinical consultations should always be individualized.<sup>242</sup> It is essential to

analyze whether the potential benefits of a procedure, whether short term or long term, outweigh the risks for a particular patient in a particular clinical setting. The core of an apheresis consultation is achieving a balance between guiding medical management toward the root causes of disease pathogenesis and judicious deployment of a “brute force” extraction of a blood constituent. Conflicting data, less convincing putative mechanisms, or symptom-based, rather than pathophysiologically based, justifications for plasmapheresis abound in the medical literature. To attempt to review all reports of a “successful” plasmapheresis case reports is beyond the scope of this chapter, but future rigorous clinical trials can hopefully test the value of TPE in diseases published in case reports or series but not discussed here.

### Specialized therapeutic plasma processing

There are 30 Category I, II, or III indications for therapeutic immunoabsorption (IA), LDL apheresis, or rheopheresis (a form of double-membrane filtration plasmapheresis or DFPP) in the current ASFA guidelines. 87 IA is essentially an alternative to TPE in these guidelines in that it shares the same designation with TPE for seven Category I, six Category II, and seven Category III indications, occasionally with a lesser grade of evidence. For one indication, dilated cardiomyopathy, it has a higher category designation than TPE (II vs. III). Lipid apheresis has six Category I, II, or III indications. In contrast to standard plasmapheresis, which nonspecifically removes plasma, the following discussion focuses on therapeutic procedures that are designed to remove a specified fraction of the plasma. The first step is the separation of plasma using a membrane followed by passing the plasma across a selective filter, which may be either single use or regenerating. A pathogenic plasma substance (e.g., a specific autoantibody) is usually present at relatively low levels in the circulation. Thus, selective extraction of pathologic plasma constituents in a way that minimizes the sacrifice of healthy plasma proteins could be a more efficient treatment of disorders with lesser theoretical iatrogenic coagulopathy than TPE.

### Immunoabsorption apheresis

Immunoabsorption systems employ the principle of affinity chromatography and make use of immobilized sorbents or ligands that have enhanced or specific binding affinity for a specific antigen, antibody, immune complex, or other substance in the patient's circulation.<sup>81</sup> Examples include (1) staphylococcal protein A or sheep antihuman immunoglobulin (IgG) for the extraction of IgG and immune complexes from the circulation, (2) sheep antihuman low-density lipoprotein (LDL) or apolipoprotein B antibody for the extraction of LDL, (3) synthetic blood group substances for the removal of ABO isoagglutinins, and (4) DNA for the removal of DNA antibody. Although no longer commercially distributed in the United States, two immunoabsorption systems that have received approval from the FDA are the staphylococcal protein A sepharose column (Immunosorba, Fresenius HemoCare, Redmond, WA) and the staphylococcal protein A silica column (Prosorba, Fresenius HemoCare).<sup>243</sup> Protein A is a cell-wall constituent of the Cowan I strain of *Staphylococcus aureus*. Mammalian IgG binds to five homologous regions at its amino terminus, but interaction of protein A with other plasma proteins is insignificant. Processing of 2.5 plasma volumes using a protein A agarose column resulted in a 97% reduction in IgG1, a 98% reduction in IgG2, a 40% reduction in IgG3, a 77% reduction in IgG4, a 56% reduction in IgM, and a 55% reduction in IgA, whereas plasma levels of albumin, fibrinogen, and

antithrombin were reduced by less than 20%.<sup>244</sup> Thus, in principle, plasma adsorption with protein A affinity columns permits the processing of more plasma than does TPE without unacceptable loss of other essential plasma constituents.<sup>245</sup> In practice, some clinical coagulation tests, particularly fibrinogen, can be statistically significantly affected by IA.<sup>246,247</sup> The use of these devices in a variety of clinical conditions in Europe and Japan has been extensively reviewed.<sup>81,248</sup>

### Dilated cardiomyopathy

Dilated cardiomyopathy (DCM) describes a cardiac disorder in which the left ventricle is dilated and exhibits impaired contraction. It may be idiopathic, familial, postviral, alcohol- or drug-induced, or related to other cardiac disease. A genetic mutation can be found in about 35% of patients.<sup>249</sup> Both ventricles may be affected. It presents with heart failure, is often progressive, and may be complicated by arrhythmias, thromboembolic events, and sudden death. It is the most frequent antecedent cause of heart transplantation throughout the world.<sup>250</sup> Approximately 60–80% of patients with DCM harbor autoantibodies directed against cardiac myosin heavy chain, myocardial  $\beta_1$ -adrenergic receptors, or other cardiac tissue with a predominance of antibodies of the IgG 3 subclass.<sup>251–253</sup>

### Immunoabsorption in the treatment of autoantibody-associated dilated cardiomyopathy

Autoantibodies targeting guanine nucleotide-binding protein-coupled receptor (GPCR) that blocks myocyte signaling cascades were first described in the late 1980s, but their potential relevance to clinical DCM was initially explored using IA in the mid-1990s using an antihuman polyclonal immunoglobulin (AHPI) system known as Therasorb.<sup>254</sup> In a pilot study of eight patients with DCM and advanced congestive heart failure who were subjected to a series of four or five IA procedures per week over two weeks,  $\beta_1$ -adrenoreceptor autoantibody levels decreased by an order of magnitude and heart failure symptoms improved in seven of the subjects. The effect was transient, however, and autoantibodies and symptoms returned to baseline 75 days after completion of IA.<sup>254</sup> A more durable reduction in  $\beta_1$ -adrenergic receptor antibodies was found in a 34-patient randomized controlled trial that correlated with improvement in both left ventricular ejection fraction (LVEF) at 12 months and 5-year survival (82% in IA-only treated group vs. 41% in controls;  $p < 0.0001$ ).<sup>255</sup> No concomitant IVIG that might confound results was given in that study. A second randomized trial using the same IA platform studied 18 patients with DCM and advanced heart failure who were assigned to either best medical therapy (control group) or best medical therapy with the addition of IA.<sup>256</sup> During the first course of IA, procedures were performed on three consecutive days, and patients received 0.5 g/kg of IVIG by intravenous infusion. Three subsequent courses of IA, performed on two consecutive days at four-week intervals, were also followed by infusions of IVIG at the same dose. After the first course of IA/IVIG, a sustained improvement in many clinically relevant parameters, including the LVEF, cardiac index, stroke volume index, and systemic vascular resistance, was reported. Improvement in symptoms and functional status paralleled the hemodynamic changes. The control group demonstrated no hemodynamic improvement at the end of the three-month study.  $\beta_1$ -Adrenergic receptor antibodies decreased by >80% after the first course of IA but tended to rise between monthly courses of treatment. There have now been multiple trials, case reports, and case series generally supporting improvement.<sup>148,257</sup>

The vast majority of studies infused intravenous IgG (IVIG), which might solely, or in concert with IA, account for clinical improvement. Unfortunately, trials of treatment with IVIG alone have been equivocal. One trial, in which patients initially received a total of 2 g/kg followed by 0.4 g/kg monthly for five months, demonstrated significant improvement in left ventricular ejection fraction in the treatment group but not the placebo group.<sup>258</sup> A second trial, in which patients received a single course totaling 2 g/kg, demonstrated no effect of IVIG on left ventricular ejection fraction after 6 or 12 months (although both the treatment and placebo groups showed improvement).<sup>259</sup> The in vitro inotropic activity of antibodies removed from patients who responded to IA was stronger than antibodies from patients who did not respond to antibody-removal therapy, supporting a pathophysiological relevance to these antibodies *in vivo*.<sup>260,261</sup> Overall, since most studies have been conducted using IA, ASFA designates DCM a Category II indication for IA and a Category III indication for TPE.<sup>87</sup>

### Familial hypercholesterolemia

Familial hypercholesterolemia (FH), an autosomal dominant disorder, is a major cause of death or early disability resulting from premature atherosclerotic heart and peripheral vascular disease.<sup>262</sup> It is caused by mutations in the LDL receptor (LDL-R), with frequencies of 1:500 for heterozygotes and 1:1,000,000 for homozygotes.<sup>263</sup> Clinical features including xanthomas, xanthelasmas, corneal arcus, and the occurrence of coronary heart disease, stroke, and death are common in the fourth or fifth decade of life.<sup>262</sup> A serum LDL cholesterol (LDL-C) level below 100 mg/dL (achieved through diet, lifestyle modification, and 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibiting drugs or statins) can lower cardiovascular morbidity and mortality in high-risk patients, but a sizable minority of high-risk patients fail to achieve LDL-C-lowering goals by this approach.<sup>264</sup> New medical therapies target the recycling of LDL-R in hepatocytes. A serine protease in plasma called proprotein convertase subtilisin-kexin type 9 (PCSK9) binds to the LDL-R, flagging the LDL-R for degradation. Lower surface receptor levels lead to increased plasma LDL-C levels.<sup>265</sup> A PCSK9 inhibitor, evolocumab, effectively decreased LDL-C by 20% in homozygous FH and by 55% in severe heterozygous FH over a four-year period, giving new hope for an effective medical therapy beyond statins.<sup>266</sup>

### LDL apheresis

Manual, then automated, plasma exchange was successfully employed as an adjunct to lipid-lowering therapy beginning over 40 years ago. However, the kinetics of restoration of plasma lipid levels and the unwanted lowering of essential plasma proteins (e.g., fibrinogen and albumin) rendered this approach challenging for long-term therapy. The development of apheresis devices that permit a more selective removal of plasma LDL cholesterol and related substances has provided a practical approach to managing statin-resistant patients in conjunction with statins and other medical therapies.<sup>267</sup>

Two apheresis systems for selective removal of LDL cholesterol are FDA approved for use in the United States. The Liposorber LA15 system (Kaneka Pharma America, New York, NY) uses dextran sulfate bound to cellulose to selectively extract LDL cholesterol from plasma. In this popular system, plasma is initially separated from the cellular components of blood by filtration through a disposable semipermeable polysulfone hollow fiber column, and the

separated plasma is then perfused over a disposable adsorption column that contains 150 mL of dextran sulfate. Dextran sulfate has strong affinity for lipoproteins and adsorbs these from the plasma. The H.E.L.P. system (B. Braun Medical, Bethlehem, PA) employs a 0.55-μm hollow fiber column to separate the plasma from the cellular elements of the blood. The plasma is acidified with 0.3 M sodium acetate buffer, and heparin is added to precipitate LDL-C. The LDL-C-heparin precipitate is filtered from the plasma using a 0.45-μm polycarbonate filter, excess heparin is adsorbed from the filtered plasma with a DEAE cellulose membrane filter, and the filtered plasma is then restored to physiologic pH by bicarbonate hemodialysis.

ASFA has designated FH a Category I indication for LDL apheresis in homozygotes and a Category II indication for LDL apheresis in heterozygotes.<sup>87</sup> The FDA-approved indications include homozygotes with plasma LDL cholesterol >500 mg/dL and heterozygotes with LDL cholesterol >300 mg/dL (or >200 with known coronary artery disease). A regimen that combines medical therapy with LDL apheresis on a biweekly schedule can effectively lower LDL cholesterol by 60–80% in otherwise treatment-resistant patients, improve the physical stigmata of hypercholesterolemia such as xanthomas and xanthelasmas, improve myocardial perfusion and coronary artery patency, and favorably affect other markers of cardiovascular risk (e.g., triglycerides, fibrinogen, homocysteine, C-reactive protein, and adhesion molecules).<sup>268</sup> Vascular access is either peripheral or by arteriovenous fistula or graft, due to the chronic treatment requirements of this genetic disease.<sup>269</sup> More recently, LDL apheresis has been found effective in ameliorating drug-refractory coronary artery disease in patients with Lp(a)hyperlipoproteinemia.<sup>270</sup>

### Conclusions

Therapeutic plasma exchange and specialized plasma therapies occupy a distinct clinical niche, thanks largely to advances in the last 50 years in biomedical engineering and transfusion medicine. On the horizon is the expanding armamentarium and applications of immunotherapies, particularly in the treatments of autoimmune diseases and immunomodulation. We hope these elegant approaches will better focus future therapy on treating the causes of disease rather than using the nonspecific apheresis-based methods described here to remove their accumulated consequences.

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## CHAPTER 26

# Therapeutic phlebotomy and cellular hemapheresis

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The eighth comprehensive, evidence-based analysis of apheresis therapies by the American Society for Apheresis (ASFA) recorded 72 disease conditions as category I (apheresis as first-line therapy) or category II (apheresis as second-line or adjuvant therapy) indications for therapeutic apheresis.<sup>1</sup> Among these, 43 (60%) were indications for plasma exchange, consistent with prior surveys that found plasma exchange to be the predominant form of apheresis therapy in North America.<sup>2,3</sup> Whereas apheresis procedures have been in wide use for decades,<sup>4</sup> many on the basis of accumulated experience, the approval of new therapeutic indications, devices, and drugs is increasingly dependent on the presentation of carefully acquired supportive evidence in randomized controlled trials.<sup>5,6</sup> This applies to phlebotomy and apheresis therapies as well<sup>7,8</sup> and ASFA presents its recommendations, and the strength of the evidence that underlies them, according to the G.R.A.D.E. system.<sup>1</sup> Accordingly, this chapter, which reviews therapeutic phlebotomy and specialized apheresis procedures that target specific cellular compartments of the blood for processing, emphasizes those procedures that are supported by evidence.

### Therapeutic red cell apheresis

*Therapeutic erythrocytapheresis* is the removal of red cells, typically using automated blood-processing instruments that are capable of selectively removing erythrocytes while returning the plasma, buffy coat cells, and additional isotonic saline to the patient.<sup>9</sup> The therapeutic replacement of patient red cells for donor red cells is more specifically referred to as *red cell exchange* (RCE) or a *red cell exchange transfusion* to contrast it with a *simple transfusion*,<sup>10</sup> although the term erythrocytapheresis is frequently used synonymously.<sup>1</sup>

### Red cell exchange (RCE)

Therapeutic RCE can be performed manually or with programmable automated blood-processing (apheresis) instruments. Manual exchange is mainly limited to neonates or resource-limited settings. Partial manual exchange (PME) transfusion is performed in some settings when access to an automated procedure is limited, uneconomical, an apheresis catheter is undesirable, or for some pediatric settings.<sup>11–13</sup> Although PME may be required due to

logistical constraints, automated RCEs are generally more efficient and patients meet therapeutic goals more consistently than with PME.<sup>14,15</sup> Thus, this discussion focuses on automated RCE.

Basic features of automated blood processors that perform therapeutic apheresis using centrifugation technology are detailed in Chapter 24. The machine operator enters the patient's gender, height, and weight into the instrument's computer to calculate total blood volume. The operator also inputs the known starting and desired ending hematocrit of the patient, the average hematocrit of red cell replacement units to be used, and the desired fluid balance (a default of 100% may be offered by the instrument). Finally, the operator can choose to enter the desired fraction of the preprocedure red cells remaining (FCR) within the patient's circulation at the end of the procedure or the volume of replacement fluid (e.g., red cells of average hematocrit as programmed into the instrument's computer) needed for the procedure.

Calculation of the desired FCR depends on the starting points and targeted endpoints of both the patient's hematocrit and the relevant therapeutic parameter (e.g., percent Hgb S or parasitemia). Most RCEs also achieve a net positive transfusion to treat symptomatic anemia, but if no increase in hematocrit is desirable (e.g., if concerns for increased whole blood viscosity in a sickle cell anemia patient were clinically relevant), then the first factor below cancels out to 1:

$$FCR = 100 \times \left( \frac{\text{starting hematocrit}}{\text{ending hematocrit}} \right) \times \left( \frac{\text{endpoint parameter}}{\text{starting parameter}} \right) \quad (1)$$

For example, a sickle cell patient with a hematocrit of 25% and 100% Hgb S, targeted to an endpoint of Hgb S 30% and ending hematocrit of 30%, would yield

$$FCR = 100 \times \left( \frac{25\%}{30\%} \right) \times \left( \frac{30\%}{100\%} \right) = 25\%$$

The replacement volume needed to reach the desired FCR is dependent on the patient's blood volume, the starting hematocrit, the target hematocrit, fluid balance, and the average hematocrit of the replacement RBC units. It is important to remember that modern automated apheresis instruments incorporate the starting and

endpoint hematocrit, and thus the machine FCR simply approximates the desired fraction of original parameter of interest. In clinical circumstances when the volume of available or appropriate donor blood may be limited, such as a highly alloimmunized hemoglobinopathy patient who requires rare, multiply antigen-negative units and an urgent procedure, the replacement volume available for the exchange can determine the maximum achievable FCR given the patient's starting Hct and the targeted end Hct.

According to ASFA, RCE is indicated as first- or second-line therapy for the management of sickle cell disease (SCD) and for the treatment of severe manifestations of the protozoal infections (e.g., malaria and babesiosis).<sup>1</sup>

### Sickle cell disease

Symptoms of SCD and traditional treatments have long existed in Africa.<sup>16</sup> Genetic analysis suggests that the origin of the genetic mutation responsible for SCD has an age of 259 generations.<sup>17</sup> The indigenous names of this malady reveal not only its enduring presence for many African peoples, but also capture the agonizing and unremitting nature of its relentless pain. The Banyagi diagnosis *adep* means “beaten up,” and both the Adagme and Ewe have captured SCD’s iterative and gnawing misery in their terms *hemkom* (“body biting”) and *nuidudui* (“body chewing”).<sup>18</sup> Specific iatrogenic limb girdle tribal markings have been applied to people with *hemkom* in the Adagme group in Ghana as described by local physicians, and the disease has been traced in one Ghanaian family to 1670.<sup>19,20</sup> The first association of recurrent bone pain with cold weather, fever, and joint pain was documented in the English literature in 1874 by Sierra Leonean physician Dr. Africanus Horton.<sup>21</sup> Sickled erythrocytes were first described in Western medicine in 1910 by Dr. J.B. Herrick, who noted the abnormally shaped red cells in the peripheral blood film of a dental student from the Caribbean island of Grenada.<sup>22</sup> An underlying mutation results in substitution of valine for glutamic acid as the sixth amino acid residue in the hemoglobin β chain and likely arose approximately 7300 years ago either in the Sahara or in west/central Africa.<sup>17</sup> Forces of balancing selection have been at play to maintain the variant in human populations due to its protective effects from severe falciparum malaria in the heterozygous state (sickle trait).<sup>23</sup> However, once at appreciable frequency in a population, the homozygous sickle state leads to SCD. The description of these countervailing forces on survival provided one of the initial and strongest examples of heterozygous advantage.<sup>24,25</sup>

At a molecular level, sickling is due to increased hydrophobic interactions between nearby Hgb S molecules whereby deoxygenated Hgb S aggregates into large inflexible polymers and inflexible sickled red cells. Increased sickled cell adhesion, inflammation, and vasculopathy lead to vaso-occlusive crises.<sup>26,27</sup> The exquisite dependence of polymerization on the proportion of HgbS<sup>28</sup> has provided a scientific basis, in concert with the strong clinical basis,<sup>29</sup> for the transfusion therapy of normal red cells in sickle cell anemia.

SCD is the most common inherited blood disorder in the United States, affecting an estimated 100,000 Americans, with an estimated 3000 neonates born with SCD each year.<sup>30,31</sup> FDA-approved medical therapies for SCD include hydroxyurea, voxelator, crizanlizumab, and L-glutamine to reduce severity or frequency of acute complications, but transfusion therapy remains a mainstay of both acute and chronic disease management.<sup>32,33</sup> For the most part, clinical studies related to transfusion management of SCD have focused on simple transfusion or manual partial exchange transfusion.<sup>29,34–37</sup> The efficacy of manual versus automated RCE in

**Table 26.1** Red Cell Exchange in Sickle Cell Disease (2019 ASFA “Strong Recommendation” Grade 1 Evidence)<sup>1,33</sup>

Clinical Setting	Diagnosis or Purpose	2019 ASFA category
Acute or emergent	Acute vaso-occlusive stroke	I
	Severe acute chest syndrome	II
Chronic/nonacute	Prevention of stroke in high-risk children	I
	Secondary stroke prevention	I

the treatment of SCD has not been directly compared.<sup>33</sup> For most SCD complications, simple transfusions may likely be as effective, but automated RCE can more rapidly decrease the proportion of Hgb S red cells during severe acute episodes.<sup>1</sup> In SCD patients who receive chronic transfusion, automated RCE can mitigate iron overload<sup>38,39</sup> while maintaining a low Hgb S level. Thus, it has entered into routine use in centers where therapeutic apheresis is available. Its indicated roles in the aspects of SCD (Table 26.1)<sup>1,33,40</sup> are discussed here. Exchanges performed using isotonic saline, rather than red cells, as the replacement fluid in the early phases of the procedure are called *depletion exchanges* or *isovolemic hemodilution* and can reduce the number of donor red blood cell (RBC) units required by reducing the removal of the initial replacement red cells.<sup>38,39</sup>

### Life- or organ-threatening complications

RCE is standard therapy (ASFA category I) for patients with acute vaso-occlusive stroke,<sup>1</sup> and although the efficacy of transfusion (either simple or exchange) has not been studied by randomized trials, emergency RCE should be performed shortly following documentation of thrombotic (rather than hemorrhagic) stroke by noncontrast computed tomography.<sup>29,33,40</sup> The treatment goal should be a hemoglobin concentration between 9 and 10 g/dL and less than 30% Hgb S. Acute intervention, followed by chronic maintenance transfusion therapy, may limit early morbidity and mortality and prevent recurrence (discussed further in this chapter).<sup>29,40</sup>

The National Acute Chest Syndrome Study Group’s (NACSSG) definition of the acute chest syndrome (ACS) remains the standard since its report in 2000,<sup>36</sup> and is based on the presentation with a new alveolar infiltrate involving one or more complete lung segments (atelectasis excluded) and accompanied by chest pain, a fever >38.5 °C, tachypnea, wheezing, or cough in a patient with SCD. ACS is the second most common cause for hospitalization and the leading cause of death in SCD.<sup>40,41</sup> Although the NACSSG report was not powered to detect a particular advantage of RCE over simple transfusion,<sup>36</sup> and there is only one small RCT addressing the use of even simple transfusion in ACS,<sup>42</sup> the consensus opinion of experts in the field is to recommend RCE for severe ACS (oxygen saturation <90% despite supplemental oxygen).<sup>43</sup> Both RCE and simple transfusion are characterized by this same expert consensus panel as “acceptable treatment modalities” for “moderate” ACS.<sup>1,27</sup>

Acute multiorgan failure syndrome is a common cause of death in SCD,<sup>33,41,44</sup> may present as an unusually severe pain episode in patients with Hgb SS or Hgb SC disease, and is characterized by fever, accelerated hemolysis with a rapid decrease in hemoglobin and platelet count, nonfocal encephalopathy, and rhabdomyolysis.<sup>44</sup> Besides the central nervous system, other organs, including liver and kidney, may be involved.<sup>28,44</sup> Standard red cell transfusion therapy is likely effective if severe anemia is present. Although evidence is limited to case

reports and series, RCE should be considered with higher hemoglobin levels.<sup>33</sup> RCE can also be considered for hepatic sequestration and intrahepatic cholestasis.<sup>40,45</sup> Splenic sequestration occurs most commonly in young children, and for patients with significant anemia on presentation, simple transfusion restores the circulating blood volume sufficiently to support remobilization from the spleen. The transfusion strategy should maintain a hematocrit low enough to avoid possible hyperviscosity syndrome when sequestered RBCs are eventually returned to the circulation. For the rare adults with this presentation or for patients without significant anemia, an exchange transfusion may be considered.<sup>46–49</sup> The role of transfusion and exchange transfusion in priapism, which occurs in approximately 30–90% of males with SCD,<sup>40,44</sup> has been debated.<sup>50</sup> However, there is increasing consensus that early transfusion is not warranted, supported by an NIH expert panel that states that transfusion should not be used,<sup>27,40</sup> although British guidelines include consideration of RCE if patients do not respond to initial shunt procedures.<sup>51</sup> Studies have shown no benefit over conventional therapies, the time to resolution is often longer for those transfused, and further interventions such as surgical decompression are often required.<sup>29</sup> In addition, severe neurologic abnormalities have been associated with RCE, as first reported in six boys with sickle cell anemia 1–11 days following partial exchange transfusion for priapism unresponsive to conservative therapy.<sup>52</sup> The syndrome was called ASPEN (Association of Sickle cell Priapism, Exchange transfusion and Neurological events) and was characterized by severe headache at the onset, often associated with increased intracranial pressure and further neurologic events ranging from seizure activity to obtundation requiring ventilatory support.<sup>52–54</sup> It is likely that ASPEN was caused by hyperviscosity since the postexchange Hct was greater than the pre-exchange values in the initial publications, and a subsequent report that did not raise the hematocrit postexchange was not associated with ASPEN.<sup>55</sup> However, the mean time to detumescence did not differ significantly between patients treated with transfusion (mean of 10.8 days) versus those treated with conventional therapies (8.0 days) in a review of 42 case reports of SCD-associated priapism.<sup>56</sup> Neurologic complications with transfusion therapy were described in nine cases, some with persistent long-term deficits. Taken together, there are many alternative treatments favored as first-line therapies for SCD-associated priapism, with a limited role of RCE.<sup>57,58</sup>

### **Primary and secondary prevention of stroke**

Approximately 5–10% of untransfused children with SCD will have a clinically evident cerebral infarction by the age of 20 years.<sup>59,60</sup> An additional 40% are estimated to experience silent cerebral infarcts (SCIs), which are radiologically indistinguishable<sup>61</sup> and lack overt signs of stroke, but which are highly morbid events associated with decreased intellectual abilities, poor academic achievement, additional SCIs, and progression to overt stroke.<sup>62,63</sup> A recent meta-analysis did not find an increased risk of stroke for patients with sickle trait,<sup>64,65</sup> although a potential interaction with chronic kidney disease may affect 30-year ischemic stroke risk in patients with sickle trait in one recent study.<sup>66</sup> There are likely many distinct but clinically coincident pathophysiological mechanisms by which stroke occurs in both children and adults with SCD, including thromboembolic mechanisms, endotheliopathy, or decreased cerebral vascular reserve or autoregulation.<sup>67</sup> Chronic transfusion therapy (CTT), given every 3–4 weeks, to maintain the level of Hgb S below 30% can improve the arteriographic appearance of affected cerebral vessels and reduce the risk of recurrent stroke from 66% to 90% to approximately 10%.<sup>33,40</sup> Chronic automated RCE can be

substituted for simple transfusion, with the added potential benefit of preventing or mitigating iron overload.<sup>38,39</sup> Reports of recurrent stroke rates of 50% or greater after discontinuation of transfusion therapy, coupled with increased mortality in the first three years when patients stop transfusion during a pediatric-to-adult care transition, have led most to recommend indefinite prophylactic transfusion regimens.<sup>33,40,68,69</sup>

The demonstration that transcranial Doppler ultrasound (TCD) was highly predictive of stroke risk in children with SCD<sup>68</sup> led to the Stroke Prevention Trial in Sickle Cell Anemia (STOP trial),<sup>35</sup> which examined the ability of CTT to prevent a *first* stroke in high-risk children with SCD. Increased cerebral blood flow (CBF) likely occurs in SCD as a compensation for reduced oxygen content due primarily to anemia from chronic hemolysis. Chronic anemia also pathologically increases the oxygen extraction fraction (OEF) of cerebral blood in SCD. Both the CBF and OEF can be markedly reduced, although not entirely to normal levels, with chronic RCE.<sup>70</sup> Importantly, there is no validated radiologic or laboratory test that predicts stroke risks in adults with SCD.<sup>71</sup> Time-averaged mean blood flow velocity of at least 200 cm/s in the internal carotid or middle cerebral artery and a stroke-free history were required for STOP study entry.<sup>35</sup> Over a period of approximately 2–3 years, transfusion therapy to maintain Hgb S below 30% without exceeding a hemoglobin concentration of 12 g/dL reduced the occurrence of stroke in the treatment group by 90% compared to the control group.<sup>35</sup> A follow-up study (STOP2)<sup>72</sup> examined the effect of discontinuation of transfusion therapy after 30 months in children from the first STOP trial whose TCD readings had reverted to normal. The study was halted when an interim analysis revealed that, of 41 children randomly assigned to discontinue transfusions, 14 had reverted to elevated TCD findings within nine months after stopping transfusions and two had suffered ischemic strokes. Neither elevated TCDs nor strokes were observed in the controls continuing transfusion. The TCD With Transfusions Changing to Hydroxyurea (TWiTCH) trial showed that children with abnormal TCDs but no history of prior stroke could transition to hydroxyurea for primary stroke prophylaxis after an initial period of transfusions (12% of which were RCEs).<sup>73</sup> Despite years of awareness of the importance of identifying children who require CTT using TCD for primary stroke prevention, failure to comprehensively screen children with SCD is a significant contributor to preventable stroke in children with SCD in the “post-STOP” trial era.<sup>74</sup> Further, the indication for transfusion in primary stroke prevention may broaden, given the lower rates of both silent and overt strokes among children with magnetic resonance imaging (MRI)-demonstrated silent infarcts, but lacking elevated intracranial arterial flow rates, who were randomized to receive chronic transfusions (the Silent Infarct Transfusion [SIT] trial).<sup>75,76</sup>

Chronic transfusion remains the mainstay of secondary stroke prevention.<sup>77,78</sup> The early closure of the Stroke With Transfusions Changing to Hydroxyurea (SWiTCH) trial, which was halted when interim analysis revealed reversion to elevated TCD and increased incidence of overt secondary stroke when transfusions stopped, reaffirmed that current medical therapy alone cannot substitute for transfusion (with chelation).<sup>79</sup> As a result of these studies demonstrating no equivalent therapy or safe endpoint for CTT, the current recommendation is that individuals with a history of elevated transcranial arterial flow or a history of stroke continue transfusion therapy indefinitely.<sup>33</sup>

Whether chronic transfusion therapy takes the form of simple or exchange transfusions is a multifactorial decision but may be

informed by distinguishing the effect of anemia (manifest by low total Hgb) from the Hgb S levels on pathogenesis. Interestingly, hemoglobin level was an independent predictor of both CBF<sup>80</sup> and OEF,<sup>70</sup> but percent Hgb S was not, suggesting that simple transfusion to treat anemia may help prevent strokes by improving cerebral metabolic reserve and RCE's rapid decrease in percent Hgb S may protect patients through different mechanisms.

### **Prevention of transfusional iron overload**

There are no randomized, prospective comparisons of simple transfusion versus automated RCE in the prevention of iron overload in children with SCD who require chronic transfusion therapy. Nonetheless, RCE results in 85% less iron loading compared to simple transfusion and may prevent iron overload if used as the initial form of CTT and particularly if combined with chelation therapy.<sup>14,81</sup> Iron accumulation will be slower but will still occur if the postprocedure hematocrit is greater than the preprocedure value, resulting in a net gain of iron; therefore, chelation therapy may still be required. In four case series of 8–14 children<sup>38,39</sup> who either converted from a simple transfusion program to monthly RCE, began on RCE early on with variable chelation therapy with deferoxamine,<sup>40</sup> serum ferritin tended to stabilize in those who were not on chelation therapy and significantly decreased in those who continued on chelation therapy. However, RCE resulted in a 25–100% increase in red cell usage and a concomitant increase in donor exposures.

### **Controversial clinical presentations in SCD patients**

In 2016, there were over 130,000 SCD-related inpatient hospitalizations in the United States.<sup>82</sup> The average length of stay was five days with 70% of these admissions in children and young adults aged less than 34 years. Adults aged 18–34 years had higher than average 30-day readmission rates, demonstrating the chronic, pernicious nature of this disease. There is variation in exchange transfusion practices<sup>83</sup> and in consensus guidelines about automated or manual exchanges,<sup>1,43,51</sup> demonstrating both the lack of conclusive data on which to make evidence-based recommendations and the requirement to take an individualized approach for consideration of RCE in these situations. Transfusion medicine practitioners, particularly those in metro areas, are likely to receive consultation requests about management (with either simple or exchange transfusion) for patients with SCD suffering from many of its complications other than those described above, such as acute or chronic vaso-occlusive pain, associated with bone marrow transplant to treat their SCD<sup>84–86</sup> or those requiring medical attention for unrelated conditions, such as pregnancy or preoperative evaluation, which we briefly explore here.<sup>33</sup>

### **Pregnancy**

Determining the potential role of chronic transfusion therapy in pregnancy is the first step to determining if that transfusion therapy should be performed via simple or exchange transfusions. A meta-analysis comparing prophylactic or chronic transfusions during pregnancy to ad hoc transfusions showed reduced maternal mortality, pain episodes, pulmonary complications, pyelonephritis, perinatal mortality, neonatal death, and preterm birth with the prophylactic transfusion strategy. However, other important outcomes, such as intrauterine fetal demise, small-for-gestational-age infants, or low-birth-weight infants, or preeclampsia did not differ between the groups.<sup>87</sup> One RCT did demonstrate fewer pain events in the chronic transfusion group, but fetal or maternal

outcomes could not be detected due to the size of the study.<sup>88</sup> The 2020 ASH guidelines support prophylactic transfusion at regular intervals at the onset of pregnancy for women with a history of severe complications of sickle cell disease or pregnancy comorbidities,<sup>89</sup> but routine implementation of red cell exchange transfusion is not indicated.

### **Preoperative**

The Transfusion Alternatives Preoperatively in Sickle cell disease (TAPS) trial randomized 67 individuals in Canada and Europe with SCD who were undergoing low- or intermediate-risk procedures to preoperative transfusion with a goal of 10 g/dL Hgb or no transfusion.<sup>90</sup> Twice as many patients in the untransfused arm experienced postoperative complications, including acute chest syndrome than the transfused arm, providing good evidence to support a hemoglobin threshold of 10 g/dL prior to low- or intermediate-risk procedures. Neither hospital stay nor readmission rates differed between the groups. Simple transfusions are an acceptable method to receive this transfusion therapy, a practice supported by a 1995 RCT that demonstrated no advantage to RCE over simple transfusion in terms of perioperative complications.<sup>34</sup> There are no data specifically favoring a particular Hgb S% prior to general anesthesia that reduces perioperative complications so simple transfusions are the preferred transfusion method for most patients.<sup>40</sup>

### **Protozoan disease**

Severe manifestations of malaria and babesiosis are ranked as category III and II indications, respectively, by ASFA, largely on the basis of anecdotal evidence.<sup>1,91–94</sup>

### **Malaria**

Globally, severe malaria is most commonly caused by *Plasmodium falciparum*.<sup>95</sup> Plasmodium infection results from injection of sporozoites into the bloodstream by the bite of a female *Anopheles* mosquito. The sporozoites migrate to and infect liver cells, where they asexually reproduce to form numerous merozoites that burst forth and invade RBCs. This initiates the erythrocytic life cycle with repeated rounds of RBC infections and increasing parasitemia, and symptoms such as recurrent fever, which can culminate in life-threatening end-organ dysfunction that defines severe malaria.<sup>96</sup>

The relative severity of *P. falciparum* malaria is due to adherence of falciparum-infected erythrocytes to glycosylated molecules on microvascular endothelium and to platelet CD36 via *P. falciparum* erythrocyte membrane protein-1 (PfEMP-1), an adhesive protein expressed on the surface of infected erythrocytes.<sup>97–99</sup> The sequestration and vaso-occlusion combined with the elaboration of proinflammatory cytokines such as tumor necrosis factor- $\alpha$  (TNF $\alpha$ ) and interferon- $\gamma$  result in the severe manifestations of the disease.<sup>99</sup> The use of exchange transfusion and automated RCE in the treatment of severe malaria is based on an ability to rapidly reduce the burden of parasitemia and the potential to thereby improve the rheologic properties of the blood and reduce the level of toxic mediators such as cytokines.<sup>1</sup> On the other hand, it is unclear what effect peripheral parasite removal has on sequestered parasites. Sporadic case reports and case series continue to be associated with rapid reduction in parasitemia and clinical improvement.<sup>100</sup> However, the evidence for improved survival is mixed, with retrospective case control series variably favoring antibiotic therapy

alone or adjunct exchange transfusion, but all studies have suffered from poor control for malaria severity.<sup>192</sup>

The US Centers for Disease Control and Prevention (CDC) no longer recommends consideration of RBC exchange in cases of severe malaria with parasitemia >10%.<sup>1</sup> This was based on the lack of high-quality evidence in the literature and analysis of their malaria reporting data from 1985 to 2010 comparing 101 exchange transfusion cases with 314 propensity-matched nonexchange cases of severe malaria.<sup>101</sup> They found no survival advantage with 17.8% mortality in the exchange-transfused group compared to the 15.9% for the controls. However, their retrospective analysis only had power to assuredly detect a very strong effect (<4.6% mortality in exchange transfused at 90% power, presuming 15.9% in controls) and lacked key data for the vast majority of cases (e.g., <10% of cases reported parasitemia levels, a key trigger for exchange per ASFA guidelines). These study limitations were cited in ASFA's response to this publication saying that the ASFA category III recommendation for RCE would stand.<sup>102</sup> What is not in dispute is that current artemisinin combination therapies lead to rapid parasite clearance, in line with the rapidity of clearance by apheresis or manual exchange.<sup>103</sup> Given that these drugs also clear sequestered parasites definitively, artemisinin-based therapy limits the rationale for adjunct RCE.<sup>101,103</sup> However, in the face of the emergence and spread of artemisinin resistance in Southeast Asia and<sup>104</sup> and the lack of another effective antimalarial,<sup>104</sup> a properly controlled prospective trial to determine the benefit of exchange (e.g., 50% improved survival) may be of value if parasite clearance by drug becomes ineffective.

### Babesiosis

Babesiosis is a zoonotic disease and is spread to humans primarily through ticks of the genus *Ixodes*.<sup>105</sup> The first reported case of human babesiosis was in an asplenic individual from Europe.<sup>106</sup> The first case in a patient with an intact spleen was reported in Nantucket.<sup>107</sup> Within the United States, the predominant organism is *Babesia microti*, the reservoir hosts are wild rodents, and the vector is the deer tick *Ixodes scapularis*, which is the same tick that transmits *Borrelia burgdorferi*, the causative agent of Lyme disease.<sup>105</sup> Over the past several decades, the endemic range has expanded, and now encompasses seven states in the Northeast and the Midwest United States, which accounted for >86% of 2161 reported babesiosis cases in 2018.<sup>108</sup>

When injected into the human bloodstream, the sporozoites of babesia, akin to plasmodium, directly invade RBCs. After asexual budding into four merozoites, the parasites perforate the erythrocyte membrane, resulting in hemolysis.<sup>109</sup> They are then free to infect other erythrocytes and transform into dividing trophozoites (ring forms and tetrads visible on peripheral blood films).<sup>105,109</sup> One to six weeks following inoculation, infected patients develop a flu-like syndrome characterized by fever, fatigue, and malaise.<sup>110</sup> Headache, chills, sweats, myalgia, and arthralgia are frequent complaints. Physical findings may include fever and splenomegaly, and jaundice and pallor may accompany marked extravascular hemolysis. Although most cases are subclinical or mild,<sup>111</sup> severe manifestations, including disseminated intravascular coagulation, respiratory failure, and renal failure, may occur. Immunocompromised or asplenic individuals are typically more severely affected.<sup>105,112</sup>

Transfusion transmission occurs in endemic areas, leading to infections of those most vulnerable: neonates, the immunocompromised, and the elderly.<sup>113</sup> Both community-acquired and transfusion-transmitted cases have increased in recent years,<sup>114,115</sup> prompting the

FDA to issue a final industry guidance in 2019 recommending blood donor testing in 14 endemic states and Washington, DC, as well as the addition of a specific babesiosis history question to donor eligibility screening across all US blood collection areas.<sup>116</sup> Although clinical trials are lacking, several case reports and case series suggest that, given the absence of a microvascular sequestration of infection, RCE, whole blood exchange, or RCE followed by plasma exchange, combined with antibiotic therapy, can rapidly reduce parasitemia and be potentially beneficial in severe cases of babesiosis with >5–10% parasitemia.<sup>1,117–120</sup> Exchange also appears effective in neonates with severe transfusion-associated babesiosis.<sup>121</sup> Current recommendations from ASFA<sup>1</sup> and ISDA<sup>122</sup> support RCE in severely ill individuals. A single one- to two-volume RCE can reduce the circulating population of parasitized erythrocytes by 85–90%.<sup>1</sup> Unfortunately, the true effectiveness of RBC exchange on morbidity and mortality has been difficult to glean and ultimately still awaits better controlled data and potentially randomized controlled trials.<sup>94,123</sup>

### Erythrocytapheresis and therapeutic phlebotomy

Therapeutic phlebotomy or venesection is a procedure that has been performed throughout recorded history. In current clinical practice, therapeutic phlebotomy is an evidence-based intervention for disorders such as polycythemia vera and hemochromatosis.<sup>124</sup> Although erythrocytapheresis has been investigated as a means of more rapid removal of red cell mass and limiting plasma loss, phlebotomy remains the therapeutic mainstay in most settings given its simplicity.<sup>125</sup>

### Polycythemia vera

Classified as a bcr/abl-negative classic myeloproliferative neoplasm showing absolute erythrocytosis,<sup>126</sup> it is further characterized by panmyelosis in the marrow and peripheral blood, splenomegaly, hyperviscosity of the blood, thrombosis, and a tendency to evolve into either acute myeloid leukemia or myelofibrosis.<sup>127,128</sup> Over 95% of cases are now known to be associated with an acquired point mutation, V617F, in exon 14 of the Janus kinase 2 (JAK2) gene on chromosome 9p24.<sup>129</sup> The remaining 5% of patients appear to have mutations in exon 12 of JAK2, some of whom may have a more benign phenotype (idiopathic erythrocytosis).<sup>129</sup> World Health Organization (WHO) diagnostic system (Table 26.2) recognizes the predominance of JAK2 mutations in polycythemia and key finding of erythrocytosis and hypercellular bone marrow.<sup>130</sup> Setting precise cutoffs for erythrocytosis is challenging, and the most recent findings lean toward sensitivity given the utility of mutational detection for specificity. Although more rarely used, The International Council for Standardization in Haematology recognizes absolute erythrocytosis in an individual whose measured total red cell volume, or *red cell mass*, is more than 25% above

**Table 26.2** Diagnostic Criteria for Polycythemia Vera. Source: Adapted from Barbui et al.<sup>130</sup>

<b>Major</b>	<b>1. Male: Hgb &gt; 16.5 g/dL (Hct 60%); female: 16.0 g/dL (Hct &gt; 48%) or other evidence of increased RBC mass</b>
	2. Bone marrow with hypercellularity for age and trilineage myeloproliferation
<b>Minor</b>	3. Presence of JAK2 V617F or JAK2 exon 12 functional mutation
	1. Serum erythropoietin level below laboratory reference range

Diagnosis: All three major criteria or first two major criteria and the minor criterion.

the mean predicted value for a person of the same body surface area.<sup>128</sup> This formed the basis for previous Hgb/Hct cutoff of  $\geq 60\%$  (Hgb  $\geq 18.5$  g/dL) and women with a hematocrit  $\geq 56\%$  (Hgb  $\geq 16.5$  g/dL). But such specific cutoffs for Hgb in the WHO criteria were not sensitive enough and missed mildly elevated RBC mass in early polycythemia vera.<sup>131</sup>

### Therapeutic phlebotomy in polycythemia vera

The increased whole blood viscosity that results from the expansion of total red cell volume in patients with polycythemia vera is the underlying basis of the life-threatening prothrombotic state and the headache, fatigue, dyspnea, cyanosis, and other signs and symptoms that characterize the disorder.<sup>130</sup> Aggressive phlebotomy to a hematocrit below 45% in males and females is indicated for the prevention of life-threatening thrombotic complications of polycythemia vera.<sup>132</sup> High-risk patients (age  $>60$  years or history of thrombosis) should also be treated with cytoreductive hydroxyurea or, if erythrocytosis is recalcitrant, a second-line agent (e.g., busulfan or interferon- $\alpha$ ).<sup>132</sup> Low-risk patients (age  $<60$  years, no cardiovascular risk, platelet count  $<1,000,000/\mu\text{L}$ ) may initially be managed with phlebotomy alone.<sup>132</sup> Low-dose aspirin (100 mg/day) is recommended for all patients without specific contraindications to its use.<sup>132</sup> In general, approved Janus kinase inhibitors are reserved as second-line therapy when standard cytoreductive therapy fails or is not tolerated.<sup>132,133</sup> The development of agents with greater JAK2 and mutant specific inhibition still holds great promise and should influence future management of polycythemia vera.<sup>133,134</sup>

### Erythrocytapheresis in polycythemia vera

According to ASFA, polycythemia vera is a category I indication for red cell volume reduction by erythrocytapheresis.<sup>1</sup> A retrospective case series of 69 patients with polycythemia vera who underwent 206 isovolemic erythrocytapheresis procedures using 4% albumin as replacement fluid reported the reduction of hematocrit from  $56.8 \pm 5.6\%$  to  $41.9 \pm 6.6\%$  after the removal of  $1410 \pm 418$  mL of red cells.<sup>135</sup> A subset of 21 patients for whom close follow-up data were available maintained a hematocrit of  $<50\%$  for a median of six months.<sup>135</sup> The volume of red cells to be removed (VR) during an erythrocytapheresis in order to achieve a desired hematocrit can be calculated as (Formula (2)):<sup>135,136</sup>

$$\text{VR} = \left( \frac{\text{starting HCT} - \text{desired Hct}}{79} \right) \times \left( \frac{\text{blood volume}}{\text{(ml/kg)}} \right) \times \left( \frac{\text{blood weight}}{\text{(kg)}} \right) \quad (2)$$

Thus, for a 70-kg person with a blood volume of 70 mL/kg whose hematocrit is to be lowered from 68% to 55%, the volume of red cells to be removed is calculated as

$$\text{VR} = \left( \frac{68-55}{79} \right) \times (70 \text{mL/kg}) \times (70 \text{kg}) = 910 \text{mL of red cells}$$

Additional studies have confirmed that erythrocytapheresis can rapidly decrease hematocrit for extended intervals relative to simple phlebotomy.<sup>136-138</sup> Costs may be more equivalent when patient time lost to increased visits with traditional phlebotomy is considered.<sup>138</sup> Automated erythrocytapheresis may have a role for patients with acute thrombotic or microvascular complications, or to avoid perioperative thrombohemorrhagic complications in a patient with an uncontrolled hematocrit who requires urgent surgery.<sup>1,136</sup>

### Secondary erythrocytosis

Secondary erythrocytosis includes conditions that result in an elevated total red cell volume but are not clonal disorders of the marrow.<sup>126,128,139</sup> Congenital and acquired causes have been described, and they are relatively rare and predominantly involve the regulation or aberrant expression of erythropoietin or abnormalities of the erythropoietin receptor (see Table 26.3).<sup>140,141</sup> The vast majority of cases are hypoxia-stimulated, usually due to chronic lung disease, smoking, or apnea. A diagnostic investigation of a patient with suspected erythrocytosis is performed in order to (1) establish that a true state of erythrocytosis exists (i.e., an elevated total red cell volume), (2) rule out polycythemia vera, and (3) determine the cause of secondary erythrocytosis, thereby leading to clinical management to alleviate the underlying cause.<sup>126,128,139-141</sup>

### Therapeutic phlebotomy in secondary erythrocytosis

The role of phlebotomy is less certain in secondary erythrocytosis than in polycythemia vera.<sup>1,141</sup> As suggested by Table 26.3, secondary erythrocytosis is generally an adaptation to the disordered regulation of erythropoietin or to hypoxemia. In some cases, the underlying cause can be treated medically or surgically, and in others the erythrocytosis represents a physiologic adaptation to a chronic condition such as hypoxia but without thrombotic risk.<sup>142</sup> For example, adults with cyanotic congenital heart disease are not considered to be at heightened risk for thrombotic stroke despite mean hematocrits of  $57.5\% \pm 7.2\%$ <sup>143</sup> and do not exhibit symptoms of hyperviscosity until hematocrits reach 65% (in the absence of dehydration or iron deficiency).<sup>144</sup> A program of therapeutic phlebotomy should not be undertaken purely for the sake of achieving a target hematocrit in an asymptomatic individual. Isovolemic phlebotomy, with saline replacement, should be reserved for patients who are neither dehydrated nor iron deficient, and who have moderate symptoms of hyperviscosity (i.e., headache, slow mentation, visual disturbance, tinnitus, dizziness, etc.).<sup>144</sup> Withdrawal of up to a unit of whole blood, replaced by 750–1000 mL of isotonic saline, has been recommended for the relief of symptoms. Similar recommendations may refer to patients with high oxygen-affinity hemoglobin levels who have symptoms such as dizziness, dyspnea, or angina, which are believed to result, in part, from an expanded total red cell volume.<sup>141</sup> There is again no formal evidence that phlebotomy is beneficial, and a modest target (i.e., a hematocrit  $<60\%$  achieved with fluid replacement) has been recommended.<sup>141</sup> Likewise, patients with chronic hypoxic lung disease and erythrocytosis or with smoker's erythrocytosis are best managed using medical therapy to deal with their underlying pulmonary disorder. Uncontrolled studies suggest that phlebotomy to a hematocrit of 50%–52% may improve exercise tolerance, alleviate headache and confusion, and otherwise ameliorate symptoms of hyperviscosity.<sup>141</sup>

Postrenal transplant erythrocytosis, defined as a persistently elevated hematocrit above 51%, occurs spontaneously in 15–20% of kidney transplant recipients in the first 8–24 months after engraftment.<sup>145-147</sup> One-fourth of cases remit spontaneously within two years of onset, with the balance persisting for up to several years until chronic graft rejection supervenes.<sup>141</sup> The major risk factors are retention of the native kidneys, male gender, smoking, a rejection-free course with a well-functioning graft, and adequate red cell production (without the need for erythropoietin or transfusion) prior to transplant.<sup>145,147</sup> Hyperviscosity symptoms such as malaise, headache, plethora, lethargy, and dizziness are described as common among patients with this condition, and 10–30%

**Table 26.3** Secondary Erythrocytosis<sup>140,141</sup>

Type	Underlying Cause
Congenital	Genetic mutations <ul style="list-style-type: none"> <li>• High oxygen-affinity hemoglobin</li> <li>• Bisphosphoglycerate mutase deficiency</li> <li>• Erythropoietin receptor mutation</li> <li>• Oxygen-sensing pathway mutations (<i>VHL</i>, <i>PHD2</i>, and <i>HIF-2a</i> gene mutations)</li> </ul>
Acquired	Hypoxia-stimulated <ul style="list-style-type: none"> <li>• Cyanotic congenital heart disease</li> <li>• Chronic lung disease</li> <li>• High-altitude habitat</li> <li>• Smoker's erythrocytosis</li> <li>• Carbon monoxide poisoning</li> <li>• Chronic hyp ventilation (sleep apnea)</li> <li>• Renal artery stenosis</li> </ul> Inappropriate erythropoietin production <ul style="list-style-type: none"> <li>• Renal cancer</li> <li>• Hepatic cancer</li> <li>• Cerebellar hemangioblastoma</li> <li>• Endocrine tumors</li> <li>• Uterine leiomyoma</li> <li>• Polycystic kidney</li> <li>• Meningioma</li> </ul> Drug-mediated <ul style="list-style-type: none"> <li>• Androgen therapy</li> <li>• "Blood doping" (surreptitious erythropoietin use)</li> </ul> Multifactorial etiology <ul style="list-style-type: none"> <li>• Postrenal transplant erythrocytosis</li> </ul>

develop significant thromboembolic complications.<sup>145,147</sup> The pathogenesis appears to be multifactorial and likely involves an interplay between endogenous erythropoietin production by the retained native kidney, the renin-angiotensin system, androgen secretion, insulin-like growth factors, and cytokines.<sup>145,146</sup> One retrospective series reported 11 thromboembolic events, including transient ischemic attacks and strokes, and venous thromboembolism in 10 of 53 (19%) patients with postrenal transplant erythrocytosis but in none of 49 control cases ( $p < 0.001$ ). This sort of experience has led to an appreciation of the need to control the red cell volume in these patients.<sup>145–147</sup> The mainstay of treatment is angiotensin-converting enzyme inhibition or angiotensin-converting enzyme receptor blockade, sometimes in combination with theophylline, which lowers hemoglobin and hematocrit within eight weeks, with peak effect seen after up to 12 months.<sup>148</sup> Phlebotomy is reserved for cases where erythrocytosis inadequately responds to drug treatment.

#### Erythrocytapheresis in secondary erythrocytosis

Automated erythrocytapheresis is seldom recommended for the management of secondary erythrocytosis,<sup>149</sup> and its optimum role has not been established, thus requiring individualized decisions.<sup>1</sup> It may be useful in circumstances where isovolemic procedures are called for, such as in cyanotic heart disease.<sup>144</sup> Erythrocytapheresis has not been reported in the management of posttransplant erythrocytosis.

#### Hereditary hemochromatosis (HH)

Hereditary hemochromatosis is an inherited disorder that, untreated, results in iron deposition in, and damage to, the liver, heart, pancreas, and other organs, including bronze pigmentation of the skin.<sup>150,151</sup> Its prevalence is approximately 1:200 among those of European ancestry.<sup>150,152</sup> The most common genetic mutation, accounting for >90% of cases (and almost all cases in persons of Northern European ancestry), is homozygosity for a missense mutation (G845A) in the *HFE*

gene resulting in tyrosine substituted for cysteine (C282Y).<sup>153</sup> HFE C282Y (as well as H63D) decreases hepcidin transcription by stabilizing the ALK3 protein that inhibits transcription.<sup>154</sup> In the absence of a physiological means of body iron excretion, the increased iron uptake resulting from these mutations leads to the slow accumulation of iron in the liver and other organs, and eventual liver failure (via cirrhosis, hepatocellular carcinoma, etc.), diabetes, hypogonadism, hypopituitarism, arthropathy, cardiomyopathy and heart failure, and skin pigmentation.<sup>152</sup> A presenting syndrome of asthenia, arthralgia, and abnormal liver function (the three As) has been described as classic for the clinical disease.<sup>151</sup> Because of the central importance of iron loading in the pathogenesis of HH, iron removal by phlebotomy is the mainstay of treatment.<sup>152,155</sup> Diagnostic elements of HH are provided in Table 26.4.

#### Therapeutic phlebotomy in HH

Therapeutic phlebotomy has been the primary mode of iron reduction in HH for over a half century.<sup>150,156</sup> Phlebotomy therapy should be started in all patients whose serum ferritin level is elevated (Table 26.4) and should not be withheld from the elderly on the basis of age or from iron-loaded patients who have not developed clinical symptoms.<sup>151,156</sup> A common treatment approach is to perform one phlebotomy per week (1 unit or 7 mL/kg of whole blood not to exceed 550 mL per phlebotomy) until the serum ferritin is below 50 ng/mL (although this endpoint is not based on clear-cut evidence).<sup>155–157</sup> Thereafter, it is usually necessary to annually remove 3–4 units of blood to maintain the ferritin between 50 and 100 ng/mL. Strict maintenance below 50 ng/mL and <50% transferrin saturation has been recommended by others as optimal.<sup>155,158</sup> While transferrin saturation control has been associated with decreased disease progression in terms of arthropathy based on retrospective data, well-controlled data for ferritin and other markers to inform best maintenance targets are lacking.

Malaise, weakness, fatigability, skin pigmentation, cardiac function, and liver transaminase elevations often improve with treatment, whereas diabetes, cirrhosis, arthropathy, pituitary dysfunction, and hypogonadism almost never improve. Importantly, the risk of hepatocellular carcinoma will persist if cirrhosis was present before the onset of phlebotomy therapy.<sup>151,156</sup>

**Table 26.4** Some Diagnostic Considerations in HH (Absent a Cause of Secondary Iron Overload)<sup>156,157</sup>

Diagnostic Tool	Factors to Consider
Clinical clues	<ul style="list-style-type: none"> <li>• Celtic ethnicity</li> <li>• Chronic asthenia</li> <li>• Arthropathy (fourth and fifth metacarpophalangeal joints: "hemochromatosis handshake")</li> <li>• Impotence</li> <li>• Hyperpigmentation</li> <li>• Liver abnormalities (transaminase elevation and hepatomegaly)</li> <li>• Diabetes</li> <li>• Cardiomyopathy</li> <li>• Transferrin saturation &gt;45%</li> <li>• Serum ferritin: &gt;200 ng/mL in premenopausal women; &gt;300 ng/mL in men</li> </ul>
Screening criteria	<ul style="list-style-type: none"> <li>• Hepatic iron index &gt;1.9</li> <li>• Hepatic iron concentration &gt;80 (µmol/g dry weight)</li> <li>• Grade 3–4 hepatic iron deposition</li> <li>• <i>HFE</i> gene analysis</li> </ul>
Diagnostic tests	

### Erythrocytapheresis in HH

This was first described in Europe, where a German group from Munich reported successful lowering of iron in 14 patients with hemochromatosis, with intervals of 2–11 months between procedures.<sup>9</sup> Their follow-up study<sup>159</sup> described prospective observations on eight patients who were treated with isovolemic erythrocytapheresis (1000 mL removed) every four weeks until serum ferritin fell below 300 ng/mL. Iron depletion, thus defined, was achieved after a mean of 8.5 months, during which a mean of 9.4 liters of red cells was removed in a mean of 8.9 procedures. Subsequent studies have supported these findings, and, based on this rapidity of response and the potential removal of 2–3 times more RBCs per procedure compared to a simple phlebotomy, it has been designated a first-line therapy (category I) in the ASFA guidelines.<sup>1</sup> Recently, there have been controlled trials comparing the relative efficacy and cost-effectiveness of erythrocytapheresis versus simple phlebotomy.<sup>160,161</sup> The first study compared 500 mL weekly phlebotomies to erythrocytapheresis every two weeks. The authors found that erythrocytapheresis patients demonstrated a more rapid decline and normalization of ferritin; however, this group started with a lower average ferritin. This higher iron load in the phlebotomy group was supported by the fact that threefold more phlebotomies were needed to normalize the serum ferritin level, even though erythrocytapheresis removed only twofold more iron-containing RBCs.<sup>160</sup> Overall, these data suggest that an erythrocytapheresis procedure every two weeks and weekly whole blood phlebotomy are equivalent, a strategy that is supported by another randomized control trial that found no significant difference in mean time to iron depletion (ferritin <50 ng/mL).<sup>161</sup> In this study, the mean technician time was increased for the apheresis group,<sup>161</sup> consistent with the added procedural complexity, and thus decisions between the two modalities should probably be determined by patient and institutional preferences related to costs and effort. Intriguingly, although on average two simple phlebotomies remove equivalent iron to one erythrocytapheresis, individuals with larger blood volumes see a greater gain from erythrocytapheresis.<sup>125</sup> This is a consequence of the lack of volume adjustment for simple phlebotomy and suggests that blood-volume-based simple phlebotomies could be an optimal solution in terms of costs and effort. Recent work examining maintenance phlebotomy suggests that there is minimal difference in procedure numbers (3.3 vs 1.9 annually for phlebotomy and cytapheresis, respectively) and thus cost of apheresis was not significantly defrayed.<sup>162</sup> A recent Cochrane review reported insufficient evidence to recommend erythrocytapheresis over therapeutic phlebotomy for HH.<sup>163</sup> Without consensus, leveraging predictive models can help determine individuals who may be most appropriate for cytapheresis.<sup>164</sup>

### Therapeutic platelet apheresis

*Thrombocytosis* (or *thrombocythemia*) is defined as a platelet count  $>450 \times 10^3$  cells/ $\mu$ L for adults, with typically higher thresholds for infants and children.<sup>165,166</sup> Elevated platelet counts can arise from primary myeloproliferative diseases or secondary to a range of conditions. Mutations in erythropoietin (*EPO*), its receptor (*MPL*), associated signaling pathways (via *JAK2*), and calreticulin (*CALR*) underlie many primary thrombocythemic states.<sup>165,167</sup> In children and younger adults, most thrombocytosis is reactive (Table 26.5).<sup>168–170</sup> Extreme thrombocytosis (EXT) is defined as a platelet count  $>1000 \times 10^3$  cells/ $\mu$ L and occurs in approximately 1% of hospitalized

**Table 26.5** Some Causes of Primary (Clonal) and Secondary (Reactive) Thrombocytosis

Type	Possible Causes
Primary thrombocytosis	Essential thrombocytosis Polycythemia vera Chronic myelogenous leukemia Myelofibrosis Myelodysplastic syndrome (5q-) Acute hemorrhage
Secondary thrombocytosis	Chronic blood loss with iron deficiency Tissue damage or trauma Malignancy Inflammatory bowel disease Acute or chronic inflammation Acute or chronic infection Physical exercise Rebound (after chemotherapy, splenectomy, or immune thrombocytopenic purpura) Medication

patients.<sup>171</sup> There is great variability and tolerance of such high counts in individuals, which is likely related to the underlying cause as well as the duration of the high count. In children, extreme thrombocytosis is almost always reactive, transient, and does not require specific medical treatment.<sup>165,172</sup> The most common setting of EXT in a recent adult series was reactive thrombocytosis in post-surgical patients (50.8%). Hematologic malignancy was a cause in 30% of all patients, but it was 3.4 times more likely to be the cause of EXT in outpatients compared to inpatients. Most cases were multifactorial and resolved by treating the underlying causes.<sup>171</sup>

*Thrombocytapheresis* is a term that describes the selective removal of platelets from a patient, for therapeutic purposes, using a blood-processing (apheresis) device.<sup>170</sup> The 2019 ASFA review of indications for apheresis therapy lists “symptomatic thrombocytosis” as a category II indication for thrombocytapheresis.<sup>1</sup> This designation refers to primary thrombocytosis as results from a clonal (myeloproliferative) disorder of the marrow.<sup>168</sup> Thrombocytapheresis for prophylaxis in asymptomatic patients or to lower the platelet count in cases of secondary or reactive thrombocytosis is listed as a category III (i.e., specific role of the procedure not determined in this condition) indication because published evidence is insufficient to establish when the procedure is of benefit in these circumstances.<sup>1</sup> Secondary thrombocytosis per se does not convey a risk of thromboembolic morbidity absent confounding factors such as malignancy or major surgery.<sup>169,173</sup> Even then, antiplatelet agents should be the first option for treatment.<sup>170</sup> In any case, the treatment of the underlying cause is the prime factor in the resolution of secondary thrombocytosis. In fact, given the absence of risk posed by the platelet count in secondary thrombocytosis, the platelet count may be considered a laboratory sign of an underlying condition that should be investigated.<sup>168</sup>

### Primary thrombocytosis (essential thrombocythemia, ET)

Thrombocytosis with thromboembolic complications is a feature common to chronic myeloproliferative disorders,<sup>66,126</sup> of which half are essential thrombocythemia.<sup>95,169</sup> Thus, the remainder of this discussion will focus on essential thrombocythemia. Both thrombotic and hemorrhagic complications result in patients with essential thrombocythemia.<sup>168,174</sup> The molecular basis of myeloproliferative neoplasms has provided new insights into the pathophysiology of this disease. Somatic mutations in *JAK2*, *CALR*, or *MPL* have been

reported in approximately 90% of patients, aid in diagnosis, and influence the clinical presentation.<sup>167,174</sup> Factors that increase risk of arterial thrombosis include age greater than 60 years; thrombosis history; cardiovascular risk factors such as hypertension, hypercholesterolemia, and diabetes; and *JAK2* V617F positivity;<sup>98,175</sup> 50–60% of patients with ET have a *JAK2* mutation, which leads to thrombopoietin-independent cell proliferation. *CALR*-mutated ET is associated with extreme platelet counts ( $>10^6/\mu\text{L}$ ), reduced risk of thrombosis, and bleeding due to an acquired von Willebrand syndrome (AvWS). AvWS results from the accelerated clearance of hemostatically competent large multimers of von Willebrand factor from the circulation, which improves with therapeutic reduction of the platelet count.<sup>175–177</sup> The *MPL* gene encodes the thrombopoietin receptor, and several mutations cause a small proportion of ET (about 5% of cases). A recent systematic review found that platelet activation is increased in ET, particularly in patients with a history of thrombosis, but other platelet attributes or function were inconsistent.<sup>178</sup> There is no evidence in favor of prophylactic apheresis treatment of low-risk, asymptomatic patients, regardless of platelet count, but high-risk and symptomatic patients may be treated to lower their platelet count to below  $400,000/\mu\text{L}$ . Low-dose aspirin is a proven treatment in all patients, although AvWS should be screened for in those with extreme thrombocytosis ( $>10^6/\mu\text{L}$ ).<sup>179</sup> The United Kingdom Medical Research Council Primary Thrombocythemia 1 Study has established hydroxyurea plus low-dose aspirin as the treatment of choice in high-risk patients.<sup>180</sup> Whereas the rate of first thrombosis in an untreated high-risk population is approximately 26% at two years, the rate decreases to about 4% at two years with hydroxyurea and aspirin.<sup>180</sup>

### **Therapeutic thrombocytapheresis in primary thrombocytosis**

Rapid lowering of an elevated platelet count, using apheresis and/or chemotherapy, is indicated for patients with myeloproliferative neoplasms who present with clinical syndromes of microvascular thrombosis such as digital or cerebral ischemia.<sup>1</sup> Case series and case reports have reported successful, rapid lowering of the platelet count in symptomatic patients in whom chemotherapy either was not an immediate option or was judged to have an insufficiently rapid effect.<sup>181</sup> Procedures in which 1.5–2.0 blood volumes are processed, and crystalloid replacement fluids are used to manage fluid balance, can lower the platelet count by approximately 40–60%,<sup>1,182</sup> but reductions as great as 65–70% have been reported with the latest apheresis instruments.<sup>183,184</sup> Like all cytapheresis, thrombocytapheresis without concomitant pharmacotherapy is not a practical means for controlling the platelet count beyond the acute setting. Although thrombocytapheresis has been used in the past for the management of a high-risk pregnant patient with essential thrombocythemia for whom hydroxyurea is contraindicated, interferon-alpha is the treatment of choice in pregnancy.<sup>1,185</sup>

### **Therapeutic white cell apheresis**

Hyperleukocytosis (white cell count  $>100,000/\mu\text{L}$ ) with symptomatic leukostasis is a category II indication for therapeutic leukocytapheresis.<sup>11</sup> Leukocytapheresis by selective adsorption techniques has shown a disappointing lack of efficacy in trials for idiopathic inflammatory bowel disease.<sup>186,187</sup> For ulcerative colitis, the remission rate was 17% for leukocytapheresis and 11% for the sham control ( $p = 0.36$ );<sup>186</sup> and for Crohn's disease, the remission rate was 18% for the leukocytapheresis and 19% for the sham control ( $p = 0.86$ ).<sup>187</sup>

Currently, ASFA rates immunoabsorption for IBD as a category III indication, except for ulcerative colitis in Japan, which is category II and may be due to the fact that TNF $\alpha$  blockade is not standard therapy in Japan.<sup>1</sup>

### **Leukocytapheresis for hyperleukocytosis**

Hyperleukocytosis is a major risk factor for early mortality, often from pulmonary and/or central nervous system hemorrhage, in adults and children with acute myeloblastic leukemia or other hyperleukocytic leukemias.<sup>188</sup> It occurs in 5–13% and 12–25% of adult and pediatric AML cases, respectively.<sup>189</sup> The reported incidence in acute lymphoblastic leukemia ranges from 10% to 30%.<sup>189</sup> Mortality rates of 20–40% have been reported.<sup>189</sup> Leukostasis represents end-organ damage due to leukocyte-mediated microvascular occlusion and damage resulting in infarct and hemorrhage. It usually does not occur until white blood cell counts surpass  $100,000/\mu\text{L}$  in acute myelogenous leukemia (AML) and  $400,000/\mu\text{L}$  in acute lymphoblastic leukemia (ALL).<sup>1</sup> Clinical features of leukostasis include respiratory distress, hypoxemia, diffuse interstitial or alveolar infiltrates on chest X-ray, confusion, somnolence, stupor or coma, headache, dizziness, tinnitus, gait instability, or visual disturbances.<sup>190</sup> Physical examination may demonstrate papilledema, dilated retinal veins and/or retinal hemorrhages, cranial nerve defects, or meningeal signs.<sup>189</sup> Metabolic derangements caused by tumor lysis may include hyperkalemia, hyperuricemia, hypocalcemia, and hyperphosphatemia and may result in renal failure and early death. Coagulopathy results from release of lysosomal enzymes from myeloid blasts, disseminated intravascular coagulation, and thrombocytopenia resulting from marrow failure.<sup>190</sup>

A standard treatment approach to hyperleukocytosis includes intravenous hydration and lowering of plasma uric acid using allopurinol or urate oxidase. Hydroxyurea may be prescribed to rapidly lower the total circulating nucleated cell count without precipitating a tumor lysis syndrome.<sup>189</sup> Induction chemotherapy may be used for this purpose but may precipitate tumor lysis syndrome and hemorrhage.<sup>191</sup> The impact of leukocytapheresis (or *leukapheresis*) on important clinical outcomes, including overall survival, is controversial. There is no doubt that processing 1.5–2.0 blood volumes by apheresis can reduce the circulating white cell count by up to 60%.<sup>1,190–193</sup> However, a recent meta-analysis across 13 retrospective studies involving 1743 patients showed that leukapheresis did not improve early mortality compared to medical therapies (RR, 0.88; 95% confidence interval, 0.69–1.13;  $p = 0.321$ ), consistent with a prior meta-analysis from 2014.<sup>194,195</sup> This lack of improved survival is seen in diverse global populations, such as a group of 40 Indonesian patients for whom there was no difference in either 7-day or 28-day survival for patients receiving leukapheresis in addition to chemotherapy compared to patients receiving chemotherapy alone, although poor survival did correlate with age  $>60$  years, creatinine  $>1.4$ , blasts  $\geq 90\%$ , and DIC.<sup>192,193,196,197</sup> Hyperleukocytosis is distinct from leukostasis since the metabolic anomalies, coagulopathy, tumor lysis, and multiorgan failure that characterize the leukostasis syndrome may occur at variable WBC counts.<sup>198–200</sup> Hyperleukocytosis without symptoms is an ASFA category III (individualize decision-making) for leukocytapheresis. Hyperleukocytosis with leukostasis is classified as category II mainly based on anecdotal reports of rapid reversal of signs and symptoms. Cytoreduction by medical therapies remains the emergent, first-line treatment for symptomatic leukostasis.<sup>1</sup>

## Extracorporeal photopheresis

Extracorporeal photopheresis (ECP or *photochemotherapy*) describes a procedure in which circulating mononuclear cells are collected by centrifugal apheresis, treated with 8-methoxypsoralen (8-MOP, a photoactivating agent that intercalates with DNA), and then exposed to ultraviolet A (UVA) light. The treated cells are then reinfused into the patient. A full procedure is completed in approximately 1.5–3 hours.<sup>201,202</sup> The mechanism of action of ECP is poorly understood. In a broad sense, DNA crosslinking in photo-treated leukocytes is thought to induce apoptosis in treated cells, which are then phagocytosed by immature dendritic cells upon reinfusion, resulting in both direct and indirect immunomodulation.<sup>202,203</sup> Although the main effects vary somewhat among diseases, they likely include the generation of tolerogenic dendritic cells, skewing toward anti-inflammatory cytokine production, and increased tolerogenic and regulatory T-cell populations.<sup>202,203</sup> Currently, erythrodermic cutaneous T-cell lymphoma (CTCL) is the only category I indication for ECP. ASFA has listed the prophylaxis and treatment of heart transplant cellular rejection, acute or chronic graft-versus-host disease (GVHD), and chronic lung allograft rejection from bronchiolitis obliterans syndrome as category II indications for ECP, with variable qualities of evidence. There is a growing body of potential applications in transplant rejection as well as in autoimmune diseases for which the ASFA recommendations are generally category III.<sup>1,204</sup> Some ECP regimens used in the treatment of the category I and II indications are described in Table 26.6.

**Table 26.6** Regimens of Extracorporeal Photochemotherapy (ECP)

Indication	Treatment
Cutaneous T-cell lymphoma	ECP cycle (two consecutive days) once or twice a month; continue for six months before declaring treatment failure
Cardiac allograft rejection	<p>Prophylaxis</p> <ul style="list-style-type: none"> <li>• Month 1 after transplant: ECP days 1 and 2, 5 and 6, 10 and 11, 17 and 18, and 27 and 28</li> <li>• Months 2 and 3 after transplant: ECP on two successive days every two weeks</li> <li>• Months 4–6 after transplant: ECP on two successive days every four weeks</li> </ul> <p>Acute (moderate) rejection</p> <ul style="list-style-type: none"> <li>• ECP procedures on two consecutive days at weekly episode intervals as needed to resolve rejection as indicated by endomyocardial biopsy</li> </ul> <p>Recurrent/refractory cellular rejection</p> <ul style="list-style-type: none"> <li>• ECP on two consecutive days weekly for one month, then every two weeks for two months, and then monthly for three months (total of 22 procedures)</li> </ul> <p>Chronic rejection</p> <ul style="list-style-type: none"> <li>• Beginning within one month of transplantation: ECP on two successive days every four weeks for twelve months, every six weeks for next six months, and every eight weeks during next six months</li> <li>• ECP two consecutive days: weekly for two months, then every two weeks for the next two months, then monthly for a total of six months</li> <li>• ECP on two consecutive days every 1–2 weeks, and then consider monthly interval; treat at least six months before declaring treatment failure</li> <li>• ECP two consecutive days weekly for two months (approximately 20 procedures), then weekly for three months, tapering to one procedure every other week, and then monthly till tapered completely</li> </ul>
Lung allograft rejection: bronchiolitis obliterans syndrome (BOS)	
Chronic graft-versus-host disease	

## Cutaneous T-cell lymphoma (CTCL)

The CTCLs are a heterogeneous group of extranodal non-Hodgkin lymphomas of T-cell origin that target the skin.<sup>205</sup> Mycosis fungoides, the most common form of CTCL, accounts for almost half of all primary cutaneous lymphomas.<sup>202</sup> It is largely a disease of adults (median age 55–60 years at diagnosis) and typically presents as an indolent disorder that progresses slowly over years from patchy skin involvement to infiltrated plaques, tumors, and widespread disease.<sup>202</sup> Whereas localized (e.g., nonerythrodermic) mycosis fungoides is adequately managed with topical therapies, the application of ECP in erythrodermic mycosis fungoides is recommended by ASFA.<sup>1</sup> Whereas limited-stage mycosis fungoides does not shorten life expectancy, advanced-stage disease may be associated with a 10-year disease-specific survival of 20%.<sup>206</sup> Sézary syndrome is defined as a triad of erythroderma, generalized lymphadenopathy, and the presence of neoplastic T cells (Sézary cells) in the skin, lymph nodes, and peripheral blood.<sup>202,207</sup> A retrospective cohort study from Stanford University of 106 patients with erythrodermic mycosis fungoides and Sézary syndrome identified age  $\geq 65$  years, clinical Stage IV, and circulating Sézary cells  $\geq 5\%$  of total lymphocytes as independent negative prognostic factors for survival.<sup>207</sup> Median survivals of patients with none, one, or more than one of these adverse prognostic factors were 122 months ( $n = 36$ ), 44 months ( $n = 39$ ), and 18 months ( $n = 31$ ), respectively ( $p < 0.005$ ).<sup>208</sup>

The 1987 report by Edelson *et al.*<sup>209</sup> of a successful pilot trial of ECP, in which 27 of 37 treatment-resistant patients with CTCL experienced an average 64% decrease in cutaneous involvement after  $22 \pm 10$  weeks, led to US Food and Drug Administration (FDA) approval of ECP for the treatment of CTCL later that year. Long-term follow-up of the original 29 patients with erythrodermic CTCL from that pilot study<sup>209</sup> reported that median survival of the treated patients was 60.33 months from diagnosis and 47.9 months from the start of ECP. Four of the six patients who achieved complete responses in the original study remained in complete remission.<sup>210</sup> Between 1987 and 2001, 21 studies reported a total of 485 patients treated using ECP.<sup>202,211</sup> Although most patients in these studies had erythrodermic CTCL, most studies did not report the response rates separately for the erythrodermic subjects.<sup>212</sup> In addition, responses were defined as  $>25\%$  skin clearing in some studies and  $>50\%$  skin clearing in others, and complete responses were defined as either 75–100% skin clearing,  $>90\%$  skin clearing, or 100% skin clearing.<sup>202</sup> Nonetheless, overall response rates reported in these studies ranged from 31% to 87.5%, and complete response rates ranged from 0% to 54% (20–30% in most studies).<sup>202,211–213</sup> The variability in response rates has been attributed to differences in entry criteria, prior or concurrent therapy, duration between diagnosis and application of ECP, the ECP protocol followed, and the definition of response.<sup>211</sup> Based on the above, ECP has been recommended by several international clinical guideline groups,<sup>213</sup> including the category I recommendation for erythrodermic CTCL from ASFA.<sup>1</sup>

## Cardiac and lung allograft rejection

In the United States, the rate of heart transplantation remained steady in the 2200–2500 range from the early 1990s to the 2010s, with an increase to 3500–3600 by 2020, but lung transplants have increased from less than 100 in 1989 to 2500 in 2020.<sup>214–216</sup> Rejection is still a serious problem for both heart and lung allografts, accounting for approximately 11% of deaths in the first three years after heart transplant, and underlies the majority of

graft failures that account for 35% of overall deaths.<sup>214</sup> Similarly for lung allografts, rejection underlies approximately 25% of mortality at five years, when bronchiolitis obliterans syndrome (BOS), the end result of chronic rejection, is included.<sup>215</sup> Immunosuppressive therapy to prevent allograft rejection has included perioperative antilymphocyte antibody (polyclonal antilymphocyte or antithymocyte globulin, OKT3, or IL-2 receptor antibodies), chronic postoperative calcineurin inhibitors (e.g., tacrolimus and cyclosporine), mycophenolate mofetil, sirolimus, and others.<sup>214,215</sup> Because of the association between rejection, graft failure, and death, evidence of rejection should result in adjustment in the immunosuppressive regimen to initially resolve the episode of rejection. For unresponsive cardiac rejection or lung transplant BOS, ECP is an appropriate adjuvant; and these are listed by ASFA as category II indications for ECP based on the evidence described below.<sup>1</sup>

### **Extracorporeal photochemotherapy in the treatment of cardiac allograft rejection**

Studies in support of ECP for treatment of cardiac allograft rejection focus on the effects of ECP on endomyocardial biopsy findings rather than on survival or graft function.<sup>211</sup> Two pilot studies have shown evidence that the risk of acute cellular rejection episodes can be decreased by incorporating ECP into the prophylactic immunosuppressive regimen of cardiac allograft recipients without increasing the risk of infection caused by immunosuppression.<sup>217,218</sup> A prospective randomized pilot trial comparing 10 cases receiving ECP and 13 controls treated with only immunosuppression reported that the development of panel-reactive antibodies (responsible for chronic antibody-mediated rejection) and coronary artery intimal hyperplasia (a pathogenetic mechanism of graft failure in chronic rejection) are mitigated by the addition of ECP to the posttransplant immunosuppressive regimen for the first two years after transplant surgery.<sup>219</sup> Dall'Amico and colleagues in Padua, Italy, reported a prospective pilot trial in eight patients, with recurrent acute rejection episodes despite immunosuppression, who were treated with two consecutive days of ECP every four weeks for six months.<sup>220</sup> Seven benefited with a reduction in the number and severity of rejection episodes; reduction in daily prednisone, cyclosporine, or azathioprine doses; and improvement on endomyocardial biopsy specimens. The proinflammatory cytokine IL-6 was decreased in 13 recipients who were treated with ECP for recurrent AMR, and ejection fraction increased from  $35 \pm 20\%$  to  $45 \pm 23\%$  ( $p = 0.004$ ) after ECP, although antibody levels were unchanged.<sup>221</sup> Other small case series and case reports have presented corroborating data supporting ECP efficacy.<sup>222</sup>

### **Extracorporeal photochemotherapy in the treatment of lung allograft rejection**

The use of ECP in lung transplant is a promising new avenue,<sup>223</sup> although mostly retrospective studies have been reported thus far. A major RCT on the topic, designed to assess the efficacy and tolerability of ECP for either refractory or newly diagnosed BOS, is due to be completed in 2025.<sup>223</sup> The first reports of treatment were in 1995 and represented a handful of cases pointing to effective improvement in clinical lung function (FEV<sub>1</sub>) and histological improvement. A number of small studies followed that generally supported these findings.<sup>222</sup> Only recently have larger studies examining ECP been reported,<sup>87,224,225</sup> encompassing a total 135 patients and supporting a role for ECP in stabiliz-

ing lung function (FEV<sub>1</sub>) with minimal procedural side effects. This trend has persisted, with nearly 200 additional patients reported in retrospective studies<sup>226</sup> and a single report of 51 patients in a prospective study also showed significantly longer survival in the 61% of patients who responded to ECP compared to both ECP-nonresponders and patients without ECP treatment.<sup>87</sup> Although the lack of adequate controls leaves a larger question as to the benefit of ECP compared to regimens that do not include ECP, there is a decrease in donor-specific antibodies in patients treated with ECP compared to controls, akin to ECP for cardiac rejection.<sup>227</sup>

### **Graft-versus-host disease**

GVHD occurs in hematopoietic progenitor cell transplant recipients when T cells of donor origin (either transplanted with, or that developed from, the graft) interact with tissue in the HLA-matched but genetically nonidentical host.<sup>228</sup> Classical acute GVHD develops within 100 days of transplantation, with skin manifestations that vary from an erythematous morbilliform rash to epidermal necrolysis, mucosal inflammation causing diarrhea and abdominal cramping, and abnormalities of liver function tests.<sup>228</sup> GVHD that develops beyond 100 days of transplantation, or persists more than three months, is traditionally referred to as chronic GVHD and is characterized by an oral, ocular, and mucous membrane sicca syndrome; skin involvement; scleroderma; bronchiolitis obliterans; joint contractures; myofascitis; esophageal stricture; or other fibrotic complications in various organ systems.<sup>228</sup> The cumulative incidence of acute GVHD is approximately 12–75%, and the cumulative incidence of chronic GVHD is approximately 15–70% after hematopoietic progenitor cell transplantation, depending on whether the donor-recipient pair are related or unrelated and whether a myeloablative or nonmyeloablative conditioning regimen was used.<sup>229,230</sup> Accurate diagnosis and staging are important in that recurrent or late-onset acute GVHD may not require prolonged therapy as is required with chronic GVHD, whereas overlap syndromes may require shorter courses of typical treatments for chronic GVHD.<sup>231</sup>

### **Extracorporeal photochemotherapy in the treatment of GVHD**

The application of ECP to the treatment of GVHD has been extensively reviewed.<sup>211,232</sup> Among the larger prospective reports is one from a London group that treated 28 patients who had developed steroid-refractory chronic GVHD following HLA-matched allogeneic marrow or peripheral blood progenitor cell transplant.<sup>233</sup> Among the patients, 27 were classified as having extensive chronic GVHD and 20 had involvement of more than 50% of their skin surface. Patients were given ECP on two consecutive days every two weeks for the first four months and monthly thereafter. ECP was initiated within a median of 34 months (range 10–167) after transplantation and 23 months (range 2–164) from the onset of chronic GVHD. Of the 21 patients with cutaneous involvement who were evaluable, a 25% reduction in skin involvement was noted in eight (38%) after three months and in 10 (48%) after six months, and a statistically nonsignificant improvement in liver function tests was noted.<sup>233</sup> There is only one randomized control study that failed to show significant improvement in the primary outcome, which was a decrease in skin involvement (14.5% for ECP arm vs 8.5% in control); however, there was significant decrease in steroid usage compared to the control arm.<sup>234</sup> Given the lack of large randomized prospective studies, a

meta-analysis was performed combining 18 prospective and retrospective studies of steroid-refractory GVHD with sufficient comparable cases.<sup>232</sup> This meta-analysis found significant efficacy with a complete response rate of 29% (CI 19–42%) along with particularly strong response rates of 74% (CI 60–85%) and 68% (CI 55–77%) for skin and liver, respectively.<sup>232</sup> Only modest activity was seen for lung or gastrointestinal manifestations. Based on the overall evidence, ASFA considers skin manifestations to be a category II indication for ECP, with a slightly better quality of evidence for chronic over acute presentations.<sup>1</sup>

### **Therapeutic apheresis of nonhematopoietic cells**

#### **Loiasis**

Loiasis is a parasitic infection by the nematode *Loa loa* that is transmitted by *Chrysops* flies via blood meals.<sup>235</sup> Adults reside in the subcutaneous tissues but produce microfilaria that are found in the blood and most body fluids. While most people are asymptomatic, the most common findings are Calabar swellings that are localized nontender sites usually on arms and legs near joints and eye worm that represents adult worm movement across the cornea. Fatigue, itching, malaise, and eosinophilia are often associated. Diethylcarbamazine (DEC) is the treatment of choice killing all life stages. However, there is a risk of neurologic complications with DEC treatment in those with high peripheral blood microfilarial levels (>8000/ $\mu$ L). Currently, while not specifically covered in the ASFA guidelines<sup>1</sup>, CDC treatment recommendations include the reduction of parasites to below 8000/ $\mu$ L with cytapheresis or initial albendazole prior to initiating DEC.<sup>235</sup> Apheresis has been used successfully in the past to significantly reduce parasite burden based on individual and series of cases, paying particular attention to the diurnal variation in parasitemia. Differences in methodology and the number of procedures make it difficult to determine the cellular constituent to target in the buffy coat region to maximize microfilarial removal. Buffy coat removal and platelet apheresis have shown good microfilarial reductions but often promote thrombocytopenia.<sup>236,237</sup> More recent mononuclear cell targeting using standard apheresis methods may work just as well as previous methods and spare platelets.<sup>238</sup>

### **Conclusion**

Refining the role of therapeutic cellular apheresis as a treatment option in diverse clinical conditions depends not only on a better understanding of the pathophysiology of the disorders in question

but also on the rapid, recent development of potent and effective medical therapies, particularly in the areas of antimicrobials, chemotherapy and chemical cytoreduction, and disease modifiers in sickle cell anemia. Evidence of the clinical impact and efficacy of apheresis therapies from properly conducted clinical studies should guide and direct the appropriate and deliberate application of apheresis technologies.

### **Disclaimer**

The authors have disclosed no conflicts of interest.

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## **SECTION VI**

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# **Blood transfusion**

## CHAPTER 27

# Patient blood management

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## Introduction

Patient blood management (PBM) is a multidisciplinary approach to improve patient outcomes using evidence-based strategies in patients who may need transfusion. PBM encompasses all aspects of patient evaluation and the transfusion decision-making process. Despite continued declines in demand for blood products, blood transfusion remains one of the most commonly performed medical procedures in the United States and other developed countries.<sup>1</sup> The goal of PBM is not only to improve outcomes by transfusing blood appropriately, but also to introduce strategies to prevent patients from needing a transfusion in the first place. Avoiding and/or minimizing transfusion certainly leads to fewer transfusion reactions, fewer donor exposures, and in some cases lower cost. It has been stated that transfusions are also associated with worse patient outcomes, including bacterial infections, increased length of stay, prolonged ventilation, and mortality.<sup>2</sup> Such statements are based primarily on observational data or meta-analyses<sup>3</sup> and are controversial because of potential confounding and/or methodologic issues.<sup>4</sup> Even in the absence of serious morbid events causally related to transfusion, if a transfusion does not confer a benefit, it should be avoided. In a 2011 survey of 1342 US hospitals, 30% of respondents stated they offered some components of a PBM program and PBM programs were reported by 37.8% of the 1068 US hospitals surveyed by the AABB in 2013.<sup>5,6</sup> The uptake of PBM has undoubtedly risen since then, but compliance with PBM standards remains suboptimal in some regions.<sup>7</sup> Several professional organizations have focused on PBM: AABB, the Society for the Advancement of Blood Management (SABM) and the Network for Advancement of Transfusion Alternatives (NATA) in Europe. Each of these organizations has annual meetings with PBM content and abundant written resources, including published PBM program guidelines or standards.<sup>8,9</sup> An effective PBM program combines multiple approaches that span patient care from prehospitalization to the inpatient setting and even after discharge. Furthermore, the techniques of PBM are not limited to surgical patients. The features of a comprehensive PBM program are shown in Figure 27.1 and are discussed in this chapter.

## Definitions of PBM

*"Patient blood management (PBM) is a patient-focused, evidence-based and systematic approach to optimize the management of patients and transfusion of blood products for quality and effective patient care. It is designed to improve patient outcomes through the safe and rational use of blood and blood products and by minimizing unnecessary exposure to blood products."*

- World Health Organization (WHO) Global Forum for Blood Safety:  
Patient blood management<sup>10</sup>

*Patient blood management (PBM) is "the timely application of evidence-based medical and surgical concepts designed to maintain hemoglobin concentration, optimize hemostasis, and minimize blood loss to improve patient outcome."*

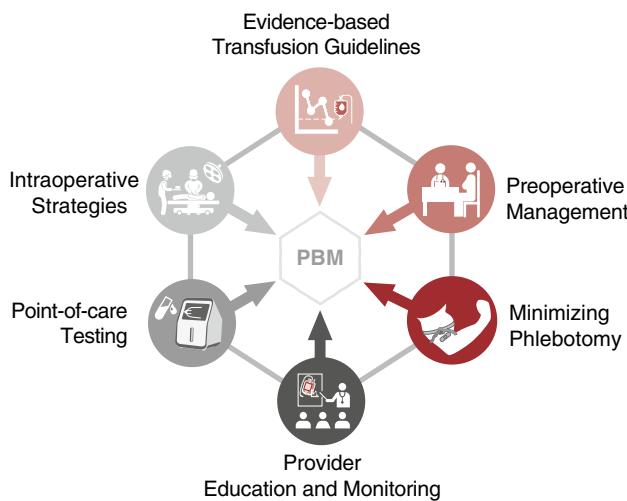
- Society for the Advancement of Blood Management (SABM)<sup>11</sup>

*"Patient Blood Management (PBM) is a multidisciplinary, evidence-based approach to optimising the care of patients who might need a blood transfusion. It represents an international initiative in best practice for transfusion medicine. It is a long-term approach to improve patient care and save money. It requires co-ordinated planning at national and local level and resources and investment."*

- National Health Service (NHS) Blood and Transplant, United Kingdom<sup>12</sup>

## Implementing a PBM program

There exists no single approach to implementing a PBM program that is appropriate for all hospitals. The approach will vary depending on the size of the hospital, patient populations, and hospital resources. Strategies common to a successful program have been described.<sup>13–15</sup> By its nature, a PBM program is multidisciplinary, and thus a program should start by identifying and recruiting the relevant physician, nursing, and administration stakeholders or "champions" from a variety of specialties. Once identified, these champions can form the basis for a PBM oversight committee with representatives from transfusion medicine, surgery, anesthesia, critical care medicine, hematology/oncology, nursing, information



**Figure 27.1** Features of a patient blood management (PBM) program: evidence-based guidelines for transfusion thresholds, indications and dose; preoperative anemia evaluation and management; physician education and monitoring; point-of-care hemostasis testing; intraoperative strategies (autologous salvage, acute normovolemic hemodilution, and use of hemostatic agents); and limiting phlebotomy blood loss for laboratory testing.

technology (IT), and data analytics. Some PBM committees also include representatives from perfusion, pharmacy, laboratory medicine, internal medicine, risk management, or hospital administration. Many institutions have created a coordinator position called a Transfusion Safety Office (TSO) or PBM coordinator. This position is analogous to an Infection Control Officer but focuses on transfusion and PBM. The TSO is typically a nurse but could be a person with a laboratory or a quality systems background. Such individuals act as a liaison between the PBM committee and clinical services focusing on education, auditing, monitoring, data collection, and reporting. The PBM committee should have a medical director or codirectors responsible for direction, organization, and oversight of committee activities and representing the committee within the hospital. Ideally, the chair should be a clinician with credibility among the physicians who order blood products. A transfusion medicine physician may also serve well in this role. The most important point is that it is a physician who is committed and passionate about creating a successful PBM program.

Essential to the success of the PBM committee is visible and vocal support of the hospital leadership. This includes the hospital chief executive officer (CEO) and senior management or administration as well as the departmental chairs of the key clinical services that utilize blood products. Effective leadership drives physicians and staff to participate in PBM initiatives. Some PBM initiatives may require financial investment, such as hiring a TSO or building IT infrastructure. Such vital investment will require institutional leaders to “buy in” to the notion of investing now in order to improve patient care and ultimately reduce future costs. A lack of hospital leadership support is the most common reason why PBM programs fail to achieve their goals.

Many hospitals have an existing transfusion committee or similarly tasked committee that reports to the hospital medical executive committee or in some cases through the hospital quality assurance structure. Traditional transfusion committee activities will include some, but not all, PBM activities. For example, transfusion

committees typically monitor blood usage and adverse event reports, but not the management of preoperative anemia or the use of point-of-care testing. It is prudent for the PBM committee to work closely with the transfusion committee to complement but not duplicate their activities. When the transfusion committee is a standing committee, it can be a useful conduit for the approval of PBM policy proposals and education, and an additional source of resources for PBM activities. It is also feasible to roll the oversight of PBM activities into the responsibilities of the existing transfusion committee.

Once the PBM committee has been constituted, it should define the scope of activities it will address. Figure 27.1 presents a comprehensive set of PBM activities, but rarely can all be addressed and almost certainly not simultaneously. The goal is to achieve measurable, impactful, and lasting success. Thus, initially it would be wise to address those aspects that are the most feasible and that will have the greatest impact on patient care. In this way, the program can attract positive attention to itself, and perhaps garner additional PBM champions, and continued administrative support. Later, as the program matures and circumstances change, other aspects of the program can be addressed. Monitoring, measurement, and metrics are vital components of a PBM program.<sup>16</sup> The metrics or key performance indicators should be meaningful, quantitative, and feasible to measure. This is where IT/ data analytics support and having a TSO/PBM coordinator to collect and analyze the data are so important. As part of defining its scope of activities, the PBM committee should define the metrics for each activity and acceptable targets for success (goals), as well as the IT resources and data analytics support (e.g., for reports) and/or other staff needed for chart review.

Several professional organizations have developed best practice guidelines for PBM, including AABB, the Society for the Advancement of Blood Management (SABM), NATA in Europe, NHS Blood and Transplant in the United Kingdom, and the National Blood Authority in Australia.<sup>8,9,12,17</sup> The Joint Commission, in partnership with the AABB, in the United States also offers a two-year voluntary certification process that provides a third party evaluation of a hospital’s PBM program; the accreditation process is based on the AABB Standards for a Patient Blood Management Program, which were originally published in 2014 and updated in 2018.<sup>18</sup>

The 2018 Frankfurt Consensus Conference catalogued the current state of the PBM evidence base for RBC transfusion by analyzing 145 studies, including 63 randomized clinical trials (RCTs) with 23,143 patients and 82 observational studies with more than four million patients. The scientific committee established 10 clinical recommendations and 12 research recommendations for management of preoperative anemia, transfusion thresholds for adults, and implementation of PBM programs.<sup>19</sup> The clinical recommendations are summarized in Table 27.1. While these recommendations focus on red cell transfusions only, they are a useful resource for institutions embarking on implementation of a PBM program.

### Evidence-based transfusion guidelines

There is no doubt that evidence-based transfusion guidelines derived from review of the current literature are a key component of a PBM program. They do not, however, solely constitute a PBM program. If the transfusion committee does not already have such guidelines in place, the PBM committee should develop a set of transfusion guidelines for the hospital. A number of organizations have conducted careful literature reviews based on Grading of

**Table 27.1** Clinical Recommendations from the 2018 Frankfurt Consensus Conference on PBM

Category	Clinical Recommendation	Level of Evidence
Preoperative Anemia	CR1—Detection and management of preoperative anemia early enough before major elective surgery	Strong recommendation, low certainty in the evidence of effects
	CR2—Use of iron supplementation to reduce red blood cell transfusion rate in adult preoperative patients with iron-deficient anemia undergoing elective surgery	Conditional recommendation, moderate certainty in the evidence of effects
	CR3—Do not use erythropoiesis-stimulating agents routinely in general for adult preoperative patients with anemia undergoing elective surgery	Conditional recommendation, low certainty in the evidence of effects
	CR4—Consider short-acting erythropoietins in addition to iron supplementation to reduce transfusion rates in adult preoperative patients with hemoglobin concentrations <13 g/dL undergoing elective major orthopedic surgery	Conditional recommendation, low certainty in the evidence of effects
Red Blood Cell Transfusion Thresholds	CR5—Restrictive RBC transfusion threshold (hemoglobin concentration <7 g/dL) in critically ill but clinically stable intensive care patients	Strong recommendation, moderate certainty in the evidence of effects
	CR6—Restrictive RBC transfusion threshold (hemoglobin concentration <7.5 g/dL) in patients undergoing cardiac surgery	Strong recommendation, moderate certainty in the evidence of effects
	CR7—Restrictive transfusion threshold (hemoglobin concentration <8 g/dL) in patients with hip fracture and cardiovascular disease or other risk factors	Conditional recommendation, moderate certainty in the evidence of effects
	CR8—Restrictive transfusion threshold (hemoglobin concentration 7–8 g/dL) in hemodynamically stable patients with acute gastrointestinal bleeding	Conditional recommendation, low certainty in the evidence of effects
Implementation of Patient Blood Management Programs	CR9—Implementation of PBM programs to improve appropriate RBC utilization	Conditional recommendation, low certainty in the evidence of effects
	CR10—Computerized or electronic decision support systems to improve appropriate RBC utilization	Conditional recommendation, low certainty in the evidence of effects

Abbreviations: CR: clinical recommendation; PBM: patient blood management; RBC: red blood cell.

Modified from Mueller *et al.* (2019).<sup>19</sup>

Recommendations Assessment, Development and Evaluation (GRADE) methodology, and published suggested guidelines.<sup>8,20–22</sup> The multidisciplinary membership of the committee lends itself to the adoption of the guidelines across the clinical services. The guidelines should address the indications for each blood component *and* the recommended dosing. Transfusing one unit of red cells at a time for an appropriate indication and reevaluating a stable patient are mainstays of optimal red cell transfusion practice.<sup>15</sup> Other chapters in this textbook provide transfusion guidelines and dosage recommendations for each component and a detailed review of the evidence for these guidelines. Once guidelines have been established, physician education, implementation, and auditing of transfusion practice are the next steps. These steps, particularly implementation and auditing, are far more difficult than developing the guidelines.

### Physician education and monitoring

To implement the various aspects of PBM, detailed information on baseline practices is required.<sup>14,23</sup> Although some of this information might be obtainable through a manual review of patient charts, large-scale data mining is really only possible by implementing electronic monitoring programs and by facilitating the storage of relevant data in a structured format. These programs can not only serve to harvest data to elucidate the state of practice, but can also be used to advise clinicians about potentially unnecessary transfusions before the order to transfuse is placed. Broadly known as clinical decision support systems (CDSS), these automated

programs can be designed to accomplish several tasks that would otherwise be impossible to achieve using a manual system once they are integrated into the computerized physician order entry (CPOE) system. Once deployed, the CDSS can operate continuously, and dispassionately provide suggestions every time that it detects a blood product order on a patient whose laboratory values do not indicate that a transfusion is necessary—a task that, although potentially successful,<sup>24</sup> would otherwise require a significant time commitment from the blood bank staff. Several large meta-analyses have shown practice improvements following the implementation of CDSS in various clinical areas.<sup>25,26</sup>

As it pertains to transfusion, a CDSS can be very basic or highly complex. A basic system would, for example, consider only one parameter (e.g., a hemoglobin or platelet value) in its analysis of whether a transfusion order was in accordance with the institutional guidelines. Several studies have reported on using a single pretransfusion hemoglobin value to evaluate the suitability of the transfusion order.<sup>27–29</sup> However, this “one-size-fits-all” or static approach to laboratory-based transfusion threshold values is not amenable to the needs of physicians who are caring for patients with specialized disorders or for patients in specific clinical situations. Different patients can benefit from transfusion at different hemoglobin concentrations.<sup>30–32</sup> Thus, the evolution of the CDSS can be toward an “adaptive alert” system. This type of CDSS often requires the prescriber to first select an indication for the transfusion; associated with each indication is an evidence-based threshold for the product (Table 27.2). If the patient’s most recent laboratory values are higher than the recommended guideline

**Table 27.2** List of Transfusion Indications in the Computerized Physician Order Entry Screen at the Authors' Institutions

**(A) Red blood cells**

- Acute bleeding with blood pressure instability\*
- Hemoglobin**  $\leq 7.0 \text{ g/dL}$  and/or **hematocrit**  $\leq 21\%$  in a hemodynamically stable patient in the ICU
- Hemoglobin**  $\leq 8.0 \text{ g/dL}$  and/or **hematocrit**  $\leq 24\%$  in a non-ICU patient with signs and symptoms of anemia
- Hemoglobin**  $\leq 10.0 \text{ g/dL}$  and/or **hematocrit**  $\leq 30\%$  associated with acute ischemic cardiovascular disease (angina pectoris and myocardial infarction)
- Hemoglobin**  $\leq 7.5 \text{ g/dL}$  and/or **hematocrit**  $<22.5\%$  in the intraoperative setting for a cardiac patient
- Hemoglobin**  $\leq 8.5 \text{ g/dL}$  and/or **hematocrit**  $\leq 25.5\%$  in an outpatient setting
- Surgical blood loss anticipated\*
- Other (alert generated if **hemoglobin**  $>8.5 \text{ g/dL}$  and/or **hematocrit**  $>25.5\%$ )

**(B) Plasma**

- Coagulation disorders associated with active bleeding and **INR**  $\geq 1.6$  and/or **abnormal viscoelastic test parameters (R time/CT, K time/CFT)**
- Coagulation disorders, with **INR**  $\geq 1.6$  and/or **abnormal viscoelastic test parameters (R time/CT, K time/CFT)**, in patients undergoing surgery or invasive procedures, where the INR elevation is not due to vitamin K deficiency/ antagonist
- To correct warfarin effect in cases of active bleeding or emergent surgery (**INR**  $\geq 1.6$ )
- Therapeutic plasma exchange\*
- Massive bleeding\*
- Other (alert generated if **INR**  $< 1.6$ )

**(C) Platelets**

- Platelet count  $<10,000/\mu\text{L}$  ( $10 \times 10^9/\text{L}$ ) in a stable patient
- Platelet count  $<20,000/\mu\text{L}$  ( $20 \times 10^9/\text{L}$ ) with evidence of platelet consumption (e.g., fever and sepsis)
- Platelet count  $<50,000/\mu\text{L}$  ( $50 \times 10^9/\text{L}$ ) with active bleeding or prior to an invasive procedure
- Active bleeding or need for emergent procedure on antiplatelet medications\*
- Platelet dysfunction documented by abnormal platelet function tests (e.g., PFA-100/200 Closure Time, VerifyNow, and viscoelastic tests)
- Massive bleeding\*
- Other

**(D) Cryoprecipitate**

- Active bleeding associated with or prior to an invasive procedure with hypo- and/or dys-fibrinogenemia: **Fibrinogen level**  $<100 \text{ mg/dL}^{**}$  and/or **abnormal viscoelastic test parameters (K time/CFT, } \alpha\text{-angle}**
- Massive bleeding\*
- Other

\* Indicates that an antecedent laboratory value is not required to order the product, and an alert will not be triggered if a blood product is ordered using this indication.

\*\* Fibrinogen level thresholds may be higher in certain settings, e.g., obstetric hemorrhage.

Abbreviations: CT: clotting time (rotational thromboelastometry or ROTEM); CFT: clot formation time (ROTEM); INR: international normalized ratio.

values (or lower, in the case of plasma transfusion and an INR threshold), an alert will appear on screen to inform the prescriber that, based on their patient's laboratory values, the transfusion does not appear to be indicated. The prescriber can again override the alert or warning and proceed to order the product or can cancel the transfusion altogether, a so-called "soft stop." "Hard stops," where the prescriber is unable to override the alert or warning, are generally not considered appropriate for PBM programs since transfusion indications are not all-encompassing and there are inherent risks associated with not providing a blood product when a clinician deems it necessary, even if the patient does not meet the transfusion indications. Baer *et al.*<sup>33</sup> reported on the implementation of a CDSS that displayed their neonatal intensive care units' (ICU) transfusion thresholds when the prescriber was ordering a blood product in the CPOE.

Compared to a period before the implementation of a CDSS, most studies have demonstrated a statistically significant improve-

ment in institutional transfusion threshold compliance after one was implemented.<sup>34–43</sup> Although the exact reason for the improved compliance is unclear, it is reasonable to assume that being repeatedly presented with the institution's transfusion guidelines at the time of blood product ordering is sufficient to change some practices. Having adaptive alerts also helps to improve compliance by facilitating a patient-centric approach to transfusion decision-making. At the authors' institutions, there were statistically significant decreases in the number of RBC orders that were placed and the number of orders that generated an alert after the "static" alert was changed to an adaptive alert. There was a clinically relevant, although not statistically significant ( $p = 0.089$ ), reduction in the median number of RBCs ordered per month after the adaptive alerts were implemented compared to the four months preceding the implementation of the adaptive alerts.<sup>44</sup> Similarly, several other studies have reported reduced RBC transfusions after a CDSS was implemented, although for many of these studies, the differences observed also did not reach statistical significance.<sup>27,29,33–35,37–40,42,43,45</sup>

There are fewer studies that have analyzed the effect of a CDSS on plasma orders, although the evidence has been accumulating recently. One single-center study in 2017 demonstrated a 17.4% reduction in plasma utilization after implementation of a CDSS, but found a relatively low alert acceptance rate of only 33% and marked variations in alert acceptance across different specialties.<sup>46</sup> Other groups have also described reductions in plasma utilization but have highlighted the importance of optimal CDSS design in order to improve compliance rates.<sup>47</sup> At the authors' institutions, when the static alert for plasma transfusion (i.e., an alert was generated for any plasma order on a patient whose most recent international normalized ratio [INR] value was  $<1.6$ ) was replaced with an adaptive alert (Table 27.2), there was a 15% decrease in the number of orders that generated an alert ( $p < 0.0001$  compared to the period when a static alert was in place), and the percentage of alerts that were heeded increased significantly.<sup>48</sup> The implementation of the adaptive alerts for plasma in a neonatal ICU led to a significant reduction in the number of patients that received a plasma transfusion.<sup>24</sup> Similarly, when the CPOE displayed one institution's plasma transfusion guidelines, there was a decrease in the total number of plasma orders, inappropriate plasma orders, and plasma units transfused,<sup>49</sup> although the CDSS was not as successful in reducing "invalid" plasma orders at a different hospital.<sup>50</sup> As mentioned above, a CDSS that made recommendations for the number of plasma units to be transfused based on the recipient's characteristics and the intended goal of the transfusion led to the transfusion of 68 additional plasma units.<sup>34</sup>

Reports on the application of CDSS to platelet and cryoprecipitate orders are limited. Two earlier studies demonstrated nonstatistically significant reductions in the number of patients who received platelet transfusion after a CDSS was implemented,<sup>33,51</sup> whereas a more recent study showed a reduction in platelet transfusion order errors in the inpatient setting from 3.10% to 0.33% after a careful redesign of the CDSS.<sup>52</sup> Another study on restrictive transfusion policies in hematology patients also demonstrated a nonstatistically significant reduction in the proportion of noncompliant platelet orders from 41.9% to 31.2% ( $p = 0.16$ ) after implementation of a CDSS.<sup>53</sup> Two studies conducted in large health systems have analyzed the effect of a CDSS on cryoprecipitate orders.<sup>33,54</sup> At the authors' health system, CDSS implementation resulted in 49% of cryoprecipitate orders generating an alert, of which 14.9% were canceled.<sup>54</sup>

In addition to potentially realizing cost savings by transfusing fewer blood products and having patients experience fewer transfusion reactions, the CDSS can also inform the selection of electronic medical records for the audits that are performed by transfusion committees. It is important to note that laboratory values alone are sometimes insufficient to indicate why a patient might require a blood product. Thus, permitting the physician to add a free-text explanation to an order that might not appear warranted based on laboratory parameters is a useful feature in a CPOE system. Explanatory comments can also suggest other legitimate blood product order indications for the CDSS. Thus, although high-quality evidence should form the basis when establishing transfusion thresholds, the input of the blood product prescribers should also be sought in order to achieve consensus threshold values for the alerts.

In the absence of a CDSS, other metrics can be followed to ensure compliance with institutional guidelines. Once a restrictive RBC transfusion threshold is in place, evaluating the proportion of single unit RBCs transfusions and the recipients' mean pretransfusion hemoglobin values over time by service or by individual provider can identify nonevidence-based practices and focus interventional efforts to achieve higher compliance rates. Benchmarking between providers of similar procedures or between hospitals or national databases can also be employed to identify nonevidence-based practices.<sup>55–59</sup> All of these methods require significant support from the institution's IT department.

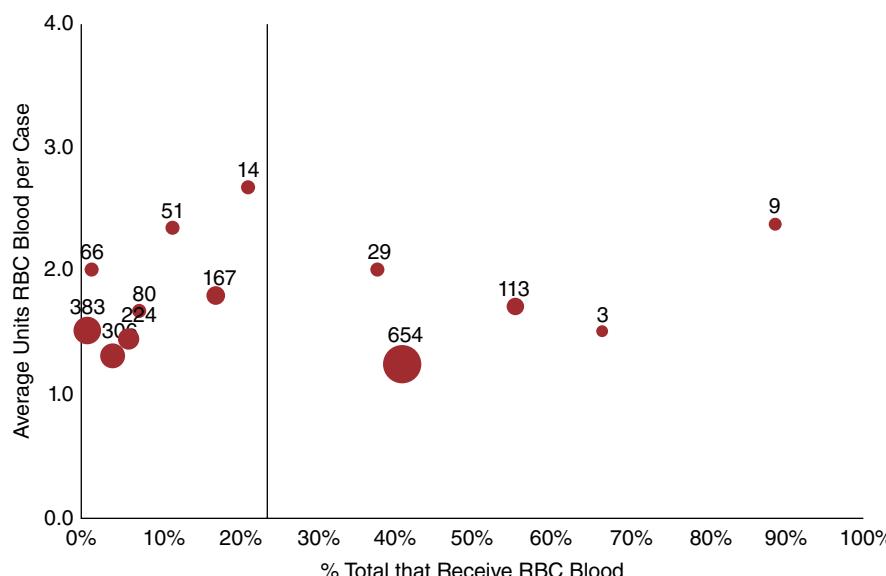
Targeted prescriber education can occur after the decision to transfuse a patient has been made, and the use of the electronic medical record can facilitate this sort of audit. Benchmarking of blood use between surgeons who perform the same procedures can be a useful way of establishing the current state of practice and permit the identification of any practitioners whose transfusion habits appear to be aberrant. For example, the RBC transfusion practice among orthopedic surgeons performing total hip arthroplasties (THAs) can be highly variable. This variability can range between surgeons who rarely transfuse their patients with RBCs (and, when they do, it is typically a small quantity) to those who routinely transfuse most of their patients with large quantities of RBCs.<sup>60</sup> The publication of the FOCUS study served as the benchmark against which

these surgeons' transfusion practice could be evaluated.<sup>32</sup> To this end, a "bubble graph" can be utilized (Figure 27.2). This graph plots all surgeons who perform THA surgeries by the frequency with which they transfuse RBCs to their patients and the mean number of RBCs that are transfused per patient. The size of the bubble reflects the number of cases. By identifying those who repeatedly transfuse greater than average quantities of RBCs, the transfusion committee or hospital transfusion service can provide focused feedback and education where and when it is required.

It is evident that automated prescriber education alone is insufficient to eliminate nonevidence-based transfusions. Surely, the best way to reduce aberrant transfusion practice is through a multimodal approach involving automated alerts, targeted education (e.g., at grand rounds), and informal discussions with the house staff and faculty when apparently nonevidence-based transfusion practices are detected, and by providing easy access to the most up-to-date and well-executed research studies. By providing a consistent message about transfusion thresholds, waste reduction strategies, and techniques to avoid transfusion, the optimal effects of PBM can be realized.

## Preoperative anemia management

The World Health Organization (WHO) defines anemia as a hemoglobin <13 g/dL in men and <12 g/dL in nonpregnant women or <11 g/dL in pregnant women.<sup>61</sup> Preoperative anemia is common in surgical patients with a prevalence ranging from 5% to 76% depending on the type of surgery, comorbidities, patient age, and gender.<sup>62</sup> A systematic review of patients undergoing hip and knee surgery reported a prevalence of 24–44%,<sup>63</sup> which is very similar to the 28–36% prevalence observed in patients undergoing coronary artery bypass grafting.<sup>64</sup> Multiple studies have shown that preoperative anemia in the orthopedic patient population is associated with a 5–12-fold higher risk of transfusion, prolonged length of stay, and higher readmission rates.<sup>63,65,66</sup> Preoperative anemia has also been associated with increased morbidity and mortality in surgical patients.<sup>63,67,68</sup> Carson *et al.*<sup>67</sup> reported a retrospective study of 1958 patients who refused blood transfusion and underwent noncardiac surgery, and found that perioperative mortality was significantly



**Figure 27.2** An example of a bubble plot. Each bubble represents a surgeon who performs total hip arthroplasties, and the size of the bubble represents the relative number of cases performed over a set time. The x-axis represents the percentage of patients who received at least one RBC unit during the case, and on the y-axis the average number of RBCs transfused is plotted.

increased when the preoperative hemoglobin was  $\leq 10$  g/dL. Wu *et al.*<sup>68</sup> reported a retrospective study of over 310,000 men  $> 65$  years old who underwent noncardiac surgery in the American Veterans' Affairs system showing that postoperative mortality and cardiac events progressively increased as the preoperative hematocrit fell below 39%. In a systematic review of the literature, Spahn *et al.*<sup>69</sup> found that preoperative anemia in orthopedic surgery patients was associated with more infections and higher mortality. Another study in orthopedic surgery patients, however, calls into question whether anemia is causally related to these outcomes.<sup>69</sup> Nonetheless, it is clear that anemia increases the risk of requiring a transfusion in the perioperative period. That reason alone would justify implementing measures to manage preoperative anemia.

PBM programs have been developed to manage preoperative anemia primarily in orthopedic surgery patients,<sup>65,70,71</sup> although these programs have also been applied to other patients who are undergoing elective surgery with expected significant blood loss. These programs consist of identifying patients undergoing elective

procedures ideally four weeks, but not less than two weeks, prior to surgery and performing a screening hemoglobin. This practice was suggested by the Joint Commission as a performance measurement initiative.<sup>72</sup> Using the WHO definition of anemia, patients would be referred to a hematologist, preoperative clinic, or other physician for the evaluation and management of the anemia. Several algorithms for the evaluation and management of preoperative anemia have been published, including one by the NATA group using the GRADE methodology (Figure 27.3).<sup>71</sup> This algorithm uses serum ferritin and transferrin saturation to triage patients to appropriate management strategies that may include oral iron, intravenous (IV) iron, folic acid, vitamin B<sub>12</sub>, and/or erythropoietin (EPO). Other algorithms use the red cell mean corpuscular volume (MCV)<sup>70</sup> or ferritin alone<sup>65</sup> as screening tests. There is no evidence that one particular strategy is superior to another.

The pharmacologic therapies for the management of preoperative anemia have been recently reviewed.<sup>71</sup> Oral iron may be sufficient to correct the anemia in patients who have iron-restricted

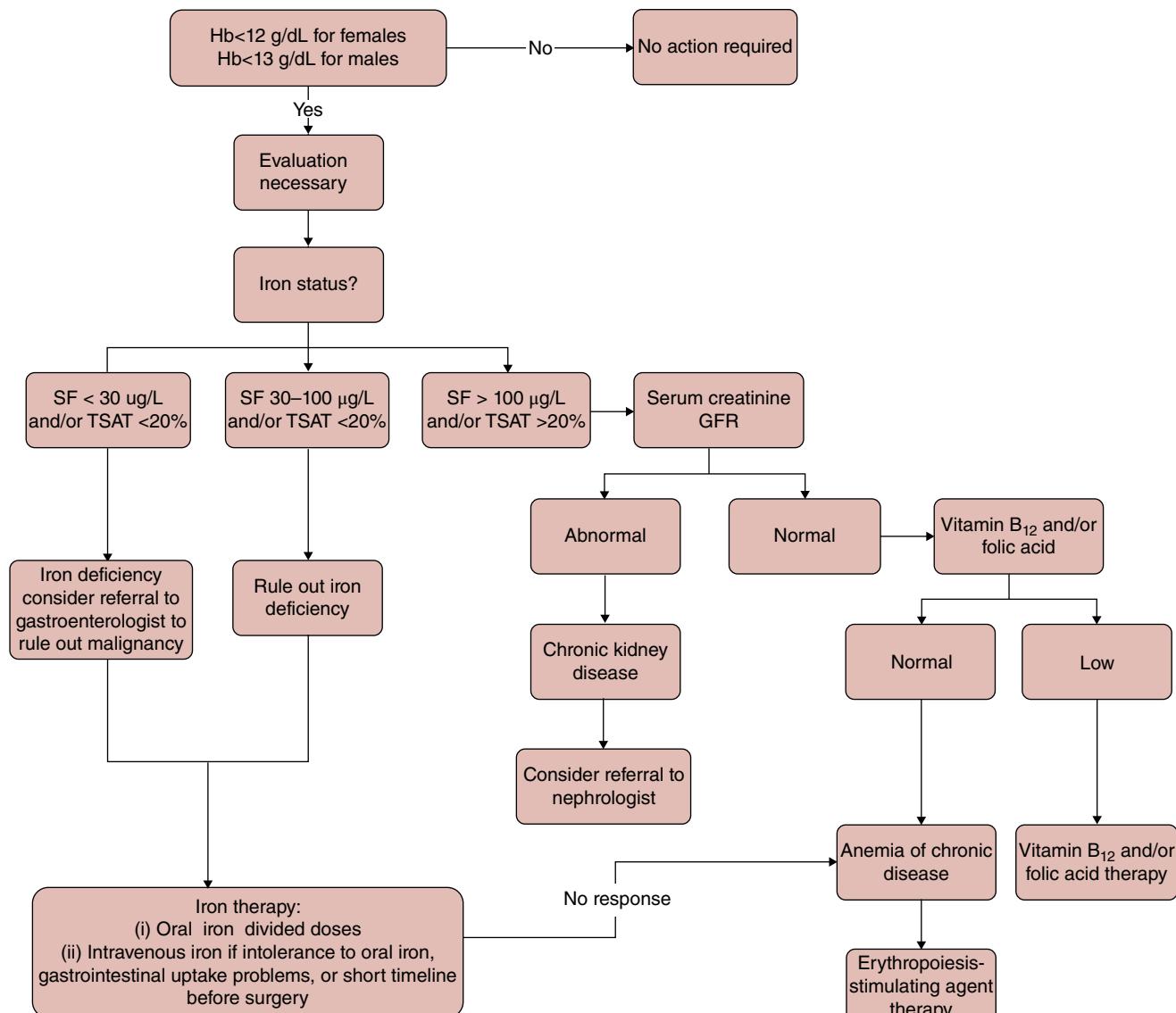


Figure 27.3 Proposed algorithm for the detection, evaluation, and management of preoperative anemia. GFR: glomerular filtration rate; SF: serum ferritin; TSAT: transferrin saturation. Source: Goodnough *et al.* (2011).<sup>71</sup> Reproduced with permission of Oxford University Press.

erythropoiesis due to absolute iron deficiency. Oral preparations include ferrous gluconate, ferrous fumarate, ferrous sulfate, and iron polysaccharide. The advantage of oral iron replacement is that it is inexpensive and easy to administer. There are several limitations, however, including gastrointestinal side effects and compliance issues. It also takes 2–4 weeks to increase hemoglobin levels. Oral iron is not very effective in patients with anemia of inflammation (chronic disease).

Intravenous (IV) iron therapy is becoming a more common strategy for the management of preoperative anemia as safer preparations are now available in the United States. IV iron bypasses absorption, tolerance, and compliance issues. It is also effective in patients with anemia of chronic disease in whom inflammation and subsequent elevated hepcidin levels prevent iron absorption.<sup>71</sup> The total dose of IV iron is typically 1 g, independent of the patient's weight. The number of doses required to deliver 1 g and achieve a therapeutic effect depends on the preparation used. An increase in hemoglobin is apparent at one week after starting IV iron therapy and reaches its apogee after two weeks.<sup>70</sup> Serious acute adverse events (i.e., anaphylaxis) have been associated with high-molecular-weight (HMW) dextran IV iron preparations but appear to be lower with low molecular-weight (LMW) dextran preparations. These iron dextran preparations have a US Food and Drug Administration (FDA) "black box" warning and require a test dose before infusion. LMW dextran preparations can be given as a single 1 g infusion and are less costly than other preparations. Other IV iron preparations that are approved in the United States include iron gluconate, iron sucrose, iron isomaltose, iron carboxymaltose, and iron carboxymethyl dextran; none of these preparations have a "black box" warning, and they do not require a test dose as they do not contain HMW dextrans. The iron gluconate and sucrose preparations are not approved for an infusion dose of more than 500 mg and thus would require the patient to return for one or more infusions. The other preparations can be given as a single total dose infusion.

Erythropoietic-stimulating agents (ESA) are FDA approved for use in patients with anemia due to chronic kidney disease (CKD), in oncology patients with chemotherapy-induced anemia, for HIV-therapy-related anemia, and in patients with anemia undergoing elective surgery.<sup>71</sup> There are three approved preparations: epoetin alfa (EPO- $\alpha$ ), epoetin beta-methoxy polyethylene glycol, and darbepoetin alfa. Epoetin beta-methoxy polyethylene glycol is only indicated for the treatment of anemia due to CKD.<sup>73</sup> Darbepoetin alfa is indicated for the treatment of anemia due to CKD and some patients with concomitant myelosuppressive chemotherapy excluding those where the anticipated outcome is cure or those in whom the anemia can be managed by transfusion.<sup>74</sup> Pregnesatide, a fourth drug approved by the FDA in 2012 for the treatment of adult patients on dialysis with anemia due to CKD, had its FDA approval withdrawn in February of 2019 following postmarketing reports of serious hypersensitivity reactions, including anaphylaxis.<sup>75</sup> EPO- $\alpha$  is the only ESA approved for use in elective surgery, and it is intended for patients with a preoperative hemoglobin >10 to ≤13 g/dL who are at high risk for perioperative blood loss from elective, noncardiac, and nonvascular surgery.<sup>76,77</sup> In this setting, EPO- $\alpha$  has been shown to be effective in reducing the need for transfusion.<sup>78</sup> Subsequent RCTs in patients undergoing hip or knee arthroplasty reported similar results<sup>79,80</sup> and did not find a difference in the rates of deep venous thrombosis between the EPO- $\alpha$  and control groups.

The preoperative dosing schedule for EPO- $\alpha$  is typically 600 U/kg weekly for three weeks and a fourth dose on the day of surgery. A daily regimen is also available but requires dosing for

10 consecutive days before surgery, on the day of surgery, and for four days after surgery. It is mainly of use in patients who have <2 weeks before scheduled surgery. The onset of action of EPO, indicated by a rise in hemoglobin, is 4–6 days. Concomitant iron therapy is necessary during EPO- $\alpha$  therapy due to increased demand for iron as it is incorporated into hemoglobin. EPO should not be used in patients with a hemoglobin >13 g/dL. Safety concerns about EPO in elective surgery patients have recently been raised. In 2007, the results of an RCT of EPO versus no EPO in 681 adult patients who underwent spine surgery without prophylactic anticoagulation were reported to the FDA.<sup>76</sup> Patients in the EPO arm received four weekly EPO doses of 600 U/kg EPO- $\alpha$  (21, 14, and 7 days before surgery, and the day of surgery). An increased incidence of deep vein thrombosis (DVT) in patients receiving EPO was observed (16 patients [4.7%]) compared to the control group (seven patients [2.1%]). Based on this study and a higher incidence of DVT in other patient populations treated with EPO, the FDA recommended that prophylactic anticoagulation be "strongly considered" when EPO- $\alpha$  is used in surgical patients. While ESA are not approved for use in patients undergoing cardiac or vascular surgery due to FDA concerns about increased mortality,<sup>76,77</sup> there is accumulating evidence that a single dose of ESA preoperatively may reduce peri-operative blood transfusion requirements in this high-risk surgical population without an increase in adverse events.<sup>81–83</sup> ESA use is also relatively contraindicated in patients with uncontrolled hypertension, recent coronary or cerebral ischemic events, and in patients who are unable to receive perioperative prophylactic anticoagulant therapy.<sup>84</sup>

## Cell salvage

The process of collecting shed autologous blood, its processing, and its readministration has been termed *cell salvage*, *autotransfusion*, *intraoperative blood recovery*, as well as *cell saving*. For the purpose of this chapter, the term *cell salvage* is used. Cell salvage can take place either in the intraoperative period or in the postoperative period. Salvage can also involve washing of the collected blood, or it can be simply readministered with microaggregate filtration.

## Unwashed cell salvage

To this day, both washed and unwashed salvage devices are used. Postoperative unwashed blood salvage has been utilized in a wide variety of surgical procedures,<sup>85</sup> but it is predominantly used after cardiac<sup>86</sup> and orthopedic procedures (Figure 27.4).<sup>87</sup> Estimates of postoperative blood loss after cardiac surgery range from 371 to 553 mL,<sup>88–90</sup> whereas volumes following total joint replacement range from 166 to 750 mL.<sup>91–93</sup> The average hemoglobin of this shed blood is reported to range from 20% to 30%.<sup>94,95</sup> At best, the volume returned to a patient would equal the red cell mass present in one unit of allogeneic red cells so the efficacy of this unwashed product in avoiding allogeneic transfusion is limited. Controversy arises as to whether the risks of retransfusion of this blood warrant the minimal amount of blood that is returned to the patient.

When one considers that this salvaged blood is laden with various inflammatory mediators,<sup>96,97</sup> fibrin split products,<sup>94,98,99</sup> complement fractions,<sup>100–102</sup> interleukins,<sup>103–105</sup> tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ),<sup>97</sup> and fat particles,<sup>106</sup> which are multiple degrees higher than circulating levels, it is easy to believe that there is risk from readministration of this shed, unwashed blood. This being said, the reported risks appear to be minimal. The most frequently reported complication following unwashed postoperative shed blood



**Figure 27.4** A salvage device for unwashed cells used for a knee replacement.



**Figure 27.5** A device for salvaging and washing blood is shown.

reinfusion relates to febrile reactions, which is a complication frequently seen following allogeneic transfusion. Dependent upon the study, the rates of febrile reactions after postoperative salvage readministration vary from 4% to 12%.<sup>92,93,107,108</sup> This complication is generally the only reported complication following readministration of unwashed, shed blood; however, all of these reports are small case series.

In a Cochrane systematic review, Carless *et al.* reviewed complications of all available studies on perioperative salvage.<sup>109</sup> They evaluated total complications, wound infections, and thromboembolic events that included nonfatal myocardial infarction. They found no difference in any of these events, but they mixed washed with unwashed salvage and the vast preponderance of studies was

from cardiac surgery. A simple solution to the perceived hazards of the contaminants of unwashed, postoperative cell salvage would be to wash the blood (Figure 27.5).

### Washed cell salvage

In a washed salvaged product, the blood is collected via suction into a reservoir where it is stored until processing. When adequate amounts of blood are collected, the blood is pumped into a centrifuge bowl where it is concentrated. During the concentration of the erythrocytes, plasma and anticoagulant are spilled into a waste bag. Following the concentration of the erythrocytes, normal saline or Ringers' Lactate solution is percolated through the red cell pack with the goal to wash out tissue factor and the inflammatory mediators mentioned previously. Following washing, the blood is leukoreduced by filtration and readministered to the patient. Washing comes with its own set of problems, which include air embolism<sup>110</sup> and exposure of red cells to nonisotonic solutions.<sup>111</sup> When the processing is completed, the blood is pumped into a reinfusion bag, and preceding the blood is a column of air. When multiple units of blood are processed, the air can accumulate in the reinfusion bag, which then presents a hazard to the patient if the patient's intravenous line is connected directly to this bag. In order to prevent this problem, the reinfusion bag should never be directly connected to the patient. Washing can also be problematic if a nonisotonic wash solution is chosen. For instance, if blood is washed with sterile water, the cells will all be lysed. If the free hemoglobin is administered to the patient, it could potentially result in renal failure or be fatal.

### Maximizing washed salvage efficiency

Washed cell salvage can provide multiple units of autologous erythrocytes for a bleeding patient.<sup>112</sup> Small changes in the collection and processing of shed red cells can make large differences in the volume of blood returned to the surgical patient. Mathematical modeling illustrates this point.<sup>113</sup> Assuming a 70-kg patient with a 5-L blood volume and preoperative hemoglobin of 15 gm/dL, and using a transfusion trigger of 7 gm/dL, a theoretical patient can avoid transfusion of allogeneic blood up to a blood loss of 9600 mL if salvages return 60% of lost red cells. However, the tolerable blood loss rises to 13,750 mL if 70% of the red cells are captured and returned. The most important factors in increasing efficiency of these systems are regulating the suction imposed on the red cells and washing bloody surgical sponges.

### Suction

For blood recovery systems to work, it is necessary to deliver shed blood to a collection reservoir where the blood awaits processing. Collection is done through suction from the site of surgery or a salvage system can be connected to a surgical drain. The way this suction pressure is applied affects the shear forces applied to the red cells. In general, turbulence and high negative pressure destroy red cells. Shear force occurs anytime a fluid moves in contact with a solid surface.<sup>114</sup> High suction pressure leads to increased mechanical force, hemolysis, and subsequently fewer salvageable erythrocytes. As such, the lowest tolerable suction pressure should be applied when removing blood from the surgical field.

Suction pressure should be regulated to 80–120 mmHg, which is generally adequate to clear blood from the surgical field.<sup>115,116</sup> If massive blood loss occurs, it is important to increase the suction pressure so that the surgeon can clearly see the surgical field. Following control of the blood loss, the suction pressure can be lowered.

### Sponge rinsing

Fully soaked surgical sponges may contain up to 100 mL of blood.<sup>117</sup> Of this blood, approximately 95% of these erythrocytes can be captured by rinsing the sponge in a basin of normal saline or Ringer's Lactate solution. The bloody rinse solution is then intermittently sucked into the salvage collection reservoir at a point when the rinse solution appears to be grossly bloody. This practice has been reported to increase erythrocyte retrieval rates by 28%.<sup>118</sup>

### Indications

Determining when to salvage erythrocytes depends upon anticipated blood loss. Prediction of anticipated blood loss is difficult. With this lack of predictability, a cell salvage program should be assessed in composite rather than for a single case. Some cases might generate 20 units of blood, whereas other cases will not generate any. If the sum of salvaged units achieves one allogeneic red cell unit equivalent on average, then the program is generally considered successful, although financial success may be dependent upon whether the service is provided in-house or whether an external perfusion service is hired.<sup>119</sup> Major vascular, cardiac, and transplantation surgery tend to be procedures that generate a large amount of blood loss.<sup>112</sup> Blood loss can also be surgeon dependent. Surgeons will have widely varying blood loss performing the same procedure. So, the type of procedure and who is performing it are primary criteria for determining need.

Given the lack of predictability of blood loss, an important feature of washed salvage is recognizing that the cost of the disposables can be staged through the use of a "standby" system. A standby system simply involves the collection of shed blood. The standby system utilizes a collection reservoir, a suction line, and anticoagulant. This represents approximately half of the cost of the disposables of a salvage system. If processing is indicated, then a processing bowl and tubing system are utilized that double the cost. In this way, half of the cost is not expended if an inadequate volume of blood is captured.

### Contraindications

Absolute contraindications to cell salvage involve contamination of the blood with anything that will lyse the erythrocytes. This would include hypotonic solutions such as sterile water, hydrogen peroxide, and alcohol. Topical collagen hemostatic agents when incorporated into a salvaged blood product can also be hazardous if the topical agent is infused. Heavy metals (chromium and cobalt) can be found in blood salvaged from metal on metal hip prostheses.<sup>120</sup> Accumulation of metal debris in the surrounding joint tissue can be suctioned into blood during replacement. Neuropathy and cardiomyopathy can result from high heavy metal blood levels.

Traditionally, cell salvage has been avoided in obstetrics where the blood might be contaminated with amniotic fluid, malignant surgery where cancer cells might be entrained into the blood, and "dirty" surgery where blood might be contaminated with bacteria. All of these areas are recommended to be contraindicated by the machine manufacturers; however, a striking lack of evidence is available to support these contraindicated areas. Theoretical concerns regarding risk of amniotic fluid embolism when using salvaged blood in obstetrical hemorrhage have not been substantiated. National and international bodies including the American College of Obstetricians and Gynecologists (ACOG)<sup>121–123</sup> have promoted the use of cell salvage in obstetrics; limitations of cell salvage in the obstetrical setting have been primarily related to the availability of appropriate staff and equipment.<sup>124</sup>

The fear of generating a diffuse metastasis when salvaging blood around a tumor site has not been borne out. In 2008, the National Institute for Health and Care Excellence (formerly National Institute for Health and Clinical Excellence) in the United Kingdom<sup>125</sup> approved the use of salvage during urological malignancies. Systematic reviews have also been performed evaluating salvage use during malignant surgery<sup>126</sup> as well as surgery involving tumors of the spine.<sup>127</sup> In neither of these reviews was there support for avoiding the salvage in these surgeries for malignancy. Lastly, blood that might be contaminated with bacteria has been a further contraindication. Surprisingly, bacterial contamination of recovered blood appears to be common. Bland *et al.*<sup>128</sup> reported that bacterial contamination of recovered blood in cardiac surgery approaches 30% of the units processed. Kang *et al.*<sup>129</sup> reported that 9% of the blood returned to liver transplant patients contained bacterial contaminants, usually of skin origin. In these circumstances of bacterial contamination, no clinical effects were seen. Although it would be prudent to avoid use of blood that has been grossly contaminated with stool, blood salvage during support of abdominal trauma would appear to be safe. In these contraindicated circumstances, the use of leukocyte depletion filters has been advocated.<sup>130</sup> Leukocyte depletion filters have been demonstrated to remove many contaminants, including cancer cells, cellular contaminants associated with amniotic fluid, and bacteria. Although these filters have not been demonstrated to produce better outcomes, theoretically additional safety may be provided by their use.

### Acute normovolemic hemodilution (ANH)

Acute normovolemic hemodilution (ANH) is a technique intended to minimize or decrease the need for allogeneic transfusion. With ANH, blood is phlebotomized from a surgical patient at the start of surgery, generally shortly after the induction of anesthesia. The amount of blood removed is that projected to leave an end of phlebotomy hematocrit of approximately 30%. The blood that is taken off is replaced with a colloid or crystalloid volume expander in order to maintain isovolemia. At the end of surgery, the phlebotomized blood is given back to the patient. The central tenet of ANH is that the patient will bleed blood that is less concentrated in terms of its erythrocytes. Although this theoretical savings sounds substantial, the savings appear to be small clinically.<sup>131</sup>

Given that the phlebotomized blood is whole blood, the effectiveness in transfusion avoidance applies to both erythrocytes and coagulation products. The real value of ANH is in the preservation of platelets and plasma. If sufficient whole blood is collected prior to surgery, a dilutional coagulopathy can be reversed.<sup>132,133</sup> Pairing of ANH and cell salvage provides a comprehensive hematologic approach to avoiding allogeneic transfusion. Cell salvage provides erythrocytes for treating anemia, whereas the ANH provides the coagulation product avoidance. In addition, the anemia created by hemodilution exposes fewer red cells to the mechanical trauma of salvage, thus providing a higher rate of erythrocyte return.<sup>134</sup>

A number of mechanisms allow the patient to tolerate aggressive phlebotomy. Increased cardiac output resulting from increases in heart rate and cardiac contractility, and a reduction in whole blood viscosity, lead to maintenance of oxygen delivery.<sup>135,136</sup> In addition, tissue oxygen needs are reduced during hemodilution from anesthesia and mechanical ventilation.

The practice of ANH is relatively limited due to a lack of understanding of how it is performed. In general, multiple units of blood are withdrawn into a standard donor bag containing 63 mL

of citrate anticoagulant. The goal is to remove 450–500 mL of whole blood into each donor bag. The 63 mL of citrate is adequate to anticoagulate this volume of blood. Typically, the blood is removed through an arterial or venous catheter. Optimally, the shortest catheter should be used in order to minimize resistance to flow and to minimize activation of platelets. Double- or triple-lumen catheters with their long catheter lengths are likely to fail because flow rates are slow. As a result of the slow flow rate, blood clotting may result. Blood flow should be greater than 30 mL/min in order to prevent clotting in the collection bag. Periodic agitation of the collected blood should take place in order to optimally mix the citrate anticoagulant with the blood.<sup>137</sup> Once the blood is collected, it should be maintained at room temperature in the operating room close to the patient. Once the blood has been mixed with the anticoagulant, there is no need to continuously agitate it.<sup>138</sup> Units should be reinfused in the reverse order of collection, i.e., first out → first in.

A systematic review and meta-analysis of randomized trials on the use of ANH in cardiac surgery, which included 29 RCTs with a total of 2439 patients (1252 patients in the ANH group and 1187 in the control group), demonstrated a reduction in the rate of allogeneic RBC transfusion (42.1% in the ANH group vs. 56.1% in the control group; risk ratio = 0.74;  $p < 0.0001$ ) and a reduction in post-operative blood loss (388 mL in the ANH group vs. 450 mL in the control group;  $p < 0.0001$ ).<sup>139</sup>

## Point-of-care testing

Point-of-care or near-patient testing involves the placement of laboratory equipment near or at the patient bedside. A number of point-of-care devices are available for the measurement of the hemoglobin concentration, prothrombin time (PT)/INR and activated partial thromboplastin time (APTT), glucose, blood gas chemistry and electrolyte concentrations, platelet function, and whole blood clotting function.

The advantage of using point-of-care devices instead of centralized laboratory testing relates to the speed of getting data for management decisions. In the acute care setting, such as the operating room or the intensive care unit where critically ill, dynamically changing patients are being cared for, the tendency is to make empiric care decisions based on clinical judgement regarding the patient condition. When laboratory information is obtained at the patient bedside, more informed patient care decisions can be made.

Another advantage of point-of-care testing involves the use of microsampling. Generally, these devices require blood samples in the microliter volume range, whereas traditional laboratory-based testing requires milliliter volume samples. It is well recognized that repeated, large-volume samples can quickly result in an iatrogenic anemia, most prominently in the critical care setting.<sup>140</sup> It has been estimated that the average ICU patient will have a fall in hemoglobin concentration of 0.5 gm/dL/day with 80% of the fall related to blood draws.<sup>141</sup>

One of the drawbacks to point-of-care testing is that the methodology for measurement is frequently different from that of laboratory-based testing, which will sometimes produce results that are not directly comparable to conventional laboratory assays. In many of these circumstances, there is no gold standard by which to say one methodology is superior to another. As such, it is important to recognize the biases of each device and react accordingly. It is also important to understand the approved label indication or intended use population for the device and/or assay before applying it to routine patient care. Robust quality management systems,

end-user trainings frequently differencing and competency evaluations, and input from laboratory medicine, where available, are critical to the proper implementation of a point-of-care testing program.

For PBM programs, whole blood viscoelastic assays using thromboelastography (TEG, Haemonetics, Braintree, MA, USA) or rotational thromboelastometry (ROTEM, TEM Systems Inc., Durham, NC, USA) have become increasingly popular in managing perioperative coagulopathic bleeding and blood product therapy. In addition to the advantage of rapid results and point-of-care availability, these assays have the theoretical advantage of providing assessment of the interactions among the various components of hemostasis. Traditional laboratory measures of hemostasis such as APTT, PT, fibrinogen, and platelet count provide a limited assessment of only that aspect of hemostasis and not their interaction. The use of TEG- and ROTEM-based algorithms for assessing hemostasis and managing blood component therapy have been shown to reduce transfusions and bleeding in the settings of massive trauma, liver transplantation, and cardiac surgery,<sup>142–155</sup> and this is discussed extensively in this volume.

## Use of hemostatic agents

The hemostatic management of patients has been advanced through better understanding of the mechanisms of coagulopathy, availability of point-of-care testing, and a growing array of hemostatic agents. The clinical use and description of these agents have been reviewed<sup>156,157</sup> and are briefly described here.

### Solvent/detergent plasma

Physicians are familiar with using plasma from a single donation of whole blood or apheresis collection to treat a coagulopathy. A pooled solvent detergent (S/D)-treated plasma called Octaplas LG (Octapharma AG, Vienna, Austria) is now approved in the United States.<sup>158</sup> This product is manufactured from plasma pools of 630–1520 donors, which are ultrafiltered (1 μm pore size) and then subjected to solvent (1% tri(*n*-butyl)phosphate [TNBP]) and detergent (1% Octoxynol-9) treatment for 1–1.5 hours.<sup>158</sup> This processing inactivates lipid membrane-bound viruses. The product is further processed through an affinity column to remove prions. Protein-coated nonenveloped viruses such as HAV or parvovirus B19 are resistant to S/D treatment. Transmission is prevented by screening for low virus loads in the starting plasma units, dilution through pooling, and the presence of neutralization antibodies introduced by pooling. The risk of transfusion-related lung injury appears to be dramatically reduced, if not eliminated, which is likely due to pooling and screening for HLA antibodies.<sup>159</sup> Octaplas LG is provided as a standard 200 mL bag of type-specific S/D plasma. It is maintained frozen at 18 °C and thawed when requested. Once thawed, Octaplas LG is stored for up to 12 hours at 2–4 °C.<sup>158</sup> The content of Octaplas LG is similar to that of plasma with the exception of lower levels of protein S activity (0.61 IU/mL; normal range, 0.56–1.68) and α2-antiplasmin activity (0.48 IU/mL; normal range, 0.72–1.32).<sup>160</sup> Prior S/D plasma formulations approved in the United States had even lower levels of protein S and α2-antiplasmin activity, and were associated with reports of enhanced fibrinolysis and thrombosis during liver transplant.<sup>161</sup> This does not appear to be the case with current Octaplas LG formulations.<sup>159</sup> Octaplas LG can be used clinically interchangeably with plasma,<sup>159</sup> including for TTP as ADAMTS13 levels are adequate.<sup>162</sup> Octaplas also appears to be safe in pediatric patients. A recently published open-label, multicenter, postmarketing study of 50 pediatric patients undergoing cardiac or

liver surgery or with liver dysfunction, who were administered Octaplas LG for the replacement of multiple coagulation factors, reported no adverse drug reactions and no serious adverse events attributable to the Octaplas LG administration.<sup>163</sup>

### **Prothrombin complex concentrates (PCC)**

Three-factor nonactivated PCC (3-PCC) containing lyophilized factors II, IX, and X but low levels of factor VII (Profilnine-Grifols, Bebulin VHBaxter) and an activated 3-PCC called FEIBA (factor VIII inhibitory bypass activity; Baxter) have been available in the United States for years. These have been used primarily for the treatment of hemophilia. In April 2013, the FDA approved the first nonactivated four-factor PCC (4-PCC) called K-Centra (CSL Behring GmbH, Marburg, Germany).<sup>164</sup> K-Centra contains hemostatic levels of factors II, VII, IX, and X. It is indicated for the urgent reversal of acquired coagulation factor deficiency induced by vitamin K antagonist (VKA; e.g., warfarin) therapy in adult patients with acute major bleeding. Dosing is based on weight and baseline INR values. The dose calculation uses the factor IX content, which is approximately 500 IU/vial. For an INR of 2–4, 25 IU/kg is used, not to exceed 2500 IU; for an INR of 4–6, 35 IU/kg is used, not to exceed 3500 IU; and, for an INR>6, 50 IU/kg is used, not to exceed 5000 IU. Patients should receive vitamin K concomitantly with K-Centra. Repeated dosing is not recommended. The primary US study upon which the US Food and Drug Administration (FDA) approval was based was an open-label RCT comparing K-Centra to plasma for urgent VKA reversal in 202 patients with major bleeding.<sup>165</sup> The study demonstrated that K-Centra was equivalent to plasma in achieving the hemostatic endpoint and did so more rapidly (17 minutes vs. 148 minutes). There was no difference in thromboembolic rates (7.8% PCC vs 6.4% FFP) or serious adverse events, although the mortality rate at 45 days was 9.7% in the PCC group versus 4.6% in the FFP group. Of note, K-Centra contains trace amounts of heparin and thus should not be used in patients with heparin-induced thrombocytopenia. The use of 3-PCC for warfarin reversal is controversial, although nonactivated 3-PCC has been used in combination with recombinant factor VIIa when there have been shortages of 4-PCC.<sup>166</sup>

There are limited studies on the use of 4-PCC for treatment of coagulopathy in other settings. A pilot randomized controlled trial (PROPHESY trial) comparing the impact of 4-PCC versus FFP on hemostasis (1 and 24 hour postintervention) in patients undergoing heart surgery showed a significant increase in factor II and factor X activity in the 4-PCC arm one-hour postadministration and no increase in thromboembolic complications.<sup>167</sup> A nationwide propensity score-matched analysis of the American College of Surgeons-Trauma Quality Improvement Program database of adult trauma patients who received 4-PCC plus FFP versus FFP alone demonstrated a lower mortality rate (17.5% vs. 27.7%,  $p = 0.01$ ), lower rates of acute respiratory distress syndrome (1.3% vs. 4.7%,  $p = 0.04$ ) and acute kidney injury (2.1% vs. 7.3%,  $p = 0.01$ ), and a decreased requirement for pRBCs (6 units vs. 10 units;  $p = 0.02$ ) in the combined 4-PCC + FFP group compared with the control group (FFP only).<sup>168</sup> Clinical trials of four-factor PCCs with regard to their efficacy, safety (thrombotic risk), and cost-effectiveness in patients not on VKAs are needed.

While 4-PCC were once used as reversal agents for direct factor Xa inhibitors, such as rivaroxaban and apixaban,<sup>169,170</sup> their use in this setting is declining due to the recent FDA approval of andexanet-alfa, a specific antidote for these anticoagulant drugs.<sup>171</sup>

### **Antifibrinolytics**

The fibrinolytic system contributes to the balance between bleeding and thrombosis by controlling clot formation, extension and remodeling, and dissolving unnecessary clots. In patients with excessive bleeding, inhibiting fibrinolysis can improve hemostasis by delaying clot dissolution. The two most commonly used antifibrinolytic agents,  $\epsilon$ -aminocaproic acid (EACA) and tranexamic acid (TXA), are lysine analogs that bind to lysine receptor sites on plasminogen, thereby slowing the rate of conversion to its active form, plasmin.<sup>157</sup> With the removal of aprotinin from the US market in 2007, these agents have been shown to be efficacious in reducing bleeding in cardiac surgery.<sup>172</sup> TXA interest was greatly promoted by the trauma study, Clinical Randomization of an Antifibrinolytic in Significant Hemorrhage (CRASH-2).<sup>173</sup> This was an RCT involving 20,211 adult trauma patients assigned to receive TXA versus placebo within eight hours of injury. The primary end point, all-cause mortality, was reduced in the TXA arm (14.5% vs. 16.0%) as was death due to bleeding (4.9% vs. 5.7%). A follow-up analysis showed that the benefit was strongest when TXA was given within one hour of injury; it was still apparent at 1–3 hours after injury, but TXA actually increased mortality if given 3–8 hours after injury.<sup>174</sup> There was no difference in bleeding or transfusions. The subsequent CRASH-3 RCT demonstrated that tranexamic acid was safe in patients with traumatic brain injury (TBI) and that treatment within three hours of injury reduced head-injury-related death.<sup>175</sup> The WOMAN trial, a large international randomized, double-blind trial of TXA versus placebo in nearly 20,000 women with postpartum hemorrhage, also showed a reduction in death due to bleeding, especially in women given TXA within three hours of giving birth (1.2% in the TXA group vs. 1.7% in the placebo group; relative risk: 0.69;  $p = 0.008$ ).<sup>176</sup> A recent review and meta-analysis of RCTs of TXA versus no TXA in surgical patients found 129 trials involving more than 10,000 patients who underwent cardiac, orthopedic, hepatic, urologic, gynecologic, cranial, or vascular surgery published between 1972 and 2011 and showed that the need for transfusion was consistently reduced by approximately one-third.<sup>177</sup> In the nonsurgical setting, TXA was not found to be effective in preventing bleeding in severely thrombocytopenic patients with hematologic malignancy.<sup>178</sup>

Antifibrinolytic use is not without risk. An RCT of TXA in cardiac surgery reported an increased risk of seizures when TXA was used at high doses (100 mg/kg of body weight).<sup>179</sup> There is also some concern that TXA may increase the risk of fibrinolysis shutdown in severely injured trauma patients, and there have been calls to limit its use to patients with evidence of hyperfibrinolysis in this setting.<sup>180,181</sup> While fibrinolysis shutdown is also amplified by TXA in surgical settings, such as oral and maxillofacial surgery,<sup>182</sup> there are no data to suggest that its use in these settings results in an increase in adverse events or poor outcomes.

### **Desmopressin**

Desmopressin (DDAVP) is a synthetic analog of vasopressin that is primarily used for the treatment of patients with von Willebrand disease (VWD), hemophilia A, some platelet disorders, and uremic bleeding. DDAVP is known to increase the plasma levels of von Willebrand factor (VWF), factor VIII, and tissue plasminogen activator. The hemostatic effect of DDAVP is well understood.<sup>183</sup> DDAVP has been used to enhance hemostasis in patients undergoing surgery with expected high blood loss. A meta-analysis of 38 trials involving 2488 patients undergoing mostly cardiac surgery but also orthopedic, vascular, and plastic surgery found a statistically

significant reduction in bleeding (80 mL) and transfusion (0.3 units), but the clinical relevance of these findings is questionable.<sup>184</sup> Due to its limited efficacy, DDAVP is not routinely used in surgical patients.

### Fibrinogen concentrates

There are two human plasma-derived, sterile, purified, virus-inactivated, and nanofiltered lyophilized fibrinogen concentrates available in the United States: RiaSTAP (CSL Behring GmbH, Marburg, Germany) and FIBRYGA (Octapharma AG, Vienna, Austria).<sup>185,186</sup> The labeled indication for both concentrates is for the treatment of acute bleeding episodes in patients with congenital fibrinogen deficiency, including afibrinogenemia and hypofibrinogenemia; neither product has a labeled indication for dysfibrinogenemia or acquired hypo-fibrinogenemia. Each bottle of RiaSTAP contains approximately 900–1300 mg of fibrinogen and each bottle of FIBRYGA contains approximately 1000 mg of fibrinogen, equivalent to 4–5 units of cryoprecipitate. Fibrinogen concentrates have been widely used in Europe for patients with perioperative bleeding and acquired hypofibrinogenemia.<sup>187</sup> The fibrinogen level at which replacement is recommended in the perioperative setting has evolved from 100 mg/dL<sup>188</sup> to more recently 150–200 mg/dL in the European trauma guidelines.<sup>189</sup> In the perioperative setting, the recommended dose is 2–4 g (25–50 mg/kg) of fibrinogen concentrate with an expected increment of 25–28 mg/dL per gram of fibrinogen concentrate.<sup>156</sup> The reported side effects include allergic reactions (chills, fever, nausea, vomiting, and thrombosis).

A recent meta-analysis of eight RCTs evaluating fibrinogen concentrate use in cardiovascular surgery patients at high risk or with evidence of bleeding found a significant reduction in transfusions but not mortality.<sup>190</sup> A later RCT of 735 patients who underwent cardiac surgery compared fibrinogen concentrates to cryoprecipitate in bleeding patients with hypofibrinogenemia, and found fibrinogen concentrates to be equivalent in blood product use and risk of thrombosis.<sup>191</sup>

### Recombinant activated factor VII (rFVIIa)

Recombinant activated factor VII (rFVIIa, NovoSeven, Novo Nordisk A/S, Bagsvaerd, Denmark) is structurally similar to plasma-derived factor VIIa and is intended to promote hemostasis through the activation of the extrinsic pathway of the coagulation cascade.<sup>192</sup> rFVIIa binds to tissue factor, which then converts factor X to factor Xa, as well as coagulation factor IX to factor IXa. The factor VII gene was cloned and expressed in Chinese hamster kidney cells where the expressed protein is autoactivated during chromatographic purification.<sup>193</sup> The labeled indication for rFVIIa is to treat or prevent bleeding in hemophilia A or B patients with inhibitors or patients with congenital factor VII deficiency.<sup>192</sup> Dosing in hemophilia patients with bleeding is 90 µg/kg every two hours until hemostasis is achieved ( $t_{1/2}$  is 2.3 hours). It is available as a lyophilized powder requiring reconstitution in 1, 2, or 5 mg vials.

There has been extensive interest in using rFVIIa off-label to promote hemostasis in trauma, surgery, intracranial bleeding, and other patient populations.<sup>194</sup> Early enthusiasm based on largely observational data or small RCTs<sup>195,196</sup> was not confirmed by RCTs in penetrating trauma,<sup>195</sup> pediatric cardiac surgery,<sup>197</sup> intracranial bleeding,<sup>198</sup> liver resection,<sup>199</sup> or variceal bleeding.<sup>200</sup> rFVIIa has also been suggested as a potential therapy for the reversal of anti-coagulants, such as warfarin, direct factor Xa inhibitors, and direct thrombin inhibitors. With the availability of four-factor

PCC, rFVIIa should not be used for warfarin reversal. Data on its use to reverse dabigatran or rivaroxaban are mixed.<sup>201–204</sup> Given the uncertainty regarding clinical effectiveness in off-label settings, safety issues must be carefully considered. A systematic review of 35 clinical trials involving 4468 patients who received rFVIIa for an off-label indication reported significantly higher risk of arterial (but not venous) thromboembolic events (CI 1.68 [1.20–2.36]).<sup>205</sup> A recent meta-analysis of six clinical trials involving 470 cardiac surgery patients reported a higher risk of stroke (OR 3.69 [1.1–12.38],  $p = 0.03$ ) in those treated with rFVIIa.<sup>206</sup> Taken together the literature suggests that the use of rFVIIa in an off-label setting has unproven efficacy, should be entertained with caution, and, if used, should be limited to patients with life-threatening bleeding.<sup>207</sup>

### Limiting phlebotomy blood loss for laboratory testing

Although it might seem like a trivial amount of blood loss, repetitive phlebotomy for laboratory testing can result in a significant decrease in the patient's hemoglobin concentration and can result in anemia. A computerized model of blood loss predicted that it would take a healthy adult of average weight and blood volume about 40–70 days of 53 mL daily blood draws to reach a hemoglobin concentration of 7 g/dL, whereas a sick patient in the ICU would require only 9–14 days to reach the same level.<sup>208</sup> The volume of phlebotomy has been correlated with the decrease in the recipient's hemoglobin or RBC transfusion requirements,<sup>209–212</sup> and one Canadian study found that for every 100 mL of blood lost by phlebotomy, the recipient's hematocrit decreased by nearly 2%.<sup>209</sup> Several studies have demonstrated that the amount of blood that is collected for diagnostic purposes is far in excess of that which is actually necessary to complete the testing,<sup>212,213</sup> suggesting that measures to reduce the volume of blood collected for laboratory testing should be implemented. To this end, the AABB has suggested several steps that can be implemented.<sup>13</sup> Some of these suggestions involve educating the providers about the potential problems caused by reduced hemoglobin and anemia that can arise from excessive phlebotomies, and changing their practices when ordering laboratory testing; limiting the number of phlebotomies to the absolute minimum number required for patient care should reduce the amount of blood lost to testing, as will eliminating standing orders for certain laboratory tests, that is, laboratory tests should only be ordered when there is a change in the patient's condition or when a diagnostic or therapeutic intervention based on their results is being considered. Other recommendations involve making changes in the way that laboratory tests are ordered, such as by electronically limiting the number of times that a test can be ordered during a defined time period<sup>214</sup> and redesigning the electronic or paper forms on which laboratory tests are ordered to discourage ordering unnecessary testing. If a laboratory test is indeed required, then the smallest volume possible should be phlebotomized. This means that special small-volume tubes must be easily accessible on the ward and that the laboratory's diagnostic equipment must be able to process these kinds of tubes, which might have different dimensions than standard tubes. Furthermore, point-of-care testing should be performed when available (and when validated) as the volume of blood required to perform the testing is usually much smaller than that required by the instruments in the main laboratory.

## Summary

PBM has become a major clinical and research focus for not only the field of transfusion medicine but also any discipline in which patients are transfused. It is designed to be patient care focused and requires a multidisciplinary approach. A PBM program requires resources, effort, and institutional support but can be implemented gradually by initially capitalizing on the obvious opportunities. IT has provided unprecedented opportunity to systematically influence ordering and monitoring of physician practices. A PBM program is one of the most powerful ways to optimize patient care in a cost-effective manner.

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## CHAPTER 28

# Clinical and technical aspects of blood administration

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## Introduction

Errors in pretransfusion specimen collection, omission of appropriate transfusion filters, or coadministration of incompatible fluids with blood products could transform blood transfusions from a lifesaving measure into a life-threatening event. This chapter summarizes key aspects of safe blood component administration and emphasizes processes and technologic advances that aim to improve the quality of patient care and patient safety. This chapter provides general guidance based on current guidelines and regulations. In addition to this information, the reader is directed to the latest version of the AABB Standards for Blood Banks and Transfusion Services, the College of American Pathologists (CAP) Transfusion Medicine Checklist, local standards of practice, and institutional requirements.<sup>1,2</sup>

## Pretransfusion considerations

Prior to transfusion, several steps should be taken to ensure safe administration of blood products for patients. First, it is essential to obtain informed consent for blood transfusion except in the setting of emergent requests for blood products. Per the AABB Standards for Blood Banks and Transfusion Services (Standard 5.28.1.1), informed consent at minimum requires the inclusion of the following: (1) a description of the risks, benefits, possible side effects, and alternatives (including nontreatment); (2) the opportunity to ask questions; and (3) the right to accept or refuse transfusion.<sup>1</sup> In some parts of the United States, there is also a legal requirement (e.g., the Paul Gann Blood Safety Act in California) to discuss alternatives to transfusion. The informed consent discussion between the health-care provider and patient should be properly documented in the patient's medical record.<sup>3,4</sup>

Second, a transfusion history should be obtained from the patient.<sup>5</sup> The history should include information about previous transfusions such as the timing and the types of blood products that were transfused and if the patient had previous transfusion reactions. If the patient has a history of transfusion reactions, the clinical team should consider whether the patient should receive prophylactic medication (see more details in Chapter 48) and if the blood component requires specific pretransfusion processing, such

as the pretransfusion modifications described later in the section "Component modification and preparation." The transfusion history should also include documentation of alloantibodies to red blood cell antigens identified in the patient to supplement the compatibility testing described below.

Third, pretransfusion compatibility testing should be performed prior to component issue unless the indication for transfusion is emergent. Determining the ABO and RhD blood type of the transfusion recipient and identifying any unexpected antibodies in the plasma of the recipient can prevent hemolytic transfusion reactions and conserve limited resources.<sup>6</sup> To begin the pretransfusion compatibility testing process, the clinical staff must obtain a blood sample from a correctly identified patient. The pretransfusion sample should be labeled legibly in the presence of the patient with a minimum of two patient identifiers (e.g., name, medical record number, and date of birth), the date and time of the blood draw, and the identification of the person who collected the sample. The blood bank must reject samples that do not strictly meet these requirements or if there are any discrepancies in labeling.<sup>1</sup> Other reasons that pretransfusion samples should be rejected include if the requisition form is missing key identifying information or if the sample was drawn in an incorrect tube.<sup>1,5</sup>

Studies have consistently shown that human errors throughout the pretransfusion process occur at substantial rates, can lead to wrong blood in tube (WBIT), and result in fatal incompatible transfusions.<sup>7–10</sup> As a consequence, both the AABB Standards for Blood Banks and Transfusion Services and CAP Transfusion Medicine Checklist require that transfusion medicine services have a policy and systems in place to reduce the risk of misidentification of pretransfusion samples and the risk of transfusion of red cells to the wrong patient.<sup>1,2</sup> Examples of processes that can reduce these risks include verifying the ABO typing of a patient with a second sample collected at a separate blood draw, using an electronic identification system that verifies at the time of transfusion that the patient about to receive a blood product is the same patient from whom that pretransfusion sample was drawn, or utilizing a mechanical barrier system that uses a code on the patient wristband to open the correctly issued blood component.<sup>2,11</sup> These protocols are necessary because the consequences of WBIT can be fatal.

## Venipuncture for intravenous (IV) access

Blood components are most often transfused intravenously through peripheral veins, but central venous access can also be used in critically ill patients.<sup>12</sup> IV line sizes typically range from 25- to 14-gauge; 20–18-gauge IV lines are usually suitable for adults, and 25–24-gauge IV lines can be used in infants and toddlers as long as an infusion device maintains constant flow.<sup>13,14</sup> Slower transfusion rates should be used with smaller gauge needles to prevent hemolysis, and special care is needed in administering red blood cells with handheld syringes through smaller needles (e.g., 23-gauge or smaller).<sup>15</sup> A previously established IV line can be used for blood transfusion. However, prior to use, the line should always be examined for patency and signs of infection. Ideally, the line should be flushed with normal saline to ensure that any incompatible fluid is removed prior to blood component infusion.

## Component issue, release, storage, and transport

### Component issue and release

Blood banks must have a formal process when issuing blood components to verify that the correct blood product is being given to the correct recipient. The verification process must check the following<sup>1</sup>:

- 1 The type of blood component
- 2 The recipient's two independent identifiers, ABO group, and Rh type
- 3 The donation identification number, donor ABO group, and if required, Rh type
- 4 The interpretation of crossmatch results, if performed
- 5 Completion of any special transfusion requirements (e.g., irradiation or washing)
- 6 The expiration date (and time, if applicable)
- 7 A check for any visual abnormalities of the unit

After this checklist is completed, the date and time of issue should be noted. Once at the bedside, the transfusionist and either another individual or an electronic identification system must identify and match the patient and the intended recipient of the issued blood product with two independent identifiers, all in the presence of the patient.<sup>1</sup>

### Component storage

Blood components must be stored within strict temperature requirements to maintain their quality and safety. All storage devices should be equipped with an emergency power supply and a continuous alarm monitoring system. In the absence of such a system, the temperature must be monitored and documented every four hours.<sup>16</sup> Alarms should continue to function even in the event of a power failure. Scheduled alarm checks should occur at least quarterly as required by the CAP Transfusion Medicine Checklist.<sup>2,17</sup>

### Red blood cells

RBCs must be stored within refrigerators in the blood bank, in refrigerators at other satellite sites, or in validated coolers monitored by the blood bank. The refrigerators must continuously maintain temperatures at 1–6 °C to limit RBC metabolic activity while ensuring cell viability.<sup>18,19</sup> During the transport process, RBCs must maintain temperatures of 1–10 °C.<sup>1</sup> Options for maintaining these temperatures for transport include bagged wet ice, commercial cooling packs, and specially designed coolers (see the section

"Component transport").<sup>5</sup> If RBCs are not maintained in these temperatures, they must be discarded and should not be transfused nor returned back to inventory.

The expiration time of RBCs is dependent on what anticoagulant is used during collection.<sup>5</sup> RBCs derived from whole blood collected in acid–citrate–dextrose (ACD), citrate–phosphate–dextrose (CPD), or citrate–phosphate–double–dextrose (CP2D) expire 21 days from collection. RBC units collected in citrate–phosphate–dextrose–adenine (CPDA-1) expire 35 days from collection. RBC units that are processed to include additive solution (AS-1) expire 42 days from collection. As discussed later, a leukoreduced RBC unit has the same expiration times as the original unit, and an irradiated RBC has an expiration of 28 days from the time of irradiation or the original expiration date, whichever is sooner.

### Platelets

Indications for platelet transfusion include prophylaxis for hypoproliferative thrombocytopenia, prophylaxis for invasive procedures in patients with thrombocytopenia or platelet dysfunction, and the treatment of active bleeding in thrombocytopenic patients. Platelets should be stored at room temperature (20–24 °C) with continuous gentle agitation.<sup>1,5</sup> During transport, platelets should also be kept at 20–24 °C but do not need to be agitated. Room temperature storage is favored because platelets stored at 20–24 °C have good post-transfusion in vivo recovery; exposure to cold temperatures results in platelet inactivation and rapid in vivo clearance.<sup>20</sup> Despite these properties, cold-stored and cryopreserved platelets are actively being investigated and are discussed in Chapter 17. Platelets are sensitive to pH changes and have storage requirements to maintain an optimal pH range. Platelets are stored in containers made of plastic that are permeable to gas, and continuous adequate agitation promotes gas exchange and helps to effectively maintain pH.<sup>21,22</sup> Platelet agitation devices have been extensively studied, and results of these studies show that horizontal agitation (circular or flatbed agitators moving side to side) maintain platelet functionality more effectively than vigorous, vertical (end over end) methods of agitation.<sup>20,23,24</sup> Studies have also demonstrated that deleterious effects on platelet components can begin to take place after one day or longer of interrupted agitation.<sup>25–27</sup> Accordingly, AABB Standards for Blood Banks and Transfusion Services permit a maximum 30-hour period during product transport when platelets are not required to be agitated.<sup>1</sup>

Room temperature storage puts platelets at increased risk of bacterial contamination, and guidelines set by the FDA and new pathogen reduction technologies address this challenge. In the FDA guidance released in September 2019 and updated in the autumn of 2021, platelets are recommended to undergo procedures that mitigate the risks of bacterial contamination. Compliant approaches include primary culture, large volume delayed sampling (LVDS), pathogen reduction, and rapid bacterial testing (see details in Chapters 17, 42, and 46).<sup>28</sup> The first two methods use standard bacterial culture methods available in microbiology laboratories but differ on sample timing and sample volume taken for culture. FDA-approved pathogen reduction technology reduces the transmission of infectious agents in platelet components by using UV-A light with a psoralen photoactive agent to crosslink DNA and/or RNA (see Chapter 42). A second technology using riboflavin and UV light is available in some countries outside the US. Finally, rapid bacterial testing utilizes either an immunoassay or colorimetric assay to detect Gram-positive and Gram-negative bacteria.<sup>29,30</sup>

## Plasma

Transfusions with plasma products are indicated for bleeding patients with coagulation factor deficiencies and patients with specific plasma-protein deficiencies. Plasma transfusions are often given to patients before invasive procedures to correct abnormalities in coagulation tests despite the lack of evidence that this practice provides real clinical benefit.<sup>31–33</sup> There are several different preparations of plasma that are differentiated based on the manner and timing of the plasma separation and freezing processes. Fresh-frozen plasma (FFP) is separated from whole blood and placed in a freezer within eight hours of collection. Generally, if plasma is frozen within 8–24 hours of collection, it can be labeled either as plasma frozen within 24 hours (PF24) or plasma frozen within 24 hours after phlebotomy held at room temperature up to 24 hours after phlebotomy (PF24RT24). FFP, PF24, and PF24RT24 can generally be maintained in a freezer at  $\leq -18^{\circ}\text{C}$  for 12 months after collection; FDA variances can be obtained to store plasma frozen for longer periods of time. Plasma that is separated from whole blood and is never frozen can be labeled as *liquid plasma*, which expires five days after the expiration date of the whole blood from which it was derived. These variations in labeling are due to concern that plasma that is not separated and frozen within eight hours could have diminished levels of coagulation factors.<sup>34,35</sup>

After thawing, FFP should subsequently be stored at 1–6 °C. Temperatures up to 10 °C are acceptable during transport. If the component is not transfused within 24 hours, it can be relabeled as *thawed plasma* and stored in a refrigerator for an additional four days.<sup>35</sup> Outside of the United States, there is limited availability of freeze-dried plasma.<sup>36</sup>

## Cryoprecipitate

Cryoprecipitate transfusions are used for fibrinogen replacement in patients with low fibrinogen as can be seen in the setting of liver failure and postpartum hemorrhage. Cryoprecipitate is derived from FFP thawed slowly in an ice bath or in a refrigerator at 1–6 °C. Cryoprecipitate must contain at least 150 mg of fibrinogen and a factor VIII activity level of 80 IU.<sup>1</sup> This component also contains other plasma proteins, including fibronectin, factor XIII, and von Willebrand factor. Cryoprecipitate, once collected from thawed FFP, must be frozen within one hour and can be maintained at  $\leq -18^{\circ}\text{C}$  for up to 12 months. When the product is requested for clinical use, it should be thawed at 30–37 °C and then stored and transported at room temperature to prevent reprecipitation of the component. A pooled component expires within four hours unless the pooling occurred with the use of a sterile connection device. In the case of single units and sterile pools, the product shelf life is extended to six hours.<sup>1,37</sup>

## Granulocytes

Granulocytes have been transfused in septic patients with severe neutropenia despite controversy over the clinical benefit of these transfusions.<sup>38</sup> From a blood component administration perspective, patients must receive the infusion of granulocytes within 24 hours of collection, and granulocytes must be irradiated because recipients are significantly immunocompromised and the product contains a substantial number of lymphocytes.<sup>1</sup> Because the RBC content of a granulocyte collection usually exceeds 2% (typically approximately 6%), granulocyte products must also be ABO and crossmatch compatible with the recipient.<sup>1</sup> Granulocytes should be maintained at room temperature without agitation and must never be leukoreduced.<sup>1,37</sup>

## Hematopoietic progenitor cells and cell-based immunotherapies

Hematopoietic progenitor cells (HPCs) can be collected from the bone marrow, from peripheral blood with stem cell mobilization, or from cord blood. These products are stored in liquid nitrogen.<sup>5</sup> Shipping and transport of HPCs are highly regulated by the FDA, AABB, FACT, and other organizations.<sup>39</sup> Hematopoietic progenitor cells that are infused must never be irradiated. To minimize hematopoietic progenitor cell loss, many transplant centers infuse hematopoietic stem cells into the recipient without the use of a standard infusion set and never with a leukoreduction filter. However, a standard infusion set can be used when debris is noted in the HPC product. After infusion, sterile saline may be injected into the empty stem cell container to rinse the bag to maximize stem cell recovery.

Cell-based immunotherapies such as CAR T cells have been FDA-approved for the treatment of a number of hematologic malignancies.<sup>40</sup> These immunotherapy products must be stored, transported, and administered according to manufacturers' instructions and product labeling and, like HPC products, they must never be leukoreduced. Before thawing, identification information on the product's label must match the patient's identity, and the infusion bag and contents should be visually inspected. Tubing should be primed with saline before infusion, and after all the contents are infused, sterile saline should be used to rinse the bag and tubing to maximize the number of cells infused into the patient.

Specific standards for cellular therapy products are established and maintained by the US Food and Drug Administration (FDA; [www.fda.gov](http://www.fda.gov)), AABB ([www.aabb.org](http://www.aabb.org)), CAP ([www.cap.org](http://www.cap.org)), and the Foundation for the Accreditation of Cellular Therapy (FACT; [www.factwebsite.org](http://www.factwebsite.org)).

## Prothrombin complex concentrates and recombinant clotting factors

Some transfusion services are responsible for dispensing prothrombin complex concentrates (PCCs)—which are used to reverse vitamin K antagonists such as warfarin—and recombinant clotting factors, which are increasingly utilized in patients with uncontrolled bleeding.<sup>41–43</sup> PCCs are also used to reverse the effects of direct oral anticoagulants in place of specific reversal medications (e.g., andexanet alfa for direct factor Xa inhibitors and idarucizumab for dabigatran) as a cost-effective alternative or when these agents are not available.<sup>44–46</sup> These products are often lyophilized and must be reconstituted with sterile water or another diluent prior to infusion. In general, recombinant factors can be administered by a slow intravenous push whereas multifactor pooled plasma products (e.g., factor VIII inhibitor bypassing agent [FEIBA] and the four-factor PCC) must be administered much more slowly. Details of preparation and administration of PCCs and recombinant clotting factors differ as some formulations include specific syringe and filter sets; in all cases, manufacturer instructions and details in product labeling must be followed. For hospitals that regularly infuse FEIBA or four-factor PCC, the use of a syringe pump or infusion pump can help to ensure that the product is administered safely and at the recommended rate.

## Storage equipment

Automated equipment for blood component storage includes refrigerators, freezers, cell washers, platelet agitators, and plasma thawing devices. Automatic refrigerators have been engineered to act as “vending machines” that store and dispense RBCs remotely.

These devices maintain appropriate temperatures for the blood components, and the computer system interfaces with that of the hospital's blood bank to perform electronic crossmatches to issue compatible blood to patients.<sup>47</sup> If a particular patient cannot receive an electronically crossmatched unit, the system also allows for the emergency release of uncrossmatched blood. These devices have been implemented to allow for quick release of blood products in settings such as the emergency room or intensive care units. Their utility is still under evaluation.<sup>48</sup>

### **Component transport**

Considerations for component transportation are critical to maintaining the integrity of the component to be transfused. Many medical centers rely on coolers with different types of cooler packs to transport blood products as long as they have been validated by the blood bank to maintain RBC and plasma temperatures between 1 and 10 °C. These coolers have been reported to be validated for up to 24 hours to maintain appropriate temperatures; however, these coolers should be used with the expectation that the transfusion should occur quickly and before substantial temperature shifts have taken place.<sup>1</sup> Temperature-sensitive adhesive labels that attach directly to the component or the containers can also be used to monitor transport temperatures. These labels have indicators that change color when the temperature limit is exceeded.<sup>49</sup> Therefore, when a blood component is not transfused, the label can be checked for a color change and/or the temperature of the unit can be taken to determine whether it can be safely returned to the blood bank inventory.<sup>2,17</sup>

### **Novel transport and storage devices**

Portable refrigerator units have been developed that are capable of maintaining a refrigerated temperature of 1–6 °C and are battery-powered, lasting up to 48 hours after leaving the blood bank according to the manufacturer. In addition, at least one model has been designed that maintains refrigerated temperatures of 1–10 °C for the transport of blood components. Although these mobile refrigerators have not been extensively studied, preliminary investigations have shown decreased wastage of blood components by allowing unused issued blood components to be returned to inventory.<sup>50,51</sup> Some models incorporate tracking devices so that the location of the cooler can be tracked by the blood bank at all times. In addition, some coolers maintain a temperature log during use or have real-time wireless temperature monitoring. One study has suggested that unmanned aerial vehicles, or drones, can be used to transport blood products from one location to another, potentially addressing challenges of transporting products in regions with geographical barriers.<sup>52</sup>

### **Return of blood components**

The blood bank must have a validated written procedure for returning blood components back into inventory after they have been issued, which should include steps to "verify the integrity and appearance of blood/blood components and maintenance at appropriate temperature."<sup>22</sup> Issued blood components may be put back into inventory if the following conditions are met<sup>1</sup>:

- 1 The container has not been opened or disturbed
- 2 The component has been maintained within the appropriate temperature range
- 3 For RBCs, at least one sealed segment remains attached to the container
- 4 Documentation that the component has been inspected and is acceptable for reissue

### **Component modification and preparation**

When ordering blood components, physicians should stipulate any specific product modification requirements. As these component modifications often influence the expiration date of the component, the time of outdate must be noted as well.

### **Leukoreduction**

Prestorage leukoreduction, the process of removing leukocytes from blood components prior to storage, is utilized to prevent transfusion-mediated transmission of cytomegalovirus (CMV),<sup>53</sup> human leukocyte antigen (HLA) alloimmunization,<sup>54</sup> platelet refractoriness,<sup>55</sup> and febrile nonhemolytic transfusion reactions.<sup>56</sup> Importantly, certain blood components, such as granulocytes and hematopoietic progenitor cells, must never be processed through a leukoreduction filter (Table 28.1).<sup>37</sup> Leukoreduction filters work by interception and adhesion of white blood cells to filter fiber media and remove >99.99% of leukocytes in RBCs.<sup>57,58</sup> These filters should not be confused with standard component filters, such as the 170–260-µm screen filters incorporated into standard blood administration tubing sets that are used for all blood component infusions and work by clot or debris interception (see Table 28.1). Additionally, 40-µm microaggregate filters are used in cell salvage devices and also work by interception.

In the United States, 95% of sampled leukoreduced components should have less than  $5.0 \times 10^6$  residual leukocytes per RBC unit or apheresis-derived platelet unit and less than  $8.3 \times 10^5$  residual leukocytes per unit of whole-blood derived platelets.<sup>1,58</sup> Notably, this requirement is less stringent than current European requirements, which stipulate that less than  $1 \times 10^6$  leukocytes should remain.<sup>5,59</sup> Many developed countries have mandated universal prestorage leukocyte reduction. Although the United States has not adopted universal leukocyte reduction, the FDA has recommended its implementation. According to 2017 National Blood Collection and Utilization Survey, 95.8% of RBCs (either apheresis or whole-blood-derived) and 99.2% of apheresis platelets transfused in the United States were leukoreduced.<sup>60</sup> Despite these high rates of leukoreduction, leukoreduction policies differ by blood bank and blood supplier, and health-care providers must be aware of their hospital-based practices.

### **Irradiation**

Irradiation of cellular blood components (i.e., granulocytes, non-pathogen-reduced platelets, and RBCs) must be performed when transfusing patients who are at high risk for transfusion-associated graft-versus-host disease (TA-GVHD). Patients at risk for TA-GVHD are usually immunocompromised due to hematologic malignancies, congenital immunodeficiency disorders, immunosuppression from myeloablative drugs, and immature immunity in neonates and fetuses.<sup>61,62</sup> Irradiation of donor units is also performed when the blood donor is known to be a blood relative or an HLA match with the recipient.<sup>1,12</sup> Ensuring proper irradiation is especially critical because TA-GVHD is generally fatal with no known effective treatment.<sup>61,63</sup> In the United States, an irradiated blood component must receive a radiation dose of 25 Gy (2500 cGy) directed at the central portion of the blood canister with no less than 15 Gy to any part of the canister and no more than 50 Gy to any part of the canister.<sup>1,61</sup> This dose of irradiation damages the DNA of T cells contained within the cellular blood component, prevents T-cell replication and engraftment, and thus eliminates the risk of TA-GVHD. Noncellular blood components—such as plasma and cryoprecipitate—and pathogen-reduced platelets do not require irradiation.<sup>35</sup>

**Table 28.1** Considerations in the Preparation and Administration of Various Blood Components

Component	Transfuse through Standard Transfusion Set and Filter	Need for Leukoreduction	Indications for Irradiation
Red blood cells	Always	Recommended—to reduce risk of CMV transmission, febrile nonhemolytic transfusion reactions, platelet refractoriness, and HLA alloimmunization	Prevention of TA-GVHD in vulnerable populations
Platelets	Always	Recommended—to reduce risk of CMV transmission, febrile nonhemolytic transfusion reactions, platelet refractoriness, and HLA alloimmunization	Prevention of TA-GVHD in vulnerable populations Not necessary for pathogen-reduced platelets
Plasma	Always	Not applicable	None
Cryoprecipitate	Always	Not applicable	None
Granulocytes	Always	Never	Always for prevention of TA-GVHD
Hematopoietic precursor cells	No	Never	Never

Sources of radiation used by blood banks are either gamma rays from cesium-137 or cobalt-60 sources or X rays from either a linear accelerator or stand-alone generators. Irradiators that utilize cesium-137 or cobalt-60 contain the isotope within chambers made of lead to prevent escape of gamma rays from the irradiator device. Linear accelerator irradiators generate a beam of X rays, allowing blood components to be irradiated.<sup>64</sup> X ray tube generating devices can also be used.

All types of irradiators require periodic maintenance and quality assurance programs to ensure that the blood container is receiving an appropriate dose of radiation. Irradiators using cesium-137 or cobalt-60 must periodically have the exposure time recalculated to account for the gradual radioactive decay of the radioactive isotopes. The FDA requires the verification of delivered doses annually for cesium-137 sources and semiannually for cobalt-60 sources.<sup>64</sup> Dose verification and adjustments for X ray irradiators should be performed per manufacturer's protocols.<sup>1</sup> In 2019, the US government set a goal to eliminate blood irradiation devices relying on cesium by the end of 2027 by working with hospitals to replace cesium irradiators with X ray devices.<sup>65</sup>

Regardless of the source of irradiation, the expiration date of irradiated RBC units is either 28 days from the date of irradiation or the original expiration date, whichever is sooner.<sup>5,66</sup> Irradiation can increase the levels of hemolysis and potassium in RBCs and so transfusions with irradiated RBCs require special consideration in vulnerable populations (e.g., the increased potassium may cause cardiac complications in small children).<sup>5,67</sup> Irradiation, however, does not alter the expiration date or the function of platelets, and as a result, some hospitals with large oncology wards irradiate platelets at the time that they are placed in hospital inventory. It should be noted that platelets processed with pathogen reduction technology do not need to be irradiated because the process crosslinks nucleic acids and effectively inactivates lymphocytes to prevent TA-GVHD.

### Washing

Cellular blood products usually contain some volume of plasma, and washing can be performed to remove plasma proteins. Washing RBCs or platelets is indicated for patients who have a history of severe allergic reactions to products containing plasma; patients with antibodies against IgA who are receiving products that are not IgA-deficient; removal of complement in products given to patients with post-transfusion purpura; and patients who cannot tolerate the potassium and preservatives in the blood component. The washing process for RBCs or platelets is usually performed with

isotonic saline in an automated, centrifuge-based machine housed in the blood bank or blood center. Once the RBCs or platelets are centrifuged into a "packed" state, isotonic saline flows through the packed cellular mass, washing out the plasma and preservative solution and taking their place. Washing can result in the loss of up to 20% of the RBCs and 33% of platelets and thus should be performed only if there is a clear indication. Furthermore, cells are generally washed in an open system, and as a result, RBCs expire 24 hours after washing, and platelets expire 4 hours after washing, regardless of the original product expiration dates.<sup>5</sup> Some washing devices may induce more RBC destruction during the washing process than others, resulting in higher supernatant potassium levels.<sup>68</sup> For these reasons, washing RBCs and platelets immediately prior to use is preferred.

### Volume reduction via aliquoting

In an effort to mitigate risk for transfusion-associated circulatory overload (TACO) in at-risk patients, RBC, platelet, and plasma units can be split into smaller aliquots using transfer bags and a sterile connecting device. Splitting units can also be utilized in times of inventory shortage in the right clinical context and under medical director guidance. Sterile connecting devices work by connecting sterile tubing segments with copper wafers heated to more than 260–300 °C, melting the plastic tubing and juxtaposing the severed ends together. The heating process prevents bacterial contamination. If the system remains closed throughout the process, the shelf life of the transfer bag will remain the same as the shelf life of the primary unit. Similar technology is employed by heat sealers, which melt plastic tubing and generate a seal that can be detached without opening the blood component.

For neonates requiring very small transfusion volumes (sometimes as little as 10–30 mL), aliquoting of cellular components with the use of connecting devices into a syringe can help to maintain an aseptic (although open system) environment and can preserve the remainder of the blood component, which can be stored properly in the blood bank for future transfusions.<sup>69</sup> Shortening of expiration dates of aliquots should be based on regulatory, institutional, and local policies.<sup>5</sup> Alternatively, RBC aliquots can be made by blood centers by using a multiple-pack system, which diverts a collection of whole blood into a primary bag with three attached smaller bags.<sup>70</sup> When this "quad pack" is centrifuged, the plasma can be separated into one bag, and RBCs are separated into the other three bags. Quad packs provide transfusion services with three RBC aliquots derived from one single RBC unit without having to use sterile connection devices.<sup>16</sup>

### **Thawing plasma**

Frozen plasma can be thawed either in a 30–37 °C water bath or in FDA-approved microwave devices. FFP and PF24 are stored at -18 °C or below and can take 20–30 minutes to thaw in a 37 °C water bath. Water baths have the potential to introduce bacterial contamination into the component if the component is not properly sealed and protected in a waterproof bag.<sup>71</sup> As such, water baths should be regularly cleaned according to the manufacturer's procedures. Water-based warmers—which circulate warm water through a plastic attachment inserted next to the plasma bag rather than requiring the plasma bag to be submerged in the water bath—may also protect against this risk.

Microwave ovens are more expensive than water baths but may be more suitable for urgent plasma requests. Specially designed microwave ovens have been shown to thaw components more quickly, in approximately 5–10 minutes.<sup>72</sup> Microwave ovens also do not carry the same risk of introducing bacterial contamination into the unit. However, microwave ovens have been reported to damage the component plasma proteins in the event of malfunction or the development of “hot spots.”<sup>73</sup> Indeed, some experiments with early microwave ovens identified areas of overheating within the plasma bag, such as at the junction where the tubing segment connects to the bag.<sup>71</sup> More recent studies with newer microwave thawing machines have not identified overheating during normal function,<sup>74</sup> and no significant effects on coagulation factor levels were observed when thawing solvent-treated plasma with modern thawing microwaves.<sup>75</sup> Radiofrequency-based thawing systems have been developed that use longer wavelengths to achieve a more uniform distribution of energy, thus minimizing temperature gradients within the plasma during rapid warming; at least one study has shown FFP thawed with these systems contains adequate coagulation factor levels.<sup>76,77</sup>

## **At the bedside: transfusion administration**

### **Pretransfusion**

Once a blood product reaches the patient's bedside, the transfusionist and either a second person or an electronic verification system must verify several pertinent pieces of information before the transfusion is initiated.<sup>1,5</sup> Transfusionists must check for a valid order for transfusion and confirm the presence of an appropriately signed consent form. They must positively identify the recipient in their presence using two unique identifiers and confirm the recipient's ABO group and Rh type. Transfusionists must confirm the following documentation on the blood bag label: donation identification number, ABO group and Rh type of the blood unit, crossmatch interpretation, documentation of special transfusion requirements and processing (e.g., irradiation), and the unit expiration to confirm it is in-date. All identification attached to the blood container must remain attached until the transfusion is terminated.<sup>1</sup> After the above steps have been completed, but before the transfusion begins, pretransfusion vital signs should be documented (e.g., temperature, pulse, blood pressure, and oxygen saturation). If the patient experiences any clinical changes during the transfusion, these initial vital signs will serve as baseline values.

### **Blood administration sets and filters**

Once blood components leave the blood bank, transfusion should be initiated as quickly as possible to avoid the risk of bacterial overgrowth.<sup>78</sup> All blood component transfusions should be completed

within four hours of the beginning of the transfusion. Institution-specific criteria should be followed, but standard blood administration infusion sets are required for the infusion of all blood products (except for hematopoietic progenitor cells). These sets consist of three main parts: inline filters, drip chambers, and tubing that can be attached directly to a previously established IV line.

Macroaggregate (standard) inline filters are typically included in the administration sets and usually have a 170–260-μm pore size to remove large fibrin clots and cellular debris from all blood components immediately before they enter the patient.<sup>1,12,79,80</sup> The filters should be used and replaced according to manufacturer recommendations. In addition, microaggregates of cellular debris and fibrin strands were historically found to potentially develop within stored blood and were thought to play a role in the development of acute respiratory distress syndrome (ARDS) despite the lack of an established causal link.<sup>80</sup> Microaggregate filters were used in the past to remove this debris but are no longer routinely used at the bedside. Instead, the majority of blood components undergo leukocyte reduction filtration at the blood center, which more efficiently filters out debris.<sup>81</sup>

The drip chamber is a standard component of the infusion set that allows the transfusionist to control the rate of infusion and importantly serves to avoid infusion of air. Prior to use, the infusion set and tubing can be rinsed or primed with either 0.9% sodium chloride or the component to be transfused.<sup>5</sup>

As previously discussed in this chapter, aliquoting for small-volume infusions in neonates utilizes a special syringe administration set, which typically includes an inline screen filter that filters the component during aliquoting.<sup>5</sup>

### **Coadministration of fluids and blood components**

In general, no IV solutions other than 0.9% normal saline should be coadministered with blood components through the same IV line. According to the AABB Standards for Blood Banks and Transfusion Services, the only compatible fluid is 0.9% normal saline, but allowances are made for drugs or solutions that have been approved by the FDA to be used with blood administration or if there is documentation that shows that coadministration is safe and does not affect the blood or component.<sup>1</sup> Hypertonic (e.g., hypertonic saline) or hypotonic solutions (e.g., dextrose 5% in water) should not be coadministered as they can cause osmotic hemolysis of the transfused blood component. Lactated Ringer's solution and other solutions that contain high calcium concentrations should be avoided as well because they can cause clotting of the blood component by overwhelming the ability of the citrate to effectively anticoagulate the product.<sup>5</sup>

### **Coadministration of medications**

Although this practice has not been extensively studied, in general, medications should not be administered simultaneously through the same IV line as the blood component.<sup>5</sup> The main reason is it would be difficult to differentiate a reaction to the medication from a reaction to the blood component in the setting of simultaneous administration of a blood component and a medication. In addition, if a transfusion needed to be stopped, then the patient may not receive the intended dose of the coadministered medication. Furthermore, some medications may not be compatible with the blood components and may cause hemolysis or clotting. For these reasons, medications should be administered with a second IV line. In the event that additional IV access has not been established, then the IV line used for the blood component should be clamped and

flushed with 0.9% saline before infusing any medications.<sup>12</sup> When blood components must be administered concomitantly, as in the setting of trauma or surgery, this can be accomplished with the use of separate IV lines. Multiple blood components can be sequentially transfused through the same tubing and IV line.<sup>5</sup>

### Patient monitoring

After the transfusion has begun, the patient's vital signs should be evaluated and documented 15 minutes after the start of the transfusion. A slow transfusion rate (e.g., 2 mL per minute) should be used because severe reactions often occur within the first 15 minutes and can be caused by volumes as small as 10 mL.<sup>5</sup> If the patient has any suspected adverse reactions, then the transfusion should be stopped until the patient can be clinically evaluated. Depending on the scenario, the transfusion may need to be discontinued altogether. If a patient has a mild allergic reaction (i.e., hives) without signs of vasomotor instability, laryngeal edema, or tongue or lip swelling, the transfusion usually can be restarted if the symptoms resolve with antihistamine therapy. However, a transfusion should not be restarted if the recipient has a change in blood pressure, fever, chills, an anaphylactoid reaction such as just described, or an increase in temperature (even if there was a preexisting fever). These symptoms should trigger a transfusion reaction workup by the physician responsible for the transfusion, the blood bank, and the blood bank consulting physician.

### Infusion flow rates

Beyond the first 15 minutes of a transfusion, optimal infusion rates vary with the component being transfused, the clinical situation, and the patient's ability to tolerate increased intravascular volume. Components should generally be infused slowly at first, and then the rate can be increased as tolerated by the patient. Patients at risk for volume overload, such as those with poor cardiac status, should receive a slow infusion rate with close monitoring, if feasible. In general, RBCs and plasma have volumes ranging from 200 to 400 mL and are typically infused over 1–2 hours. Platelets range in volume from 200 to 300 mL and are also generally infused over 1–2 hours.<sup>5,37</sup> Cryoprecipitate can be given as rapidly as tolerated and should be infused as soon after the thawing procedure as is clinically acceptable.<sup>5</sup> Transfusions for all blood components should be completed within four hours of the start of transfusion.<sup>35</sup>

There is a paucity of evidence to make specific recommendations regarding blood component flow rates in neonatal and pediatric patients. Although concern exists that increased rates can cause intraventricular hemorrhage or electrolyte imbalances in neonates, there is limited high-quality evidence to support this theory. Therefore, in this patient population, for routine blood component administration, transfusions are usually administered over 2–4 hours.<sup>5</sup> Some authors recommend infusion rates of 5 mL/kg/hour for RBCs and 10–20 mL/kg/hour for platelets, but local and institutional policies should be consulted regarding infusion rates.<sup>82</sup>

In certain clinical scenarios, there can be a need to transfuse RBCs, plasma, or platelets at a slower rate than would allow for the completion of transfusion in a four-hour period. As mentioned in the "Volume reduction via aliquoting" section, one strategy to accomplish this relies on the blood bank's ability to use a sterile tubing welder to split and aliquot units in a sterile manner. These aliquots can be issued to the patient, and the remaining sterile unit can be properly stored in the blood bank. In the case of split units, the transfusionist can administer the split unit over a four-hour period and then contact the blood bank to request the other half of

the unit. This practice allows for a unit of RBCs, plasma, or platelets to be divided and effectively infused over eight hours.

### RBC salvage devices

RBC salvage refers to techniques for reclaiming and processing blood lost in the surgical field for intraoperative autologous transfusion. The blood salvage device takes suctioned blood and separates, concentrates, and washes RBCs to provide a red blood cell suspension that can be reinfused into the patient.<sup>83</sup> Blood is suctioned from the surgical field and mixed with either heparin or citrate to prevent coagulation. The mixture is fed into a storage chamber until sufficient volume is collected for processing, and then it is centrifuged to separate the RBCs and remove all other components. The RBCs are washed to remove free hemoglobin, cellular debris, and anticoagulants, and then passed through a 40- $\mu\text{m}$  filter when they are infused back into the patient. In settings of rapid blood loss, the salvaged blood can be reinfused into the patient without the centrifugation and washing steps. However, the risks of infusing unwashed blood stem from the added anticoagulant, thrombogenic substances, free hemoglobin, and other debris, and thus salvaged RBCs should be washed except for severe emergencies.<sup>84</sup> Salvage devices have been shown by some studies to reduce allogeneic RBC transfusions for both adult and pediatric patients undergoing cardiac surgery.<sup>85,86</sup> Due to the relatively large volume needed to prime and effectively run RBC salvage, these devices are not useful in children under six months of age.<sup>87</sup>

### Rapid infusion practices

Blood products are occasionally requested on an urgent basis when a patient is deemed to have a life-threatening condition that does not allow for sufficient time for a full crossmatch prior to blood component issue. Conspicuously labeled uncrossmatched units can be stored in blood-bank-monitored refrigerators in critical areas throughout the hospital such as the emergency department, operating rooms, labor and delivery suites, and intensive care units. When uncrossmatched units are issued, there must be record of a signed statement from the ordering physician indicating a sufficient clinical requirement for the release of blood components before the completion of compatibility testing.<sup>1</sup> Although specific protocols can vary with institutional and local policies, the following should be followed when uncrossmatched units are released<sup>5</sup>:

- 1 Uncrossmatched type O RBC units should be issued, with preference to giving RhD-negative units to women of childbearing potential, infants, and individuals with a history of anti-D antibodies. Other groups may receive RhD-positive units, particularly if there are inventory limitations.
- 2 ABO- and RhD-compatible units should be issued if results of patient blood typing are available.
- 3 The unit should be labeled conspicuously on the tag or label that compatibility testing was not completed at the time of issue.
- 4 Compatibility testing should be performed and completed as soon as possible. If the uncrossmatched units are found to be incompatible, both the transfusion medical director and the patient's physicians should be alerted immediately.

In the setting of massive transfusion, transfusion services are encouraged to develop standardized protocols that rapidly deliver blood components in a balanced ratio of RBCs to plasma to platelets (discussed in more detail in Chapter 40). Before ABO typing is completed, group O RBCs should be issued. Consideration should be given to using group AB or A plasma. Transfusing group A plasma to patients with an unknown ABO type in trauma settings

has been demonstrated to be safe.<sup>88</sup> When RhD-negative trauma patients receive RhD-positive blood components, they are at risk of alloimmunization to the D antigen, and so policies should be developed to identify when RhD-positive RBCs can be issued (e.g., to all adult males and females with no childbearing potential).<sup>5,89</sup> However, if the patient's ABO and Rh type are known, then the patient should reasonably receive type-specific products rather than defaulting to universally compatible products.

In urgent circumstances, blood components often need to be transfused as quickly as possible, and large-bore intravenous catheters can be used for this purpose. When blood losses are substantial, blood infusers can be utilized for rapid transfusion. Rapid infusers can deliver warmed blood components at rates as fast as 500–1500 mL per minute through a pump, which is far faster than the rate that a standard infusion can be delivered.<sup>90</sup> Given this rapid rate of infusion, it is essential that blood components are warmed in order to prevent hypothermia-induced coagulopathy.<sup>91</sup> Rapid infusers are frequently credited with providing lifesaving delivery of blood, but care should be taken to avoid air emboli.<sup>92</sup> Some models of infusion pumps have improved safety features that automatically stop transfusion when air emboli are detected.<sup>93</sup>

### Blood warming and bedside blood pumps

Stand-alone blood warmers may be utilized when large volumes of blood components are transfused (without a rapid infuser) or when patients have cold agglutinins detected during compatibility testing. Only validated, FDA-approved blood warmers should be used for this purpose to ensure patient safety. The AABB Standards for Blood Banks and Transfusion Services require that warmers have a temperature-sensing device and a system that will warn about malfunctions and will prevent hemolysis to the blood component.<sup>1</sup> Blood warmers often consist of tubing coiled around a central heating core that is temperature-monitored. Blood must never be warmed by holding the unit in a hot water stream under a faucet or by heating it in a standard microwave oven because these methods can cause clinically significant hemolysis. Monitored blood

warmers are frequently used during lengthy apheresis procedures to promote patient comfort. Importantly, nonwarmed blood has been historically shown to lower the core temperature of transfusion recipients, possibly contributing to cardiac arrest and hypothermia.<sup>94</sup> Blood warmers must be maintained regularly to prevent malfunction and possible hemolysis. Blood can also be administered through medication infusion pumps if they have been shown not to cause shear-stress-induced hemolysis. These devices are often used for neonatal or pediatric transfusions when precise infusion volumes are needed. Not all pumps are compatible with red cell infusions, so each device model should be evaluated to ensure red cell compatibility.

### Post-transfusion

At the completion of the transfusion, the patient should be checked again for any signs of an adverse reaction to the blood component, and a final set of vital signs should be taken. Because transfusion reactions can occur several hours after the completion of the transfusion, inpatients should be monitored for at least 4–6 hours after the infusion is completed. If a patient will be discharged from an ambulatory care setting after receiving a transfusion, then written documentation of signs and symptoms of transfusion reactions and a telephone number of a responsible practitioner should be provided to the patient prior to leaving the clinic.<sup>5</sup>

### Key references

- A full reference list for this chapter is available at: [www.wiley.com/go/simon/Rossi6](http://www.wiley.com/go/simon/Rossi6)
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## CHAPTER 29

# Anemia and red blood cell transfusion

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## Introduction

Red cell transfusion is an extremely common medical intervention. In the United States in 2019, 11,590,000 RBC units were collected and 10,852,000 RBC units were transfused, a 2.5% increase from 2017.<sup>1</sup> Worldwide, 118.5 million units of blood are collected per year.<sup>2</sup> In Northern England, about 50% of units are given to medical patients and 40% to surgical patients; hip replacement and coronary artery bypass graft (CABG) procedures were the most common surgical indications.<sup>3</sup> RBC units are also frequently administered to critically ill patients, as a supportive therapy to patients receiving chemotherapy and marrow transplants, and to patients with blood loss from medical conditions such as gastrointestinal bleeding.<sup>4</sup> Approximately 25% of all red cells transfused are given to patients with a primary diagnosis of cardiac disease,<sup>5</sup> and 8% of all cardiology admissions are transfused RBCs.<sup>6</sup> In recent years, blood use has declined as clinicians adopt a more restrictive approach to transfusion.<sup>7</sup> Estimates of the cost of an RBC unit in different health systems range from \$522 to \$1183 (mean: \$761 ± \$294).<sup>8</sup>

This chapter reviews the current knowledge about red cell transfusion, their risks and benefits, as well as the risk posed by anemia, all in the hopes of providing the reader an evidence-informed approach to understanding at what hemoglobin concentration a transfusion will mitigate the effects of anemia and result in improved outcomes. In order to provide a more comprehensive overview, sections on physiological effects of transfusion and anemia give readers an indication of changes related to anemia and its treatment. This overview also summarizes observational studies. Clinical recommendations in this chapter are largely based on randomized clinical trials identified and synthesized in systematic reviews. Clinical guidelines on transfusion practices also guided some recommendations. We do our best throughout the chapter to point out where evidence or guidelines are strong. Importantly, we also emphasize where data are much weaker leading to recommendations primarily based on our experience.

## Adaptive mechanisms in anemia

In anemia, oxygen-carrying capacity decreases, but tissue oxygenation is preserved at hemoglobin levels well below 10 g/dL. After the development of anemia, adaptive changes include a shift in the

oxyhemoglobin dissociation curve, hemodynamic alterations, and microcirculatory alterations (Table 29.1). The shift to the right of the oxyhemoglobin dissociation curve in anemia is primarily the result of increased synthesis of 2,3-diphosphoglycerate (2,3-DPG) in red cells.<sup>9–11</sup> This rightward shift enables more oxygen to be released to the tissues at a given PO<sub>2</sub>, offsetting the effect of reduced oxygen-carrying capacity of the blood. *in vitro* studies have shown rightward shifts in the oxyhemoglobin dissociation curve with increases in temperature and decreases in pH.<sup>12</sup> Although clinically important shifts have been documented in a number of studies, hemoglobin oxygen saturation generally is measured in arterial specimens processed at standard temperature and pH. Therefore, current measurement techniques do not reflect oxygen-binding affinity or unloading conditions in the patient's microcirculatory environment, which may be affected by temperature, pH, and a number of disease processes. The shift in the oxyhemoglobin dissociation curve caused by decreases in pH (increase in hydrogen ion concentration) is termed the Bohr effect.<sup>13</sup> Because changes in pH rapidly affect the ability of hemoglobin to bind oxygen, this mechanism has been postulated to be an important early adaptive response to anemia.<sup>14</sup> However, the equations describing the physical process indicate that a very large change in pH is needed to modify the partial pressure of oxygen at which hemoglobin is 50% saturated with oxygen ( $P_{50}$ ) by a clinically important amount (~10 mmHg). As a result, the Bohr effect is unlikely to have significant clinical consequences.<sup>12</sup>

Several hemodynamic alterations occur after the development of anemia. The most important determinant of cardiovascular response is the patient's volume status or more specifically left ventricular preload. The combined effect of hypovolemia and anemia often occurs because of acute blood loss. Acute anemia can cause tissue hypoxia or anoxia through both diminished cardiac output (stagnant hypoxia) and decreased oxygen-carrying capacity (anemic hypoxia).<sup>15</sup> The body primarily attempts to preserve oxygen delivery to vital organs by compensatory increases in myocardial contractility and heart rate as well as increased arterial and venous vascular tone mediated through increased sympathetic discharge. In addition, a variety of mechanisms redistribute organ blood flow. The adrenergic system plays an important role in redistributing blood flow between and within specific organs; increased sympathetic tone diverts an

**Table 29.1** Physiologic Changes Associated with Normovolemic Anemia**Oxyhemoglobin Dissociation Curve**

- Oxyhemoglobin curve shifted to the right because of increased 2,3-diphosphoglycerate levels.
- Rightward shift in the oxyhemoglobin curve is not a result of the Bohr effect in patients.
- The shift in the oxyhemoglobin curve has been clearly established in many forms of anemia (excluding hemoglobinopathies).
- Shift in the oxyhemoglobin curve has been clearly established in a number of human diseases.

**Hemodynamic Alteration**

- Changes in blood viscosity result in many of the hemodynamic changes.
- Increased sympathetic activity resulting in increased heart rate and contractility.
- Increased stroke volume more than vascular resistance
- Redistribution of cardiac output toward the heart and brain and away from the splanchnic circulation.
- Systemic oxygen delivery is optimized at hemoglobin values of 10–11 g/dL.
- Global oxygen delivery declines above and below hemoglobin values of 10–16 g/dL.
- Microvascular hematocrit and regulation of RBC distribution is loosely correlated with systemic hematocrit over wide range of values

**Cardiac Output**

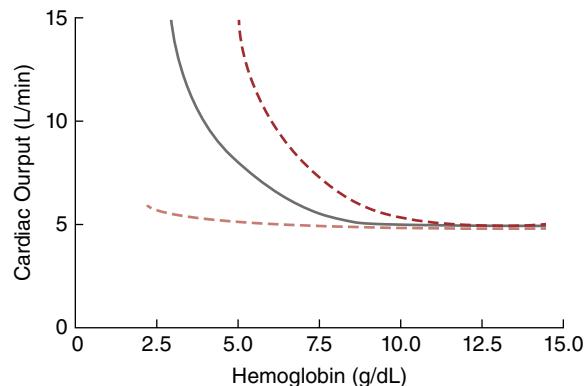
- Cardiac output increases with increasing degrees of anemia.
- Increased cardiac output is a result of increased stroke volume.
- The contribution of increased heart rate to the increase in cardiac output is variable.

**Coronary and Cerebral Blood Flow**

- Coronary and cerebral blood flow is increased.
- Coronary artery disease in the presence of moderate degrees of anemia (hemoglobin values below 9 g/dL) results in impaired left ventricular contractility or ischemia.
- Moderate anemia does not aggravate cerebral ischemia in patients with cerebrovascular disease.

ever-decreasing global blood flow (cardiac output) away from the splanchnic, skeletal, and cutaneous circulation toward the coronary and cerebral circulation. The renin–angiotensin–aldosterone system is stimulated to retain both water and sodium. Losses ranging from 5% to 15% in blood volume result in variable increases in resting heart rate and diastolic blood pressure. Orthostatic hypotension often is a sensitive indicator of relatively small losses in blood volume not sufficient to cause a marked decrease in blood pressure.<sup>16</sup> Larger losses result in progressive increases in heart rate and decreases in arterial blood pressure accompanied by evidence of organ hypoperfusion. Once vital organ systems such as the kidneys, the central nervous system, and the heart are affected, the patient is considered in hypovolemic shock. Although the American College of Surgeons Committee on Trauma<sup>17</sup> has categorized the cardiovascular and systemic response to acute blood loss according to degree of blood loss, many of these responses are modified by the rapidity of blood loss and patient characteristics, such as age, coexisting illnesses, preexisting volume status, hemoglobin value, and the use of medications having cardiac effects ( $\beta$ -blockers) or peripheral vascular effects (antihypertensives).

The compensatory changes in cardiac output most thoroughly studied are the cardiovascular consequence of normovolemic anemia. When intravascular volume is stable or high after the development of anemia (as opposed to hypovolemic anemia and shock), increases in cardiac output have been consistently reported. Indeed, an inverse relationship between hemoglobin level (or hematocrit) and cardiac output has been clearly established in well-controlled laboratory studies (Figure 29.1).<sup>18–20</sup> Similar clinical observations have been made in the perioperative setting<sup>21</sup> and for chronic anemia.<sup>18</sup> Unfortunately, the strength of inferences from clinical studies is limited by confounding factors arising from major coexisting illnesses such as cardiac disease, a lack of appropriate control patients,



**Figure 29.1** The theoretic effect of hemoglobin concentration on cardiac output. The curves illustrate how cardiac output increases as hemoglobin concentration decreases. The solid curve is meant to describe the increase in a healthy adult. The dashed line on the top shows how the cardiac output response can be accentuated in a young athlete, and the lower dashed line might correspond to someone with poor cardiovascular function.

and significant weaknesses in study design. Researchers have attempted to determine the level of anemia at which cardiac output begins to rise. Reported thresholds for this phenomenon identified in primary clinical and laboratory studies have ranged from 7 to 12 g/dL of hemoglobin.<sup>18</sup> Two major mechanisms are thought to be responsible for the physiologic processes underlying increased cardiac output during normovolemic anemia:<sup>1</sup> reduced blood viscosity and<sup>2</sup> increased sympathetic stimulation of cardiac effectors.<sup>22</sup> Blood viscosity inversely affects both preload and afterload, two major determinants of cardiac output,<sup>22–24</sup> whereas sympathetic stimulation primarily increases the two other determinants, heart rate and contractility. Unlike the situation for hypovolemic anemia, in this setting the effects of blood viscosity appear to predominate.<sup>23,24</sup>

Complex interactions exist among blood flow, blood viscosity, and cardiac output. In vessels, blood flow alters whole blood viscosity, and blood viscosity modulates cardiac output. Under experimental conditions in a rigid hollow cylinder, blood flow is directly related to the fourth power of the diameter and to driving pressure. It is inversely related to the length of the vessel and to blood viscosity (Poiseuille–Hagen law).<sup>22,25</sup> If cardiac function is normal, the increase in venous return or left ventricular preload is the most important determinant of increased cardiac output during normovolemic anemia. The conclusion is based on experiments in which viscosity was maintained during anemia by means of high-viscosity colloidal solutions. In such studies, the cardiovascular effects of hemodilution were attenuated<sup>23</sup> compared with similar levels of hemodilution accompanied by reduced whole blood viscosity. Decreased left ventricular afterload, another cardiac consequence of decreased blood viscosity, may also be an important mechanism for the increase in cardiac output as anemia worsens.<sup>23</sup>

Sympathetic stimulation can result in increased cardiac output through enhanced myocardial contractility<sup>26</sup> and increased venomotor tone.<sup>27,28</sup> The effects of anemia on left ventricular contractility in isolation have not been clearly determined, given the complex changes in preload, afterload, and heart rate. Only one before and after hemodilution study was performed with load-independent measures to document increased left ventricular contractility.<sup>26</sup> Chaper and Cain<sup>28</sup> summarized several well-controlled animal studies indicating that venomotor tone increases as a result of stimulation of the aortic chemoreceptors. If sympathetic stimulation is

significant in the specific clinical setting, contractility is increased from the stimulation of the  $\beta$ -adrenergic receptors.<sup>22,29</sup>

Fick's law states that global oxygen delivery is related to the product of cardiac output and blood oxygen content (overwhelmingly determined by hemoglobin). Yet, the inverse relationship between cardiac output and hemoglobin level (via changes in blood viscosity) has led investigators to attempt to determine the hemoglobin level that maximizes oxygen transport within the systemic circulation. Richardson and Guyton<sup>30</sup> evaluated the effects of hematocrit on cardiac performance in a canine model. They established that the hematocrit range that optimized cardiac efficiency and global oxygen transport was 40–60%. Others determined maximum oxygen delivery to be in the lower end of the range, at a hematocrit of 40–45% (hemoglobin 13–15 g/dL).<sup>31,32</sup> However, in one of the most widely quoted studies addressing this topic,<sup>33</sup> investigators found that peak oxygen transport as determined by the Fick equation occurs at a hematocrit of 30% (hemoglobin concentration, 10 g/dL). Unfortunately, global indices of oxygen delivery mask any differences in blood flow between specific organs<sup>34,35</sup> and do not reflect the net impact on microvascular oxygen delivery (see below). In addition, these studies attempted to identify a single optimal hemoglobin concentration that maximizes oxygen delivery in the equivalent of young healthy patients with anemia, an approach that neglects the large number of factors interfering with adaptive mechanisms during the management of patients with disease.

Will the transfusion of allogeneic RBCs reverse any adaptive response to acute or chronic normovolemic anemia? Assuming that oxygen-carrying capacity is not impaired during storage and that hematocrit is restored after a transfusion, the cardiovascular consequences should be reversed. However, the storage process alters the properties of red cells. These alterations may impair flow and oxygen release from hemoglobin.<sup>9</sup>

## **Microcirculatory effects of anemia and red cell transfusion**

In the systemic circulation, blood is considered a homogenous medium operating under the classical laws of fluid dynamics. By contrast, blood flow in the microcirculation—defined as arterioles, capillaries, and venules with diameter less than 300  $\mu\text{m}$ <sup>36</sup>—is governed by unique biophysical properties that impact oxygen delivery.<sup>37</sup> The reason for these discrepancies is the *two-phase nature* of blood in the microcirculation, whereby RBC and plasma flow are treated as distinct components. In microvessels, RBCs migrate away from the vessel wall, causing differential flow velocities and unequal partition of RBC and plasma at microvascular bifurcations.<sup>38</sup>

Most strikingly, these properties synergize within large microvascular networks to result in a marked reduction in hematocrit within microvessels of decreasing diameter. These phenomena partly explain why a significant proportion of microvascular hematocrits are less than 20% under normal conditions and can even be as low as 5% within capillary networks.<sup>39,40</sup> This reduced hematocrit, in combination with cell–cell interactions, also causes significant reduction in microvascular blood viscosity—well below the systemic circulation—that counteracts the impact of reduced flow velocities in the microcirculation.<sup>37</sup>

Furthermore, if the ultimate goal of RBC transfusion is to increase tissue oxygen delivery, attention then logically turns to the physiology of RBC distribution in capillary networks as the terminal site of oxygen exchange. As mentioned above, capillaries have the lowest hematocrits in the microcirculation, but also the most functional

variability in hematocrits, RBC velocity, and RBC supply rate.<sup>41</sup> While Krogh<sup>42</sup> originally proposed that capillary recruitment (i.e., the number of flowing capillaries) increases to support oxygen delivery, it has now been recognized that the overwhelming majority of capillaries are perfused at rest.<sup>43</sup> Increased oxygen demand is therefore supported by increasing RBC flux through capillaries that are already flowing, as well as increasing oxygen extraction.

The degree of anemia, the specific tissue bed, and a variety of disease processes affect microcirculatory blood flow and oxygen supply.<sup>25,44</sup> Animal studies of acute normovolemic hemodilution illustrate the ability of the microcirculation to dynamically adapt to changes in systemic hematocrit. In the cerebral circulation, capillary hematocrit was maintained even when the systemic hematocrit was diluted to 15%, and RBC velocity and supply rate increased.<sup>45</sup> In the mesentery<sup>46</sup> and skeletal muscle,<sup>47</sup> hemodilution resulted in minor reductions in capillary hematocrit (much less than the systemic drop), and these were offset by increases to RBC supply rate to maintain oxygen delivery. Moreover, the ratio of microvascular hematocrit to systemic hematocrit increased, suggesting that upstream arterioles adjusted their tone throughout the vascular network to accommodate the drop in systemic hematocrit. Taken together, these studies highlight two important physiological concepts to consider: (a) the relationship between systemic and microvascular hematocrit is nonlinear and (b) microvascular hematocrit is actively regulated to ensure adequate oxygen delivery. In the setting of microvascular injury (e.g., sepsis, diabetes), the ability to regulate hematocrit may be disrupted, which further weakens the ability of systemic transfusion to influence tissue oxygen delivery. These concepts may partly explain why the modulation of systemic hematocrit via RBC transfusion has not consistently improved clinical outcomes (see the below sections).

Stored red cells have properties that differ from those of their *in vivo* counterparts; many are related to the duration of storage (see Chapter 14). Characteristically, older RBC units have lower levels of 2,3-DPG. The result is a leftward shift in the oxyhemoglobin dissociation curve, which can impede delivery of oxygen to the tissues.<sup>14</sup> In addition, the storage of red cells decreases the deformability of their membrane.<sup>48</sup> As a consequence, stored red cells may become so rigid as to impede flow in the microcirculation<sup>49</sup> and may have limited ability to release oxygen to tissues. However, these storage lesions are reversible within 24–48 hours after transfusion.<sup>50</sup> There are also reports<sup>51</sup> suggesting that disease processes such as sepsis impair red cell deformability. In conjunction with significant systemic microcirculatory dysfunction, the decrease in red cell deformability may dramatically affect tissue oxygen delivery in sepsis and septic shock.<sup>51,52</sup> However, despite potential laboratory concerns, clinical trials have not demonstrated increased harms related to the use of standard issue red cells, averaging 20 days of storage.<sup>53</sup> Similarly, a large randomized controlled trial transfusing the shortest storage red cells did not show any benefit for clinical outcomes.<sup>54</sup> However, there are no clinical trials comparing red cells stored more than 35 days to standard issue red cells to better understand the consequences of red cells stored for prolonged intervals.

Bedside tools to assess microvascular oxygenation have been evaluated in the setting of RBC transfusion. The two most common modalities being used are near-infrared spectroscopy (NIRS) of the brain and skeletal muscle, and handheld vital microscopy (HVM) of the sublingual microcirculation. Generally, studies confirm the theory of dissociation between the microcirculation and systemic hemoglobin levels. In a small study of traumatic brain injury patients, NIRS was unable to detect a difference in cerebral oxygen

saturation despite an appropriate increase in systemic hemoglobin post-transfusion.<sup>55</sup> In other studies, when critically ill patients receiving RBC transfusion were monitored with both HVM and NIRS,<sup>56,57</sup> the change in systemic hemoglobin could not reliably predict microvascular response to transfusion. A systematic review<sup>58</sup> also found a lack of microvascular response to RBC transfusion but suggested that patients with poor microvascular indices at baseline may derive more benefit; these findings should be interpreted with caution given the heterogeneity and methodology of studies. Overall, more research is required to determine whether microvascular monitoring can guide RBC transfusions in select patient populations.

### Interaction between pathophysiologic processes and anemia

Several disease processes affecting either the entire body or specific organs potentially limit adaptive responses and make patients more vulnerable to the effects of anemia. Specifically, heart, lung, and cerebrovascular diseases have been proposed to increase the risk of adverse consequences of anemia. Age, severity of illness, and therapeutic interventions may also affect adaptive mechanisms.

The heart, specifically the left ventricle, is particularly prone to adverse consequences of anemia. This is because the myocardium consumes 60–75% (extraction ratio) of all oxygen delivered by the coronary circulation.<sup>31,34,59</sup> Such a high extraction ratio is unique to the coronary circulation. As a result, oxygen delivery to the myocardium can increase substantially only with an increase in blood flow.<sup>60</sup> In addition, most left ventricular perfusion is restricted to the diastolic period because pressures inside the left ventricle are too high to allow adequate coronary blood flow during systole. Thus, any shortening of its duration (e.g., tachycardia) decreases blood flow. Laboratory studies have been performed to investigate the effects of normovolemic anemia on the coronary circulation.<sup>32,59,61</sup> There appear to be minimal consequences of anemia with hemoglobin levels in the range of 7 g/dL if the coronary circulation is normal.<sup>19,26</sup> However, myocardial dysfunction and ischemia either occur earlier or are greater in anemic animal models with moderate- to high-grade coronary stenosis compared with controls with normal hemoglobin values.<sup>62</sup>

Unfortunately, data from studies with human participants are inconsistent on these points. Several clinical studies involving patients with coronary artery disease undergoing normovolemic hemodilution have not shown any increase in cardiac complications or silent ischemia during electrocardiographic monitoring.<sup>63</sup> In addition, a retrospective analysis involving 224 patients undergoing CABG surgery did not show a significant association between the hemoglobin level and coronary sinus lactate level (an indicator of myocardial ischemia).<sup>64</sup> However, in two recent cohort studies, moderate anemia was poorly tolerated by perioperative<sup>65</sup> and critically ill patients<sup>66</sup> with cardiovascular disease. Thus, retrospective studies seem to support preclinical reports. It is also plausible that anemia results in considerable increases in morbidity and mortality among patients with other cardiac diseases, including heart failure and valvular heart disease, presumably because of the greater burden of the adaptive increase in cardiac output.

During normovolemic anemia, cerebral blood flow increases as hemoglobin values decrease. Investigators have observed increases ranging from 50% to 500% of baseline value in laboratory studies<sup>67</sup> and in one study with human subjects.<sup>68</sup> Cerebral blood flow increases because of overall increases in cardiac output, which is

preferentially diverted to the cerebral circulation. As oxygen delivery begins to decrease, cerebral tissues can increase the amount of oxygen extracted from blood. A number of factors, including degree of hemodilution, type of fluid used for volume expansion, volume status (preload), and extent of cerebrovascular disease, are capable of potentially modifying global or regional cerebral blood flow during anemia.<sup>69</sup> The increase in global cerebral blood flow combined with the potential for improved flow characteristics across areas of vascular stenosis (improved rheologic properties of blood because of decreased viscosity) prompted a number of laboratory and clinical<sup>69–72</sup> studies to investigate the use of hemodilution as therapy for acute ischemic stroke.<sup>70–72</sup>

The results of laboratory studies suggest that moderate degrees of anemia alone should rarely result in or worsen cerebral ischemia. However, none of the randomized clinical trials demonstrated that hemodilution in acute ischemic stroke improved clinical outcomes in patients.<sup>73</sup> Because of the variety of variables that affect clinical outcomes, the negative findings may not fully rule out the possibility that hemodilution offers therapeutic benefit. Thus, the currently available evidence indicates that cerebrovascular disease does not appear to predispose patients to serious morbidity from anemia.

Changes in oxygen delivery to the brain during normovolemic anemia (either increases or decreases in blood flow) do not uniformly affect various cerebral pathologic conditions. For example, patients with high intracranial pressure from traumatic brain injury may be adversely affected by increased cerebral blood flow. On the other hand, after subarachnoid hemorrhage, mild degrees of normovolemic or hypervolemic anemia may improve overall oxygen delivery, possibly by overcoming the effects of cerebral vasospasm and thereby improving cerebral blood flow through decreased viscosity.<sup>74</sup> However, the effects of moderate to severe anemia in subarachnoid hemorrhage have not been assessed in laboratory or clinical studies.

One of the major consequences of redistributing some of the available cardiac output toward the coronary and cerebral circulation during normovolemic anemia is the shunting of flow away from other organs, including the kidneys and intestines. Critically ill patients may be adversely affected by this redistribution,<sup>75</sup> which could result in increased intestinal ischemia, bacterial translocation, and multiple-system organ failure.<sup>76</sup> Critical illness can also tax many of the body's adaptive responses. Specifically, cardiac performance may be impaired<sup>77</sup> or may already be at maximal capacity in response to increased metabolic demands. Pathologic processes affecting the microcirculation, particularly prevalent among critically ill patients, may also affect the patient's response to anemia and transfusions.

### Clinical outcomes of anemia and red cell transfusion

The decision to administer RBC units must weigh the risks of blood transfusion (see Chapters 43–51 for risks of transfusion), the ongoing risk of uncorrected anemia, and the level of anemia at which blood transfusion is expected to result in clinical benefits that outweigh the risks of associated adverse outcomes.

#### Risk of anemia

##### Preclinical laboratory studies

The critical hemoglobin threshold is similar in different animals.<sup>78</sup> Results of studies suggest that healthy animals can tolerate hemoglobin levels as low as 3–5 g/dL after normovolemic hemodilution.

Electrocardiographic changes consistent with ischemia occur at hemoglobin levels less than 5 g/dL, whereas lactate production, depressed ventricular function, and death have occurred at hemoglobin levels of 3 g/dL or less. While some animals survive with hemoglobin levels as low as 1–2 g/dL,<sup>79</sup> studies suggest a decreased ability to tolerate anemia in the presence of cardiac disease. In dogs with experimentally induced coronary stenosis varying from 50% to 80%, ST-segment changes or locally depressed cardiac function occurred at hemoglobin levels in the range of 7–10 g/dL.<sup>80,81</sup>

### Human studies

Studies involving patients who refuse blood transfusion for religious reasons provide critical insight into the effect of anemia on humans. The largest study was performed with 1958 adult surgical patients who refused transfusion for religious reasons.<sup>65</sup> The mortality was greatest among patients with the lowest preoperative hemoglobin concentrations. Among patients with underlying cardiovascular disease, the risk of death was markedly greater than for patients without cardiovascular disease, especially in those patients with a hemoglobin concentration less than 10 g/dL. Among patients without underlying cardiovascular disease, the difference in mortality at hemoglobin levels greater than or less than 10 g/dL was not as great (Figure 29.2). These results, as well as data on animals and physiologic data, suggest that anemia is not tolerated as well in the presence of cardiovascular disease.

Two studies in patients who declined blood transfusion for religious reasons evaluated the mortality and morbidity associated with nadir postoperative hemoglobin concentrations less than 8 g/dL (Table 29.2).<sup>82,83</sup> Mortality was very low (0.9%) in patients with hemoglobin concentrations between 7 and 8 g/dL but steadily rose with lower hemoglobin levels. The risk of death increased sharply below a hemoglobin concentration of 5 g/dL.

In a series of studies, the effect of anemia was evaluated among healthy volunteers who underwent isovolemic reduction of hemoglobin level to 5 g/dL. Transient and asymptomatic electrocardiographic changes were found in 5 of the 87 volunteers included in two studies.<sup>86,87</sup> These changes occurred when the hemoglobin level was between 5 and 7 g/dL and in patients with faster heart rates.<sup>87</sup> Changes in critical oxygen delivery were not measured. Subtle but reversible changes in cognition were identified in nine volunteers

younger than 35 years at a hemoglobin level between 5 and 7 g/dL.<sup>88</sup> Self-rated fatigue was found in eight volunteers when the hemoglobin level decreased to 7 g/dL. Fatigue increased as hemoglobin levels decreased to 5 g/dL.<sup>89</sup> The results of these studies suggest that important clinical effects can be detected in young, healthy humans with hemoglobin levels between 5 and 7 g/dL.

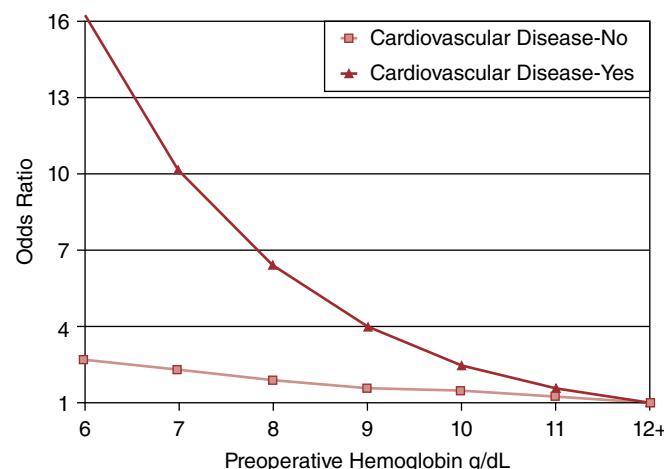
It is uncertain how these results apply to older patients with comorbid factors who are also under stress from surgery or acute illness. An analysis of 31,000 patients 65 years of age or older undergoing major noncardiac surgery examined the association between preoperative hematocrit and mortality or cardiac morbidity (cardiac arrest or Q-wave myocardial infarction).<sup>90</sup> Mortality rose when the hematocrit was less than 36%, and cardiac events were more frequent when the hematocrit was less than 39%. These results, in contrast to experimental data, suggest that mild anemia is associated with increased mortality and morbidity. However, anemia may be only a marker of underlying disease, and studies are needed to demonstrate improved outcome if anemia is corrected by transfusion.

### Clinical trials establishing overall risks and benefits of various transfusion strategies

In this section, we describe the many clinical trials that compare two transfusion strategies where one group received red cells at a higher hemoglobin concentration while another group was transfused at a lower hemoglobin concentration. In doing so, we can determine how best to administer red cells because such studies allow for an unbiased comparison of all clinically important risks of anemia, risks of transfusion, and the benefits of transfusion. We first review overall estimates of treatment effects by aggregating evidence from all trials and then examine clinically important subgroups to better understand if effects are consistent even when the benefits and consequences may vary.

### Meta-analyses

Through January 2021, there have been 48 published randomized clinical trials enrolling 21,433 patients that have contrasted the effects of different transfusion thresholds.<sup>91–137</sup> The clinical settings varied, although each trial randomly assigned patients to transfusion based on a restrictive (less transfusion) or a liberal strategy (more transfusion). In general, the restrictive triggers (specified hemoglobin



**Figure 29.2** Association between the preoperative hemoglobin level and mortality among patients with and without cardiovascular disease.<sup>47</sup> In a population of patients who refused blood transfusion, the risk of death was higher among patients with cardiovascular disease (top line) than among patients without cardiovascular disease (bottom line) for each preoperative hemoglobin level. (Source: Modified from Carson *et al.* (1996).<sup>47</sup>)

**Table 29.2** Unadjusted Rate of Death by 1 g/dL Decrement of the Nadir Postoperative Hb Level in the Two Series of Bloodless Surgery Patients<sup>83,84</sup>

Postoperative Hemoglobin	Carson et al. (2002) <sup>84</sup> N = 300		Shander et al. (2014) <sup>85</sup> N = 293		Totals N = 593	
	N	Mortality N (%)	N	Mortality N (%)	N	Mortality N (%)
1.1–2.0	7	7 (100%)	0	—	7	7 (100%)
2.1–3.0	24	13 (54.2%)	6	3 (50%)	30	16 (53.3%)
3.1–4.0	28	7 (25.0%)	16	3 (18.8%)	44	10 (22.7%)
4.1–5.0	32	11 (34.4%)	25	6 (19.4%)	57	17 (29.8%)
5.1–6.0	54	5 (9.3%)	49	7 (14.3%)	103	12 (11.7%)
6.1–7.0	56	5 (8.9%)	58	3 (5.2%)	114	8 (7.0%)
7.1–8.0	99	0 (0%)	133	2 (1.5%)	232	2 (0.9%)

concentrations that had to be attained) ranged from 7 to 9 g/dL, and liberal transfusion triggers ranged from 9 to 10 g/dL. Additionally, other transfusion strategies were used as well, and there was overlap between the liberal and restrictive transfusion groups in these trials. Overall, compared with liberal transfusion, patients in the restrictive transfusion group received 41% less transfusions, 1.21 fewer units of blood, and had a lower mean hemoglobin concentration of 1.28 g/dL.<sup>138</sup>

Importantly, the results of the meta-analysis found no clinical benefits or harm attributed to either a restrictive or liberal transfusion strategy. The primary outcome was designated as 30-day mortality as it is a clinically important end point and was adopted in the largest and highest quality trials. In the 30 trials that evaluated 30-day mortality in 16,729 participants, there was no difference between the restrictive versus liberal transfusion groups; relative risk = 0.99; 95% confidence interval [CI] 0.86–1.15 (Figure 29.3).

Other important clinical outcomes were assessed and reported in the meta-analysis.<sup>138</sup> None of the morbidity outcomes found significant differences between liberal and restrictive transfusion strategies (Table 29.3).

### Clinical settings of special interest

Transfusion thresholds could have differential effects in alternative clinical settings. As a consequence, clinicians often consider individualizing transfusion decisions in selected patients. Specific populations where risk–benefit ratios may not be the same as other patient populations have included cardiovascular diseases such as myocardial infarction and cardiac surgery. Other studies have been undertaken in orthopedic surgery and vascular surgery patient populations. A few studies have examined transfusion strategies following acute blood loss including from gastrointestinal (GI) bleeding. There have also been additional studies in subpopulations of critically ill patients including septic shock and various forms of brain injury.

### Critical care

The Transfusion Requirements in Critical Care (TRICC) trial was the first well-powered trial to evaluate clinically important outcomes associated with selected transfusion triggers.<sup>94,98</sup> In the main study, the investigators randomly assigned 838 volume-resuscitated ICU patients to a restrictive strategy in which patients received allogeneic RBC transfusions at hemoglobin levels of 7 g/dL (and were maintained between 7 and 9 g/dL) or to a liberal strategy of receiving RBCs at 10 g/dL (and were maintained between 10 and 12 g/dL). Average hemoglobin levels (8.5 g/dL vs. 10.7 g/dL) and RBC units transfused (2.6 units vs. 5.6 units) were significantly lower in the restrictive as opposed to the liberal group, respectively. The 30-day mortality was slightly lower in the restrictive transfusion group

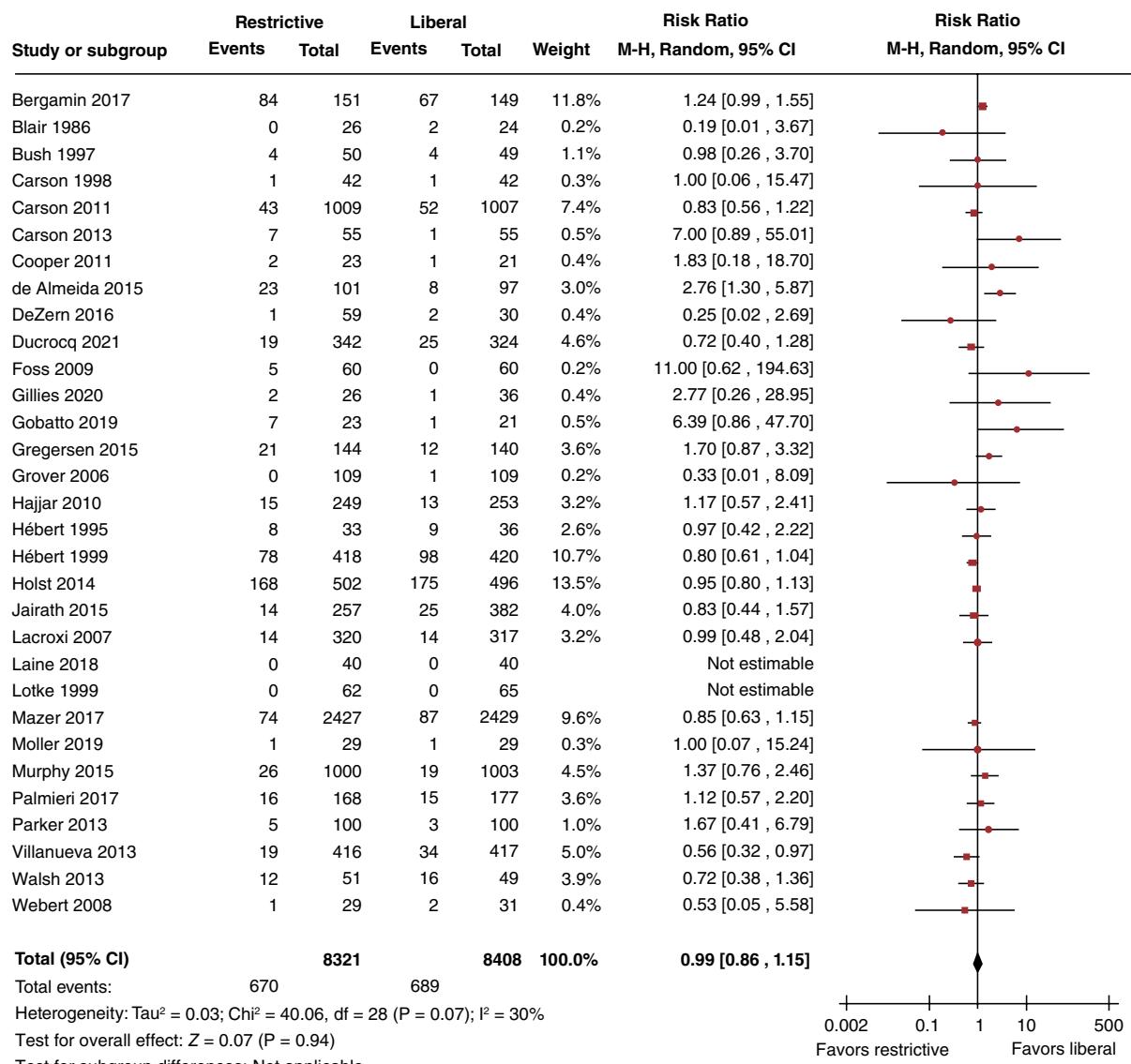
(18.7% vs. 23.3%), although the finding was not statistically significant ( $p = 0.11$ ). Mortality was lower in the restrictive transfusion group in patients less than 55 years of age ( $p < 0.02$ ) and less ill patients defined by APACHE II scores less than 20 ( $p < 0.02$ ). Furthermore, the restrictive transfusion group had fewer patients with myocardial infarction (0.07% vs. 2.9%;  $p = 0.02$ ) and congestive heart failure (5.3% vs. 10.7%;  $p < 0.01$ ).

Following the TRICC trial, there were ongoing concerns in the critical care community regarding the safety of a restrictive strategy in patients with septic shock. This is because disease processes such as overwhelming infections activate inflammatory processes that cause severe microcirculatory abnormalities, resulting in end-organ damage and ongoing oxygen debts as evidenced by high serum lactate concentrations. To address this issue, 1005 patients with septic shock and a hemoglobin level less than 9 g/dL were enrolled in a trial comparing a higher transfusion threshold (9 g/dL) to lower transfusion threshold (7 g/dL).<sup>139</sup> The median number of units transfused in the lower threshold group was 1, and in the higher transfusion group it was 4. Ninety-day mortality occurred in 43% of the lower transfusion group and 45.0% of the higher threshold group (relative risk, 0.94; 95% CI 0.78–1.09;  $p = 0.44$ ). There were no significant differences in secondary outcomes. Overall, ischemic events including myocardial infarctions and stroke, as well as circulatory overload, were not different between the study arms. Similarly, secondary outcomes related to end organ failure and organ support such as mechanical ventilation and renal replacement therapy were no different between groups.

Seven other studies contributed a total of 3529 patients included in trials conducted in critical care settings.<sup>101,113,121,124,127,130</sup> The overall relative risk comparing a restrictive to liberal transfusion strategy was 1.06, 95% CI 0.85–1.32.

### Cardiac surgery

Five transfusion trigger trials have been conducted involving 7869 cardiac surgical patients. This is a patient population where anemia and transfusions are very common, with unique clinical features that may affect the risk–benefit ratio of transfusions. Specifically, as described in the section on physiology, the cardiac muscle is supply dependent, that is, more than 70% of the oxygen delivered is consumed as compared to 30–40% in other tissues. Thus, factors that reduce blood flow (severe coronary artery disease) or oxygen delivery (transfusion restrictions) may adversely affect heart muscle. The largest trial evaluating transfusion thresholds in cardiac surgery enrolled 5243 patients who were randomly allocated to transfusion for a hemoglobin of <7.5 g/dL or <8.5 g/dL if on the non-ICU ward or <9.5 g/dL if in the operating room or ICU.<sup>126</sup> The primary outcome was a composite of death, nonfatal myocardial infarction, stroke, or renal failure with dialysis. This



**Figure 29.3** Effect of restrictive transfusion triggers on 30-day all-cause mortality. Summary of data from randomized clinical trials comparing effect of restrictive versus liberal transfusion strategies on mortality in adults (expressed as risk ratio). (Cochrane Database of Systematic Reviews 2021).

**Table 29.3** Morbidity Outcomes in Cochrane Meta-Analysis 2021

Outcome	Number of Studies	Number of Participants	Risk Ratio [95% Confidence Interval]
Myocardial infarction	23	14,370	1.04 [0.87 – 1.24]
Congestive heart failure	16	7247	0.83 [0.53 – 1.29]
Cerebral vascular accident	19	13,985	0.84 [0.64 – 1.09]
Rebleeding	8	3412	0.80 [0.59 – 1.09]
Infections	25	17,104	0.97 [0.88 – 1.07]
Thromboembolism	13	4201	1.11 [0.65 – 1.88]
Renal failure	15	12,531	1.03 [0.92 – 1.16]
Mental confusion	9	6442	1.11 [0.88 – 1.40]

composite outcome occurred in 12.3% of participants in the restrictive arm and 12.9% in the liberal arm (odds ratio 0.95; 95% CI -2.38 to -1.27). Mortality at 30 days was similar at 3.0% versus 3.6%. At six months, the primary composite outcome, mortality, and other secondary outcomes were also similar between the two arms of the trial.

A second influential trial, The Transfusion Indication Threshold Reduction (TITRe2) trial, enrolled 2007 patients undergoing cardiac surgery with hemoglobin concentration less than 9 g/dL. Participants were randomly allocated to a liberal transfusion threshold of 9 g/dL or a restrictive transfusion threshold of 7.5 g/dL.<sup>120</sup> The primary outcome was a composite of serious infection and ischemic events

(stroke, myocardial infarction, gut infarction, or acute kidney injury) within three months of enrollment in the trial. The primary outcome occurred in a similar number of patients in both groups. However, there were more deaths at 90 days in the restrictive group (4.2%) than the liberal group (2.6%) (hazard ratio = 1.64; 95% CI 1.00–2.67;  $p = 0.045$ ). At 30 days, the differences in mortality was small and nonsignificant (2.6% for the restrictive group vs. 1.9% for the liberal group). There were no differences in infectious events, sepsis, or wound infections.

Three other trials in cardiac patients found no differences in outcomes between groups. In a study of 428 patients undergoing CABG who were randomized to postoperative transfusion at a hemoglobin <8 g/dL versus <9 g/dL, there was no difference in morbidity, mortality, or self-assessment for fatigue or anemia between the two groups.<sup>125</sup> In a second trial of 502 cardiac surgery patients comparing patients transfused at hematocrit at 30% or 24%, there was no difference in composite end point of 30-day all-cause mortality, cardiogenic shock, acute respiratory distress syndrome, or acute renal injury requiring dialysis or hemofiltration.<sup>104</sup> Finally, a trial of 722 cardiac surgery patients found no differences in a composite end point of postoperative in-hospital morbidity and mortality between transfusion thresholds.<sup>97</sup>

From the four cardiac surgery trials involving 7441 participants, the 30-day mortality relative risk ratio was 0.99 (95% CI 0.74–1.33).<sup>138</sup>

### Orthopedic surgery

There have been eight trials that enrolled 3111 participants undergoing orthopedic surgery. Most of the trials enrolled patients undergoing repair of hip fracture; these patients have frequent comorbidity and are elderly. Transfusion Trigger Trial for Functional Outcomes in Cardiovascular Patients Undergoing Surgical Hip Fracture Repair (FOCUS) enrolled 2016 patients undergoing surgical repair of hip fracture.<sup>105</sup> Patients had a history of cardiovascular disease (myocardial infarction, stroke or transient ischemic attack, congestive heart failure, or peripheral vascular disease) or cardiovascular risk factors (diabetes mellitus, hypertension, renal insufficiency, smoking, and hypercholesterolemia) and hemoglobin concentration less than 10 g/dL. The patients were randomly allocated to a liberal transfusion trigger where blood was administered to maintain the hemoglobin above 10 g/dL, or restrictive transfusions in which blood was administered when the hemoglobin concentration was less than 8 g/dL or for symptoms. The symptoms of anemia included chest pain thought to be cardiac in origin, congestive heart failure, or unexplained tachycardia or hypotension unresponsive to intravenous fluids. The primary outcome was inability to walk across the room without human assistance or death 60 days after randomization, which occurred in 35.2% in the liberal transfusion trigger and 34.7% in the restrictive transfusion trigger (odds ratio in the liberal transfusion trigger, 1.01; 95% CI 0.84–1.22). In-hospital acute coronary syndrome or death occurred in 4.3% in the liberal transfusion group and 5.2% in the restrictive transfusion group, respectively (absolute risk difference, 0.9%; 99% CI 3.3–1.6). Mortality was similar between the groups at 30 days, 60 days, and a median of 3.1 years. The causes of death were also similar. The frequencies of other complications, including delirium,<sup>140</sup> were similar in the two groups.

The other seven trials included another 1000 patients of the total 3111 participants.<sup>96,99,100,103,109,118,132</sup> The results of those trials were mostly consistent with the FOCUS trial. When the 30-day mortality results were combined in meta-analysis, the relative risk was 1.16,

95% CI 0.75–1.79. None of the trials found differences in activities of daily living or function.

### Upper gastrointestinal bleeding

This patient population often becomes anemic because of active bleeding from several potential causes. Bleeding may have resolved, may be ongoing, and may even become massive. We should also note that red cells transfusions may be accompanied by approaches to correct a coagulation disturbance and the use of blood conservation treatments such as tranexamic acid.

In terms of transfusion strategies, a total of 921 patients with severe upper gastrointestinal bleeding were enrolled in a trial comparing a liberal transfusion strategy (defined as a transfusion threshold of 9 g/dL) versus a restrictive transfusion threshold of 7 g/dL.<sup>112</sup> Patients were excluded if they had massive exsanguinating bleeding, acute coronary syndrome, symptomatic peripheral vascular disease, stroke or transient ischemic attack, high risk of further bleeding, recent history of trauma or surgery, and other less common reasons. All patients at the time of enrollment were transfused one unit of red blood cells. Survival at six weeks was higher in the restrictive transfusion group (95%) compared to the liberal transfusion group (91%; 0.55; 95% CI 0.33–0.92;  $p = 0.02$ ). Recurrent bleeding occurred more frequently in the liberal transfusion group (16%) than the restrictive transfusion group (10%) ( $p = 0.01$ ). In a subgroup of patients, portal pressures were higher in the liberal transfusion group than the restrictive transfusion group.

A multicenter cluster trial randomly allocated 936 patients with acute upper gastrointestinal bleeding to a restrictive or liberal threshold (8 g/dL vs. 10 g/dL) in six university hospitals.<sup>119</sup> Mean difference in hemoglobin levels was only 0.2 g/dL but fewer patients received transfusion in restrictive group (33%) compared to liberal group (46%). There were no differences in clinical outcomes; 28-day mortality occurred in 5% in restrictive threshold and 7% in liberal threshold, and rebleeding occurred in 5% in restrictive and 9% in liberal transfusion strategy. The other trial only included 50 patients.<sup>92</sup>

An important finding, which has not been found in other clinical settings, was that the 30-day mortality was significantly lower using the restrictive strategy compared with the liberal strategy (RR = 0.65; 95% CI 0.43–0.97) in the 1522 participants in GI bleeding trials.<sup>138</sup>

### Acute myocardial infarction

Much like patients undergoing coronary artery bypass grafting, patients who have an acute coronary syndrome may either require a higher transfusion threshold to overcome the acute ischemia caused by occluded coronary arteries or may be more susceptible to volume overload from red cells. Three trials enrolled patients with acute myocardial infarction. The REALITY trial randomly allocated 668 patients with acute MI and hemoglobin less than 10 g/dL to a restrictive transfusion trigger of <8 g/dL or liberal transfusions at ≤10 g/dL.<sup>137</sup> The primary clinical outcome of MACE (consisting of death, stroke, recurrent MI, or emergency revascularization) at 30 days occurred in 11.1% of participants in the restrictive group and 14.2% in the liberal group; relative risk 0.78; 95% CI 0.00–1.17. At one year, MACE occurred in 32.4% in restrictive threshold and 28.1% in liberal threshold (hazard ratio = 1.16; 95% CI 0.88–1.53).

In addition to REALITY, two smaller trials have been published in patients with acute MI and anemia. A pilot trial of 110 patients found that compared with transfusion for a hemoglobin <8 g/dL (restrictive strategy), transfusion to raise the hemoglobin ≥10 (liberal strategy) was associated with greater survival at 30 days (98% vs.

87%).<sup>42,108</sup> Another trial of 44 patients reported one death in the liberal transfusion group and two deaths in the restrictive strategy group.<sup>106</sup>

In aggregate, the 30-day mortality risk was higher in the restrictive strategy group than in the liberal strategy group (RR 1.61; 95% CI 0.38–6.88) although the confidence intervals are wide.<sup>138</sup> A 3500-patient trial called MINT is ongoing which will provide much needed data in this important group of patients.

### Oncology patients

There are several clinical settings in which oncology patients are frequently transfused with red cells. These include patients undergoing chemotherapy, stem cell transplantation, chronic anemia in patients with cancer, and surgery for cancer. One trial has been published in which cancer patients who underwent surgery were randomized to liberal versus restrictive transfusion triggers.<sup>124</sup> Three hundred patients undergoing abdominal surgery at a cancer hospital with septic shock and admitted to intensive care unit were randomly allocated to a 9 g/dL threshold (liberal) or a 7 g/dL threshold (restrictive). The primary outcome was 28-day all-cause mortality. Unexpectedly, the liberal transfusion group had superior outcomes to the restrictive transfusion group (liberal, 45%; restrictive, 56%;  $p = 0.08$ ). At 90 days' post-randomization, mortality was also lower in the liberal (59%) versus restrictive group (70%) (hazard ratio = 0.72; 95% CI 0.53–0.97).

Several trials have compared restrictive versus liberal transfusion thresholds in patients with hematological disorders. One trial enrolled patients undergoing autologous or allogeneic hematopoietic cell transplantation for any hematological malignancy.<sup>136</sup> There were no differences in the primary outcome of health-related quality of life. Three small trials evaluated transfusion in patients with leukemia and myelodysplasia but were not powered to examine clinical outcomes.<sup>102,122,135</sup>

### Children

Children have unique age-dependent adaptations to anemia. Similarly, very young children and those undergoing procedures such as cardiac surgery may also have different risk–benefit ratios for transfusion at different thresholds. As a consequence, investigators undertook several studies in this patient population. Of note, blood is frequently transfused in critically ill infants and children. In a recent survey, 14% of patients in pediatric ICUs received blood transfusion.<sup>141</sup> There have been five clinical trials evaluating liberal versus restrictive transfusion thresholds in this population. In one study, 100 hospitalized preterm infants with birthweights between 500 and 1300 g were randomly assigned to two transfusion levels.<sup>142</sup> The transfusion protocol adjusted the hematocrit level for transfusion based on the respiratory status of the infant. A primary outcome was not designated among the 15 clinical events evaluated. Infants in the restrictive group received a median of two units less than the liberal group during the study, and the mean difference in hemoglobin concentration was ~2 g/dL. There were no differences between the liberal and restrictive transfusion groups for most outcomes, including survival, patent ductus arteriosus, retinopathy, or bronchopulmonary dysplasia. Infants assigned to the restrictive group had more apneic events and more neurologic events (combined parenchymal brain hemorrhage or periventricular leukomalacia). These differences in outcomes should be interpreted as hypothesis-generating because the composite neurologic outcomes were not designated a priori,<sup>143</sup> apnea was assessed by an unblinded

nurse<sup>143</sup> and the differences were small, and the large number of outcomes increase the risk of false-positive results.

In the second study, 451 infants weighing less than 1000 g, whose gestational age was less than 31 weeks and who were less than 48 hours old, were randomly assigned to receive transfusion at either a low or high transfusion threshold.<sup>144</sup> The thresholds used varied by respiratory support using a prespecified transfusion algorithm. The primary composite outcome was in-hospital death, severe retinopathy, bronchopulmonary dysplasia, or brain injury on cranial ultrasound. The mean hemoglobin concentration was ~1 g/dL different between groups, and the number of RBC transfusions was not significantly different. There was no difference in the composite outcomes (low threshold [74.0%] vs. high threshold [69.7%]). This trial did not confirm the hypothesis that restrictive transfusion is associated with brain injury in preterm infants.

The largest study involved 637 children admitted to pediatric ICUs.<sup>101</sup> When a 7 g/dL threshold was compared to a 9.5 g/dL threshold, the mean hemoglobin concentration was ~2 g/dL lower in the restrictive group. RBC units were transfused to 46% of patients in the restrictive group and 98% in the liberal group. The frequency of new or progressive multiorgan dysfunction (primary outcome) was 12% in both groups, and there were no significant differences in any of the secondary outcomes including death, which occurred in 4% of both groups.

The fourth trial enrolled 107 children from six weeks to six years of age with noncyanotic congenital defects undergoing corrective cardiac surgery.<sup>145</sup> The patients were randomly allocated to either a liberal transfusion group with a hemoglobin threshold of less than 10.8 g/dL or restrictive transfusions for hemoglobin levels less than 8 g/dL. The length of stay was statistically significantly although only slightly shorter in the restrictive group (median = 8 [IQR 7–11] days) versus liberal group (median = 9 [IQR 7–14] days;  $p = 0.047$ ). There were no differences in other outcomes.

A fifth trial randomized children from sub-Saharan Africa with hemoglobin less than 6 g/dL and severity symptoms including respiratory distress or reduced consciousness to receive immediate transfusion of 20 or 30 mL/kg.<sup>146,147</sup> A total of 3196 children with median age of 37 months were enrolled. Malaria was the most common cause of anemia in 64.1% of participants. The primary outcome was 28-day mortality. Overall, there was no difference in the primary outcome of 28-day mortality between the low and higher doses of RBC transfusion (hazard ratio = 0.76; 95% CI 0.54–1.08). However, in a prespecified subgroup of febrile children with temperature greater than 37.5 °C, there was significant heterogeneity ( $p = 0.001$ ). In the children without fever, mortality was lower in the higher dose transfusion group (hazard ratio = 0.43; 95% CI 0.27–0.69). In the group with fever, mortality was elevated in those that received a higher transfusion dose (hazard ratio = 1.91; 95% CI 1.04–3.49).

### Transfusion guidelines

Since the National Institutes of Health Consensus Conference developed landmark guidelines for the transfusion of blood components in 1988,<sup>148</sup> many organizations have issued guidelines with the intent of aiding clinical decisions. The administration of RBCs is based on the balance of benefits, risks, and costs. Physicians making transfusion decisions are sometimes faced with massive amounts of conflicting information. Since 1983, concerns about the transmission of human immunodeficiency virus and other viruses through blood components have significantly modified both real and perceived risks and benefits. Many organizations responded by issuing clinical

practice guidelines advocating a more restrictive approach to the use of allogeneic RBC units and other components. In recent years, there have been additional guidelines that benefit from accumulating clinical trial evidence.

Although some of the specific recommendations varied among the guidelines, most of the recommendations were similar.<sup>149–159</sup> Based on results of the multiple trials and other evidence, all the guidelines published in the past few years recommended a 7 g/dL threshold in intensive care unit patients and often in other patient populations. Many guidelines suggested 8 g/dL threshold in patients with preexisting cardiac disease. Most of the guidelines also recommended that transfusion be guided by clinical judgment rather than hemoglobin level.

The AABB guideline recommendations (chaired by Dr. Carson) emphasized that the results of clinical trials should serve as the basis of all recommendations.<sup>160</sup> Further, the guidelines began with a good practice statement: “When deciding to transfuse an individual patient, it is good practice to consider not only the hemoglobin level, but the overall clinical context and alternative therapies to transfusion. Variables to take into consideration include the rate of decline in hemoglobin level, intravascular volume status, shortness of breath, exercise tolerance, light-headedness, chest pain thought to be cardiac in origin, hypotension or tachycardia unresponsive to fluid challenge, and patient preferences. This practice guideline is not intended as an absolute standard and will not apply to all individual transfusion decisions.” The recommendations were: (1) restrictive transfusion is not indicated until the hemoglobin level is 7 g/dL for hospitalized adult patients who are hemodynamically stable, including critically ill patients. (2) A restrictive RBC transfusion threshold of 8 g/dL is recommended for patients undergoing orthopedic surgery, cardiac surgery, and those with preexisting cardiovascular disease. (3) A restrictive transfusion threshold of 7 g/dL is likely comparable with 8 g/dL, but RCT evidence is not available for all patient categories. These recommendations do not apply to patients with acute coronary syndrome, severe thrombocytopenia (patients treated for hematological or oncological reasons who are at risk of bleeding), and chronic-transfusion-dependent anemia due to insufficient evidence.

Additional authoritative evidence-based recommendations for RBC transfusion were provided by the 2018 Frankfurt Consensus Conference,<sup>161</sup> including the use of the following hemoglobin thresholds: (1) <7 g/dL in critically ill but clinically stable intensive care patients, (2) <7.5 g/dL in patients undergoing cardiac surgery, (3) <8 g/dL in patients with hip fracture and cardiovascular disease or other risk factors, and (4) 7–8 g/dL in hemodynamically stable patients with acute gastrointestinal bleeding.

Clinical Practice Guidelines on Patient Blood Management in Cardiac surgery was published by the joint effort of STS/SCA/AmSECT/SABM.<sup>162</sup> These guidelines advise using a restrictive perioperative transfusion strategy to reduce the transfusion rate and units of allogeneic RBCs administered without adversely impacting mortality or morbidity.

## **Decision-making in red cell transfusion**

### **An approach to evaluating a bleeding patient**

In the care of a bleeding patient, the physician first must determine the degree of blood loss and the rate of bleeding. As part of the initial assessment, the healthcare team must rapidly address the patient's ABCs (airway, breathing, and circulation). Although blood loss usually leads to circulatory compromise, the severe

form, termed *hemorrhagic shock*, often leads to airway and breathing concerns as well due to altered mental status, metabolic (acid-base) derangement, and physiological stress. Once airway and breathing have been assessed and cleared, the assessment of the heart rate and blood pressure—in addition to a rapid scan of the scene and the patient—will reveal obvious threats such as dramatic blood loss. During the initial assessment, patients must be completely uncovered and rapidly examined to rule out rapidly bleeding wounds, obvious signs of massive bleeding such as a rapidly expanding abdomen in patients with a ruptured abdominal aortic aneurysm, or severe hematochezia in patients with severe lower gastrointestinal bleeding. In the trauma patient, significant blood loss can occur in the hemithorax, pelvis, retroperitoneum, or long bones.<sup>163</sup> The focus is on possible causes of bleeding and important comorbid conditions, such as cardiovascular disease that could exacerbate any injury. An emergency complete blood cell count, type and crossmatch, prothrombin time, and partial thromboplastin time should be obtained. From these data and the clinical examination, the source and rate of bleeding are estimated; note that in the exsanguinating patient, point measurement of the hemoglobin level may be falsely normal. A decision can be made about the rate of RBC unit transfusion as well as any curative and supportive measures. For patients with gastrointestinal bleeding, consultations may be requested from the gastroenterology and possibly surgery departments. In the care of patients with postoperative bleeding, measures such as angiography or exploratory surgery should be considered.

In patients with active bleeding, the hemodynamic status and need for emergency intervention must be determined. Crystalloid is administered to maintain intravascular volume only if RBC transfusion is unavailable or bleeding is relatively mild; otherwise, red cell transfusions are administered rapidly to maintain adequate oxygen-carrying capacity, sometimes in an emergent fashion with uncross-matched O-negative blood. Clinical judgment is needed to estimate how much more bleeding may occur and how much lower the hemoglobin level will likely decrease, and then to perform expectant transfusion. Vital signs are examined for a decrease in blood pressure and tachycardia. The patient is asked about symptoms that can result from anemia, including cardiac ischemia (chest pain), congestive heart failure (dyspnea, paroxysmal nocturnal dyspnea, and edema), fatigue, dizziness, weakness, and orthostatic hypotension unresponsive to intravenous fluids. The hemoglobin level is measured at regular intervals.

It is important to determine the presence of coronary artery disease because results of studies with animals and with human subjects suggest that patients with coronary artery disease may be less tolerant of anemia. Review of the medical history for angina, myocardial infarction, and CABG surgery is essential. The electrocardiogram is examined for evidence of old myocardial infarction or ischemic changes. The chest radiograph is examined for cardiomegaly and other changes consistent with congestive heart failure. Patients with a history of peripheral or cerebral vascular disease are more likely also to have asymptomatic coronary artery disease.

In the care of patients with moderate to severe blood loss that has abated, there is more time for complete assessment of the source of bleeding, development of a course of management, and thorough evaluation of the patient. After bleeding has stopped and equilibration has occurred, the hemoglobin level stabilizes and the nadir hemoglobin level can be determined. Then, transfusion decisions can be made accordingly.

## Surgical patient

Patients undergoing major surgical intervention often experience hemodynamic instability and frequently require RBC unit transfusion as a consequence of considerable blood loss. Various aspects of transfusion management for surgical patients are discussed in Chapters 39 and 40.

## Chronic anemia

In chronic anemia, there is time for compensation to develop and for careful observation of the patient. Anemia is associated with an increase in blood flow resulting from decreased viscosity, greater release of oxygen caused by higher levels of 2,3-DPG, and an increase in cardiac output. There is time to determine whether these physiologic events are clinically important in the individual patient and carefully determine whether the patient has symptoms. Besides cardiac symptoms (angina and dyspnea), anemia can lead to nonspecific symptoms, such as fatigue, weakness, dizziness, reduced exercise tolerance, and impaired performance of activities of daily living. Two small pilot trials in patients with myelodysplastic syndrome were focused on function.<sup>133,135</sup> There also may be time to implement alternative treatments to correct anemia, depending on the cause. Iron, vitamin B<sub>12</sub>, and folate can be replaced. Erythropoietin can be administered. It is important to correct preoperative anemia because the hemoglobin level strongly correlates with the probability of transfusion.<sup>164</sup>

## Transfusion threshold

Recent clinical trials have assessed the optimal threshold for transfusion, and there are reliable data for many clinical settings. A 7 g/dL threshold should be used in critical care patients because multiple trials have confirmed that it is safe to withhold blood until the hemoglobin level decreases to less than this level (see the critical care summary above). A 7 g/dL threshold should also be used in patients with acute gastrointestinal bleeding because trials found a lower mortality than patients transfused at 9 g/dL.<sup>112</sup> However, it can be difficult to anticipate how low the hemoglobin level might fall in rapidly bleeding patients. Thus, blood pressure and pulse along with clinical judgment must be strongly considered in an actively bleeding patient. In postoperative cardiac surgical patients, a 7.5 g/dL threshold should be used<sup>126</sup> (see the cardiac surgery summary), while an 8 g/dL threshold is appropriate in orthopedic surgery<sup>105</sup> (see the orthopedic surgery summary) as it has been proven to be as safe as a 10 g/dL threshold. The weight of the evidence suggests asymptomatic patients with preexisting cardiovascular disease safely tolerate an 8 g/dL threshold. However, it is unclear what transfusion threshold is appropriate for patients with acute coronary syndrome as the trials have not excluded a clinically significant benefit with liberal transfusion to 10 g/dL (see the summary of acute MI above). At this time, we have no specific recommendation in this group of patients, and it is necessary to rely on clinical judgment. Patients with symptoms of anemia should be transfused as needed. No set of guidelines applies to every patient. In the end, careful clinical assessment of each patient with thoughtful consideration of risks and benefits should guide the transfusion decision.

## Dose and administration

In patients who experience acute blood loss, the rate of administration of RBCs is guided by the rate of bleeding and hemodynamic compromise. In the treatment of rapidly bleeding patients, RBCs should be given at the rate necessary to maintain oxygen transport while definitive therapy is instituted. Rates may range from 5 to 10 units over 10–15 minutes in patients who are exsanguinating to rates

in the range of one unit every 2–4 hours (1 mL/kg/hour) in patients with severe left ventricular dysfunction. When blood loss is predictable and not massive, RBC units should generally be administered one at a time. In the care of patients with chronic anemia, enough blood should be given to control symptoms. In most adult patients, 1 RBC unit increases the hemoglobin level approximately 1 g/dL and hematocrit approximately 3%. Ordinarily, 1 RBC unit is given over 1–2 hours. After transfusion of each RBC unit, the measurement of the hemoglobin level is repeated, and the patient reassessed.

## Future

During the next decade, advances and risks may dramatically alter transfusion practice. Research is underway to develop safer red cell components. Further clinical trials should be performed to better describe the clinical effect of different transfusion strategies. Trials are especially needed in patients with acute coronary syndrome, neurological disorders, and acute and chronic hematological disorders. It is possible that within the next one or two decades a cost-effective oxygen carrier will be developed that will reduce the use of allogeneic RBC units.

## Summary

Red cell transfusion is an extremely common intervention in modern medical care. Blood is administered to improve oxygen delivery, but the connection between systemic hematocrit and the microcirculation is not linear. Patients compensate for anemia through increased cardiac output, through redistribution of blood flow to the cardiac and cerebral circulation, and through microvascular regulation of capillary RBC flow. Particular attention must be paid to maintaining adequate oxygen delivery to the heart. The preponderance of evidence suggests that a hemoglobin level of 7–8 g/dL is a safe threshold for red cell transfusion. For patients with acute cardiovascular disease, a higher transfusion threshold should be considered. RBC units should usually be administered one at a time, and the patient and hemoglobin level should be reassessed after each transfusion. Transfusion decisions should also consider the rate of bleeding, the presence of underlying medical problems, the hemodynamic status of the patient, the presence of symptoms, and whether anemia is acute or chronic. Further evidence in the support of a transfusion strategy is required in patients with acute brain injury and coronary ischemia as well as whether transfusion improves subjective symptoms in chronic complex diseases.

## Disclaimer

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## CHAPTER 30

# Sickle cell disease, thalassemia, and hereditary hemolytic anemias

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## Introduction

Each year, an estimated 300,000 infants are born with either of the two most common hemoglobinopathies: sickle hemoglobinopathies or thalassemia.<sup>1</sup> Sickle cell disease (SCD) makes up 85% of these infants, and thalassemias the remaining 15%.<sup>1,2</sup> These inherited diseases are the most prevalent monogenetic disorders worldwide. It is increasingly apparent that SCD and thalassemia have become a major health challenge in emerging countries as more infants with hemoglobinopathies survive beyond infancy and into adulthood. Sickle hemoglobin (HbS) is a structural variant causing severe disease in the homozygous state and when in combination with other hemoglobin variants. The  $\alpha$ - and  $\beta$ -thalassemias are genetically diverse with more than 200 mutations accounting for decreased production of  $\alpha$ - or  $\beta$ -globin chains, respectively, in those affected. The thalassemia phenotype varies from mild to severe states of anemia. Either chronic or episodic red blood cell (RBC) transfusions are indicated for both hemoglobinopathies. In SCD, transfusions prevent and/or treat complications and ameliorate anemia by decreasing HbS concentration. In the thalassemias, transfusion prevents the anemia associated with decreased or absent hemoglobin production.

The prevalence of significant hemoglobin traits (HbS, HbC, HbE, HbD, etc., and  $\beta$ -thalassemia and  $\alpha^0$ -thalassemia) varies from about 1% in all of Europe to as high as 18% in all of Africa; the prevalence in the American population is 3%. The presence of disease is 10.8/1000 in Africans, 0.6/1000 in Americans, and 0.2/1000 in all of Europe. Half of the people in the world with SCD live in three countries: Nigeria, Democratic Republic of Congo, and India. In West Africa, about 1000 infants a day are born with SCD.<sup>2</sup>

In the world's population, 1.5% are  $\beta$ -thalassemia mutation carriers.<sup>3</sup> Hemoglobin E-beta<sup>0</sup> thalassemia (Hb E/ $\beta^0$ -thalassemia) is the genotype responsible for approximately one-half of all severe  $\beta$ -thalassemia worldwide.<sup>4</sup> The distributions of the phenotype and genotype of North American thalassemia patients today—as well as their transfusion management—are dramatically different from those in the past decades. The majority of patients, previously of Mediterranean descent, are now largely of Asian, Indian, and Middle Eastern origin.<sup>3</sup> In Thailand, almost 4000 affected children are born annually, with estimates of about 55,000 living patients

with transfusion-dependent thalassemia.<sup>5</sup> In southern China, the gene frequencies for  $\beta$ -thalassemia and for HbE are over 4%, resulting in thousands of annual births of  $\beta$ -thalassemia major and hemoglobin Hb E/ $\beta^0$ -thalassemia.<sup>6</sup>

In addition to the hemoglobinopathies, other hereditary hemolytic anemias resulting from erythrocyte membrane abnormalities (e.g., hereditary spherocytosis, elliptocytosis, pyropoikilocytosis, stomatocytosis, and ovalocytosis) or enzyme defects (i.e., glucose-6-phosphate dehydrogenase [G6PD] deficiency, and pyruvate kinase deficiency) contribute to global hematological disease burden. However, the need for transfusions, and especially chronic transfusion therapy, is often less frequent in these disorders due to the decreased prevalence of phenotypes associated with severe hemolytic anemia.

## Malaria, hematologic diseases, and blood groups

Malaria is a devastating parasitic disease in humans in which the clinically symptomatic stage involves sequential rounds of parasite invasion, multiplication, maturation within RBCs, and erythrocyte rupture upon release. Malaria infection results in particularly high mortality rates among children and pregnant women, and has been the largest cause of childhood mortality globally for much of the last 5000 years.<sup>7</sup> Due to the high infection and mortality rates, malaria is an extremely strong force for natural selection. It is estimated that there are 228 million cases of malaria worldwide, with over 90% of cases in Africa, followed by 5% in Southeast Asia, and 2% in the Eastern Mediterranean region. Although global mortality rates have improved over the last two decades, the World Health Organization still records over 400,000 malaria deaths per year worldwide, with 90% of deaths occurring in sub-Saharan Africa.<sup>8</sup> Numerous polymorphisms resulting in changes to the red cell membrane, blood group antigens, and hemoglobin have evolved and have been selected for in regions where malaria is most predominant because of survival advantages in individuals with these variants.<sup>9</sup> Subsequently, due to past and ongoing global migration, many of these polymorphisms including those that result in SCD, thalassemia, congenital red cell disorders, and

expression of certain blood group antigens can now be found in high prevalence in areas that are free of malaria. As such, an understanding of malaria and the mutations it has caused lays a foundation for understanding SCD, thalassemia, various other congenital red cell disorders, as well as transfusion therapy.

Currently, there are five species of malaria parasites infecting humans: *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale*, *Plasmodium malariae*, and most recently *Plasmodium knowlesi*. *P. falciparum* causes the largest disease burden, followed by *P. vivax*. *P. falciparum* predominates in sub-Saharan Africa, New Guinea, and Hispaniola (Haiti and the Dominican Republic), and is responsible for two-thirds of cases on the Indian subcontinent, eastern Asia, and Oceania.<sup>10</sup> *P. vivax* is much more common in the Americas and the western Pacific, although a significant number also occurs in Africa and South America.<sup>11</sup> *P. falciparum* accounts for the vast majority of severe disease and deaths.<sup>12</sup> Because of this fact, the selective pressure for evolutionary adaptations in the human population is most obvious with *P. falciparum* infection.<sup>12,13</sup>

The association between hematological disease and malaria was first noted in the 1940s in the Mediterranean by Haldane and in Africa by Beet.<sup>14,15</sup> Since then, it has been established that malaria parasites have exerted selective pressure on several genes. This evolutionary process has produced alleles that confer resistance to infection, but unfortunately concomitantly produce novel diseases due to altered forms of hemoglobin ( $\alpha$ - and  $\beta$ -thalassemia, hemoglobin S, and hemoglobin C), RBC enzymes (glucose-6-phosphate dehydrogenase deficiency), and RBC membrane proteins (Southeast Asian Ovalocytosis). Malarial resistance has also selected for a series of benign blood group antigen polymorphisms within the Duffy, Gerbich, MNS, Knops, ABO, and Lewis blood groups.<sup>7</sup>

## Structural hemoglobin mutations

### Hemoglobin S

Hemoglobin S ( $\beta$  Glu6Val, GAG>GTG) in the heterozygous form is a benign carrier condition, usually with none of the symptoms of SCD. Sickle cell trait (HbAS) is extremely common in much of sub-Saharan Africa where malaria is endemic. Hemoglobin S has become the dominant mutation due to superior protection against *P. falciparum* in the heterozygote. Sickle cell trait (HbAS) is the strongest protection against infection, demonstrating 90% efficacy in preventing severe and/or complicated malaria (cerebral malaria and severe anemia) in multiple studies.<sup>7,13,16</sup> In addition, a birth cohort study of 1022 Kenyan children demonstrated that HbAS confers 60% protection against mortality in children between ages 2 and 16 months when compared with HbAA, whereas HbSS showed no survival advantage.<sup>17</sup>

Most of the research on protective mechanisms against severe malaria has been carried out in areas where *P. falciparum* is prevalent. Although not entirely understood, there are several proposed mechanisms of HbAS protection against *P. falciparum*:

- **Increased sickling of infected erythrocytes:** Increased sickling of infected HbAS erythrocytes leads to increased RBC clearance within the spleen, promoting removal of infected cells from the circulation.<sup>18,19</sup>
- **Impaired intraerythrocyte parasite growth:** HbAS red cells infected with *P. falciparum* show increased polymerization of hemoglobin and have an intracellular environment that decreases parasite growth due to the enhanced polymerization as well as high levels of potassium and osmotic loss of water.<sup>20-22</sup> Additionally, dysregulated microRNA composition has been

shown to contribute to resistance against *P. falciparum*. Two microRNAs (miR-451 and let-7i) are highly enriched in HbAS and HbSS erythrocytes, which upon translocating into the parasite result in translational inhibition.<sup>21</sup>

- **Impaired intraerythrocyte actin cytoskeleton network:** It has been shown that HbS and HbC alter actin polymerization and disrupt the intracellular trafficking system required to direct parasite proteins to the surface of infected erythrocytes. This impairment results in less Pf-EMP-1 (*P. falciparum* erythrocyte membrane protein-1) on the red cell plasma membrane,<sup>23</sup> which in turn reduces cytoadherence of parasitized RBCs to the vascular endothelium as well as binding of parasitized RBCs to uninfected RBCs to form rosettes.<sup>24-26</sup>
- **Enhanced acquired immune response to malaria:** Immune-mediated protective mechanisms have also been associated with HbAS (as well as thalassemia and G6PD deficiency). Enhanced phagocytosis by monocytes of ring-parasitized erythrocytes from sickle trait (and  $\beta$ -thalassemia trait) individuals has been demonstrated in vitro, possibly due to increased production of reactive oxygen species intrinsic to these variant RBCs.<sup>7,27</sup> Others have shown increased lymphoproliferative responses to malaria antigens in children with HbAS compared to HbAA.<sup>28</sup>

Although these effects lead to plausible explanations for protection from severe malaria in HbAS individuals, it remains unclear which mechanisms are significant in vivo. Many of these protective mechanisms against severe malaria have also been observed in other hemoglobinopathies, RBC enzymopathies, and RBC membrane disorders.

### Hemoglobin C

Hemoglobin C (HbC) results from a mutation where lysine is substituted for glutamate in the sixth position of the  $\beta$ -globin chain. The variant HbC is less soluble than HbA, forming hexagonal crystals (in contrast to the long polymers seen in SCD), and induces red cell dehydration.<sup>29</sup> Individuals with hemoglobin C trait (HbAC) are phenotypically normal and do not show any symptoms, while persons with hemoglobin C disease (HbCC) may have a mild degree of hemolysis with splenomegaly and mild to moderate anemia. Whereas individuals with HbCC do not experience sickling events, individuals coinherit HbC and HbS (HbSC) may have serious sickle-related consequences. Hemoglobin C occurs almost exclusively in West Africa, having a frequency of 50% in the Ivory Coast. Unlike HbS, where there is a disadvantage for the homozygote, there appears to be no selective disadvantage in individuals with HbCC with respect to malaria.

HbC is not as effective in the prevention of severe malaria as HbS, which may explain why it is not more prevalent in sub-Saharan Africa. For example, HbC does not appear to protect against uncomplicated malaria, although it has been shown to safeguard against the more severe forms of the disease. One case-control study performed in Burkina Faso involving 4348 subjects demonstrated a reduction of 29% in risk of clinical malaria in individuals with HbAC and 93% in those with HbCC.<sup>30</sup> These findings were corroborated by a case-control study of 2397 children in Ghana, where a 47% reduction in risk of severe malaria was observed for children with HbAC compared to a 90% risk reduction in children with HbAS. The sample size was unfortunately insufficient for a meaningful statistical analysis of HbCC and HbSC.<sup>31</sup> The presence of HbC in the erythrocyte also appears to alter the red cell membrane as well as hinder the development of *P. falciparum* within the cell.<sup>32</sup> Similarly to HbS, protection from severe malaria conferred by HbC is largely due to

changes in the plasma membrane expression of PfEMP1 in HbCC and HbAC erythrocytes. This reduction in erythrocyte surface PfEMP1 leads to decreased cytoadherence, impaired rosetting, and possibly an increase in acquired immunity against *P. falciparum* variant surface antigens on the membrane of infected erythrocytes.<sup>33</sup> However, the enhanced immune response theory has been questioned in a report demonstrating no increase in antibody response to a diverse array of *P. falciparum* antigens in children with HbAS and HbAC infected with malaria.<sup>34</sup>

## Thalassemia

Thalassemia refers to a group of hereditary hemolytic diseases characterized by reduced or absent production of one of the globin chains which disrupts the balance between the alpha and beta or gamma globin chains. The two most common forms, alpha thalassemia and beta thalassemia, have also been associated with malarial resistance.

### Alpha thalassemia

Alpha thalassemia is the most common single-gene disease in the world and results from impaired or absent production of alpha globin chains. This results in fewer normal tetrameric hemoglobin molecules due to a relative excess of gamma globin chains in the fetus and newborn, and beta globin chains in children and adults.

Alpha thalassemia has been found to be protective against severe malaria in multiple case-control studies in Africa<sup>35–38</sup> and the southwest Pacific.<sup>39</sup> This protective effect is stronger in homozygous α<sup>+</sup>-thalassemia (α-/α-) compared to heterozygous α<sup>+</sup>-thalassemia (α-/αα).<sup>38,39</sup> An interesting phenomenon of negative epistasis has been observed where the antimalarial protective effects seen with sickle cell trait and alpha thalassemia individually are lost when α-thalassemia and sickle cell anemia are coinherited.<sup>35</sup> Although the mechanism is unclear, this phenomenon is likely the reason why the incidence of homozygous α<sup>+</sup>-thalassemia is significantly higher in parts of India and Asia (up to 80%) compared to sub-Saharan Africa (≤50%) where the beta<sup>s</sup> globin allele is more prevalent despite endemic severe malaria.<sup>35</sup> It may also explain why the beta<sup>s</sup> globin allele is infrequent in the Mediterranean where thalassemia is common.<sup>40</sup>

Proposed mechanisms for the protective effects of thalassemia include the inhibition of *P. falciparum* growth within infected red cells particularly in individuals missing three α-chain genes (i.e., HbH);<sup>41,42</sup> increased phagocytosis in *P. falciparum* infected α-thalassemia RBCs;<sup>43</sup> reduced surface expression of Pf-EMP1 resulting in less microvascular endothelial cell adherence and impaired rosetting;<sup>44</sup> and reduced α-thalassemia erythrocyte expression of complement receptor 1 (CR1), which is responsible for RBC rosetting and *P. vivax* invasion.<sup>45</sup> Additionally, thalassemic erythrocytes may demonstrate increased susceptibility to non-lethal *P. vivax* infection, particularly in young children with α-thalassemia, resulting in limited cross-species protection against severe *P. falciparum* infection.<sup>46,47</sup> Lastly, it has been postulated that increased erythrocyte count and microcytosis in children with homozygous α<sup>+</sup>-thalassemia may contribute to their resistance to severe malarial anemia.<sup>48</sup> (See further discussion on α-thalassemia in the “Thalassemia” section.)

### Beta thalassemia

β-Thalassemia results from mutations in the beta globin locus which lead to absent (β<sup>0</sup>) or decreased (β<sup>+</sup>) production of beta globin chains, and an excess of alpha globin chains. Although β-thalassemia is prevalent in malarial areas, the evidence

for β-thalassemia’s protective effect against malaria is less convincing than for other hemoglobin mutations, primarily due to the fact that the areas around the Mediterranean where β-thalassemia has been prevalent are now free of the malaria parasite. In Africa there are only a few studies investigating β-thalassemia protective effects against malaria, none of which are case-control studies on the scale of those completed in HbAS or α-thalassemia.<sup>49</sup> In a population survey in northern Liberia conducted in 1983, prevalence rates of *P. falciparum* were similar in normal children and those with β-thalassemia trait; however, the β-thalassemia trait group had lower parasite densities.<sup>50</sup> Consistent with reduced malaria burden, in vitro studies have shown protective effects. Coculture experiments have demonstrated impairment of *P. falciparum* growth in β-thalassemic erythrocytes; however, impairment was less prominent in β-thalassemia than in α-thalassemia RBC populations.<sup>42,51</sup> Further studies have shown that *P. falciparum*-infected β-thalassemic erythrocytes have marked reductions in the cytoadherence and rosette formation in the trophozoite/schizont (but not young ring) stage;<sup>52</sup> and ring-parasitized β-thalassemia trait erythrocytes undergo increased phagocytosis by human monocytes (likely due to increased membrane-bound hemichromes, IgG and C3c fragments, and aggregated band 3).<sup>27</sup> Transgenic β-thalassemic mice models have demonstrated lower and delayed peak parasitemia after infection with *P. chabaudi adami* (which causes a non-lethal infection mainly in mature red cells).<sup>53</sup> (See further discussion on β-thalassemia in the “Thalassemia” section.)

## Hemoglobin E

Hemoglobin E (HbE) is the most common hemoglobin structural mutation in Southeast Asia. HbE results from a point mutation at codon 26 of the β-globin gene (Glu>Lys). This mutation results in marginally reduced expression of β-globin chains and a mild β-thalassemia phenotype.<sup>54</sup> Although this thalassemia is common in malarial areas, there has not been direct evidence for a protective effect in large case-control population studies in Southeast Asia. The only epidemiological data come from an analysis showing higher prevalence of antimalarial titers (indicative of previous malarial exposure) in Southeast Asian refugees with HbAE or HbEE compared to refugees with HbAA.<sup>55</sup> In vitro studies have demonstrated decreased growth of *P. falciparum* in HbE-containing erythrocytes with a more pronounced effect as the concentration of HbE increases within the RBCs.<sup>55</sup>

## RBC enzymopathies

### Glucose-6-phosphate dehydrogenase (G6PD) deficiency

The gene for glucose-6-phosphate dehydrogenase (G6PD) is located on the X chromosome (band X q28). G6PD is expressed in all cells but is particularly important in erythrocytes because it is the sole source of NADPH which is necessary for the regeneration of reduced glutathione, a key metabolite in the control of oxidative damage. The global prevalence of G6PD deficiency has been estimated at 4.9% (both males and females included) with prevalence reaching 20% in some regions in Africa and Asia.<sup>56,57</sup> The mutant allele (A-), encoding 10–50% of normal G6PD enzyme activity, is the most frequent variant in western and central Africa.<sup>58</sup> The Mediterranean variant is found in the Mediterranean, India, and Indonesia, and results in only approximately 3% G6PD activity; hemolysis in people with this variant can be severe. Haplotype

analysis of both the A– and Mediterranean variants indicates increased frequency in areas where malaria is prevalent.<sup>58</sup> Case-control studies in Kenyan and Gambian children have demonstrated that G6PD deficiency (G6PD A–) is associated with a significant reduction in risk of severe malaria in male hemizygotes and conflicting evidence of risk reduction in heterozygous females.<sup>59,60</sup> These results have been questioned because of the finding that the DNA methodology used for G6PD typing likely misclassified a large proportion of the samples from the Gambia cohort.<sup>61</sup> The processes responsible for resistance remain unresolved; however, proposed mechanisms include inhibition of parasite growth<sup>62</sup> and increased phagocytosis of infected G6PD-deficient red cells due to parasite-induced oxidative modifications to RBC membranes.<sup>63</sup> (See further discussion on G6PD deficiency in the “Red cell enzymopathies” section.)

### RBC membrane mutations

The most common erythrocyte membrane disorders include hereditary spherocytosis (HS), hereditary elliptocytosis (HE), and hereditary pyropoikilocytosis (HPP), which result from mutations leading to abnormal expression of alpha spectrin, beta spectrin, ankyrin, protein 4.1, band 3, or glycophorin C. Southeast Asian ovalocytosis, dehydrated hereditary stomatocytosis (also referred to as hereditary xerocytosis), and overhydrated hereditary stomatocytosis are less common RBC membrane disorders, and result from mutations leading to abnormal expression of band 3, PIEZO1 (mechanosensitive cation channel), and Rhesus-associated glycoprotein (RhAG), respectively.<sup>64</sup> Southeast Asian ovalocytosis (SAO), hereditary elliptocytosis, and possibly hereditary spherocytosis and hereditary xerocytosis may confer protection against malaria. SAO may confer protection against cerebral malaria in patients infected with *P. falciparum*,<sup>65</sup> and may also provide resistance to high levels of parasitemia with *P. falciparum*, *P. vivax*, and *P. malariae*.<sup>66,67</sup> Evidence for protection against malaria by HE largely comes from epidemiological data that HE is found in high prevalence in regions of Africa where malaria is endemic.<sup>68</sup> In vitro data have demonstrated diminished invasion and impaired parasite growth of infected HE erythrocytes possibly due to abnormal protein 4.1/glycophorin C/p55 complexes in HE.<sup>69,70</sup> There is no published epidemiological evidence for a protective role against malaria conferred by HS. In vitro data and ankyrin/spectrin deficient mouse model experiments have demonstrated impaired parasite invasion and/or growth in HS erythrocytes largely attributed to α-spectrin deficiency.<sup>71,72</sup> It has recently been demonstrated that a novel gain-of-function PIEZO1 polymorphism (E756del) with an allele frequency of 18% in the African population is associated with xerocytosis-like RBC dehydration and protection from *P. falciparum* in vitro.<sup>73</sup>

### Blood groups

Some human blood group antigens serve as either receptors for the malaria parasite or are involved in the rosetting of infected erythrocytes, which is responsible for red cell sequestration in the microvasculature and severe malaria. The antigens which have been shown to have an effect on malaria infection include the Duffy blood group as well as those on glycophorins (i.e., MNS, Gerbich), the Knops blood group, and the ABO blood group system.

### Duffy blood group

The Duffy glycoprotein, also known as the Duffy antigen receptor for chemokines (DARC), is a receptor that binds C-X-C and C-C class chemokines as well as expresses six separate blood group antigens on the RBC surface. The Fy<sup>a</sup> and Fy<sup>b</sup> antigens are encoded by codominant *FY\*A* (*FY\*01*) and *FY\*B* (*FY\*02*) alleles. Fy<sup>a</sup> and Fy<sup>b</sup> differ by one amino acid at position 42 (Fy<sup>a</sup>: glycine; Fy<sup>b</sup>: aspartic acid), which defines the three predominant phenotypes: Fy(a+b–), Fy(a–b+), and Fy(a+b+).<sup>74</sup> A fourth common Duffy phenotype, Fy(a–b–), is the result of a mutation (–67T>C) in the erythroid-specific promoter of the GATA-1 transcription factor region; this mutation blocks the expression of the Duffy antigen specifically on red cells. The mutation is in the *FYB* allele, designated erythroid silent: *FYB<sup>ES</sup>* (ISBT allele: *FY\*02N.01*). The erythroid silent mutations does not affect Duffy antigen expression on other somatic cells. As a result, patients carrying *FYB<sup>ES</sup>* are not at risk for anti-Fy<sup>b</sup> and do not require Fy<sup>b</sup> negative RBCs for transfusion. The Fy(a–b–) phenotype is rare in Caucasians but has a prevalence of 67% in African Americans and reaches 100% in much of sub-Saharan Africa<sup>75</sup> (see Table 30.1). The Duffy antigen is the receptor for *P. vivax* (also known as the *P. vivax* Duffy binding protein [PvDBP]) and *P. knowlesi* allowing entry of the parasite into the red blood cells. As such, Fy(a–b–) individuals have been shown to be resistant to infection.<sup>76–78</sup> Recently, it has been appreciated that *P. vivax* is able to induce clinical malaria in Duffy-negative people, although the underlying mechanisms are still not understood.<sup>79</sup>

### Glycophorin C and D (Gerbich blood group)

The Gerbich (Ge) blood group includes 11 antigens and is expressed on glycophorin C and glycophorin D. Both glycophorins are encoded by the *GYPC* gene that is located on chromosome 2, 2q14>2q21. There is no homology with the glycophorins of the MNS system. There are two initiation codons: initiation at the first codon results in glycophorin C, while initiation at the second codon results in glycophorin D. Deletion of exon 3 in the *GYPC* gene results in Ge negativity. This phenotype has reached almost 50% prevalence in coastal areas of Papua New Guinea where malaria is endemic, likely due to the fact that glycophorin C is one of the receptors of *P. falciparum* (EBA-140).<sup>80</sup> Glycophorin C also interacts with the 4.1R protein, stabilizing the red cell membrane. A reduction of these antigens leads to hereditary elliptocytosis, which has also been associated with protection against malaria (see above). Furthermore, decreased intraerythrocyte parasite growth and adhesion of malaria-infected red cells to the vascular endothelium may be compromised in Ge negative individuals due to destabilization of the protein 4.1/glycophorin C/p55 complex.<sup>69,70</sup>

**Table 30.1** Prevalence of Duffy Antigens

Red Cell Phenotype	Prevalence %		
	Caucasians	African Americans	Allele
Fy (a+b–)	20**	10**	<i>FY*A/FY*A</i> (or <i>FY*01/FY*01</i> )
Fy (a–b+)	32	20	<i>FY*B/FY*B</i> (or <i>FY*02/FY*02</i> )
Fy (a+b+)	48	3	<i>FY*A/FY*B</i> (or <i>FY*01/FY*02</i> )
Fy (a–b–)	Rare	67%	<i>FY*02N.01/FY*02N.01</i>

\*\* 70–90% of Asians have this phenotype; *FY\*02N.01* is the notation for the erythroid silent GATA mutation *FYB<sup>ES</sup>* primarily seen in individuals of African descent.

## Glycophorin A and B (MNS blood group system)

The MNS blood group system includes three red cell antigens on two glycophorins. M and N antigens are found on glycophorin A, and S/s and U antigens are on glycophorin B. These two glycophorins carry two receptors for *P. falciparum*: EBA-175 on glycophorin A and EBA-1 on glycophorin B (see Table 30.2). The MNS system is second in complexity only to the Rh system, with unequal crossovers, deletions, gene conversions, and point mutations leading to 46 different antigens. The S-s-U-phenotype is found in up to 5% of Africans. Glycophorin B-deficient S-s-U- erythrocytes have been shown to be relatively resistant to *P. falciparum* invasion,<sup>81</sup> strongly suggesting malaria may have provided a selective force driving the expansion of this polymorphism. A novel malaria resistance gene in the glycophorin gene cluster has been described, which results in the loss of GYPB and gain of two GYPB-A hybrid genes encoding the Dantu blood group antigen. This antigen results from a glycophorin protein that contains the extracellular domain of glycophorin B and the transmembrane and intracellular domains of glycophorin A.<sup>82</sup> Dantu antigen homozygosity confers a strongly protective effect against severe malaria from *P. falciparum* due to inhibition of parasite invasion mediated by increased red cell membrane tension rather than receptor-ligand interactions.<sup>7</sup> Interestingly, the Dantu polymorphism is found at highest frequencies in East Africa, but is rare or absent in other malaria endemic regions within Africa, suggesting that the glycophorin gene may be subject to a number of opposing evolutionary driving forces.<sup>7,16</sup>

## Knops blood group

The Knops blood group is found on the complement receptor 1 (CR1), a glycoprotein present on the erythrocyte as well as other circulating blood cells. CR1 is one of a group of membrane proteins termed the *regulators of complement activation*. Other members of this group include decay accelerating factor (DAF/CD55), membrane cofactor protein (CD46), and membrane inhibitor of reactive lysis (MIRL/CD59). CR1 also binds C4b/C3b immune complexes for transfer to the liver or spleen where the immune complexes are ingested by macrophages, returning the red cells to the circulation. CR1 has a binding site for *P. falciparum* (through the PfRh4 parasite protein), allowing malaria entry into the host cell through a glycophorin-independent pathway.<sup>83</sup> CR1 is also involved in red cell rosetting of uninfected red cells to *P. falciparum*-infected cells via interactions with PfEMP1.<sup>84</sup> McCoy (McC) and Swain-Langley (SL) are two antigens within the Knops system which have been associated with mutations of CR1 and show marked frequency differences between Caucasians and Africans.<sup>85</sup> The McCb and the SL2 antigens are present almost exclusively in individuals of African ethnicity and may provide protection from *P. falciparum*.<sup>86,87</sup>

## ABO, Lewis blood groups

There is strong evidence that *P. falciparum* has influenced the distribution of the ABO blood groups to favor group O in regions where malaria is endemic. Blood group O is more common in equatorial

endemic malarial regions, whereas group A is more common in northern, nonendemic latitudes.<sup>88</sup> Multiple case-control studies in Africa<sup>16,89-93</sup> and a recent meta-analysis<sup>94</sup> have conclusively shown that blood group O confers protection from severe malaria from *P. falciparum*; in contrast, group A has been associated with the highest risk for severe disease.<sup>95</sup> This effect is due to the fact that A and B blood group antigens serve as coreceptors for PfEMP-1 on infected erythrocytes. PfEMP-1 binding to group A (or to a lesser extent, group B) contributes to rosetting of parasitized and uninfected red cells, and vascular cytoadherence. In vitro data have demonstrated higher *P. falciparum*-induced rosette number, size, and stability in blood groups A, B, and AB compared to blood group O red cells.<sup>96,97</sup> Inhibition of this effect can be demonstrated in vitro using soluble A and B antigen, blocking the PfEMP-1 receptor.<sup>96</sup> Inhibition may also occur in vivo due to the effect of the Lewis and Secretor gene transferase phenotype in non-O blood type individuals.<sup>88</sup> These transferases directly influence the quantity of soluble A and B antigens found in the plasma of group A and B individuals. The highest levels of soluble A and B antigens, a phenotype that may confer protection from *P. falciparum*, are seen among those expressing the combination of Le(a-b-) and Secretor-positive (Se) genes. This may account for the threefold higher prevalence of Le (a-b-) phenotype observed in individuals with African ancestry.<sup>88,98</sup>

## Sickle cell disease

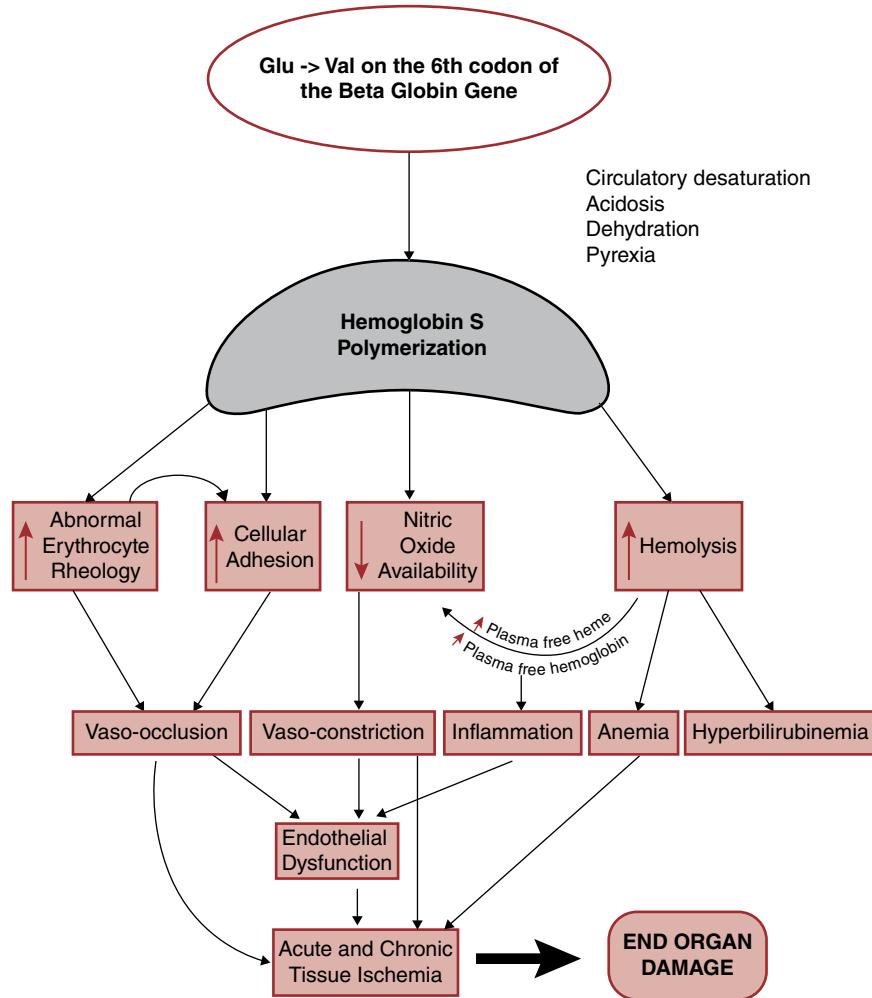
### Pathophysiology

Homozygous HbS results in hemoglobin polymerization at low oxygen tension, low pH, and with temperature elevation. HbS polymerization disrupts normal red cell membrane structure, leaving phosphatidylserine exposed on the surface of the red cell which promotes erythrocyte adherence to the vascular endothelium.<sup>99,100</sup> The distorted red cell is also rigid, predisposing to hemolysis. When sickle erythrocytes lyse, they release arginase, free hemoglobin, and heme (hemin) into the plasma, depleting nitric oxide.<sup>101</sup> As endogenous nitric oxide plays an important role in maintaining vessel wall patency, and nitric oxide depletion leads to vascular dysfunction in people with SCD.<sup>102</sup> The hemin released during hemolysis also activates toll like receptor 4 (TLR-4) and other signaling molecules, leading to proinflammatory responses.<sup>103</sup> Altered interactions between the vascular endothelium, neutrophils, platelets, and sickled erythrocytes have been identified as direct promoters of sickle-related vaso-occlusions and inducers of a chronic inflammatory state in people with SCD.<sup>104</sup> These changes in the red cell membrane, the activation of cellular elements, and changes in the vascular endothelium account for many of the clinical findings in SCD (see Figure 30.1).

HbS can also polymerize in the presence of other hemoglobin variants. These combinations are collectively referred to as SCD if they cause or have the potential to cause clinical symptoms. The most common forms of SCD are HbSS, HbSC, HbS $\beta^0$ -thalassemia, and HbS $\beta^+$ -thalassemia. SCD severity varies among these genotypes, with HbSS and HbS $\beta^0$ -thalassemia (sickle cell anemia [SCA]) classically having more serious complications compared to patients with HbSC or HbS $\beta^+$ -thalassemia. There are at least 14  $\beta^+$ -mutations commonly occurring in African Americans, which can produce a wide range of hemoglobin A concentrations in the red cell and influence the clinical disease phenotype.<sup>105</sup>

**Table 30.2** Plasmodium Receptors

Plasmodium sp.	Parasite Receptor	Red Cell Receptor
<i>P. vivax</i>	PvDBP	Duffy A, Duffy B
<i>P. falciparum</i>	EBA-175	Glycophorin A (MN)
<i>P. falciparum</i>	EBA-140	Glycophorin C (Gerbich)
<i>P. falciparum</i>	EBA-1	Glycophorin B (Ss, U)
<i>P. falciparum</i>	PfRh4, PfEMP1	Knops (CR1)
<i>P. falciparum</i>	PfEMP-1	ABO (A, B, AB)



**Figure 30.1** Pathophysiology of sickle cell disease. The pathophysiology of sickle cell disease is complex and stems from the polymerization of HbS that occurs during periods of hypoxemia, dehydration, acidosis, and pyrexia. Polymers of sickle hemoglobin cause the characteristic shape change of the erythrocyte and lead to hemolysis, abnormal rheology, cellular adhesion, and decreased nitric oxide availability. These changes result in vaso-occlusion, vaso-constriction, anemia, and chronic inflammation that contribute to SCD-associated end organ damage.

Furthermore, clinical complications can vary dramatically even among patients with the same genotype,<sup>106</sup> which has led to a decades-long ongoing search for global SCD severity predictors. Modifiers of SCD include  $\alpha$ -thalassemia, fetal hemoglobin (HbF), nutritional, and environmental factors.<sup>107-109</sup> In a recent systematic review of SCD severity predictors,  $\alpha$ -globin gene number was the most commonly studied severity predictor, followed by HbF and reticulocyte count. Coinheritance of  $\alpha$ -thalassemia trait was protective against overt stroke and abnormal transcranial Doppler (TCD) in all but one study, but not frequency of painful crisis or silent cerebral infarct. Fetal hemoglobin (HbF) is the only hemoglobin that is known to inhibit HbS polymerization, and thus its expression can reduce disease severity.<sup>110</sup> Two-thirds of the HbF studies reported beneficial effects with increasing HbF levels; however, increased HbF levels were not associated with lower hospitalization or stroke rates in other reports. The ability to predict SCA complications was mixed for all variables, except TCD and absolute reticulocyte count,<sup>107</sup> and TCD is the only currently available validated predictor of severe outcomes in SCA.<sup>111</sup> TCD, however, only identifies children with SCA who are at the highest risk of stroke; thus far predic-

tors for recurrent painful episodes or acute chest syndrome are not available. An elevated absolute reticulocyte count (ARC) between two and six months of age was associated with cerebral vasculopathy (including stroke), early hospitalization for VOE, and splenic sequestration.<sup>112-114</sup> Genome-wide association studies have looked for single nucleotide polymorphisms associated with severity, but most of these polymorphisms do not appear clinically relevant.<sup>107</sup>

## **Indications for transfusion**

Red cell transfusion is an effective therapy for SCD, but there is a paucity of randomized clinical trials to guide practice. The transfusion complications outlined later in this chapter highlight the need for evidence based indications for transfusion in SCD. Three sets of guidelines have been released in the past seven years from the National Heart Lung and Blood Institute (NHLBI),<sup>115</sup> the British Committee for Standards in Hematology (BCSH),<sup>116</sup> and the American Society of Hematology (ASH)<sup>117,118</sup> to guide transfusion decisions in individuals with SCD. Table 30.3 provides a summary of these consensus guidelines for acute and chronic transfusions.

**Table 30.3** SCD Transfusion Indications

Indication	Transfusion Method	Guideline
<b>Generally Accepted Indications for Transfusion</b>		
• Acute ischemic stroke	Exchange transfusion	BCSH, ASH <sup>N</sup> , NHLBI
• Primary stroke prevention	Chronic simple or exchange transfusion	NHLBI, ASH <sup>N</sup> , ASH <sup>T</sup> , BCSH
• Secondary stroke prevention	Chronic simple or exchange transfusion	BCSH, ASH <sup>N</sup> , ASH <sup>T</sup> , NHLBI
• Acute chest syndrome (acute)	Simple or exchange transfusion <sup>a</sup>	BCSH, ASH <sup>T</sup> , NHLBI
• Acute splenic sequestration	Simple transfusion	BCSH, NHLBI
• Acute splenic sequestration, recurrence	Chronic simple transfusion, prior to splenectomy <sup>b</sup>	NHLBI
• Preoperative	Simple transfusion	BCSH, ASH <sup>T</sup> , NHLBI
• Transient aplastic crisis	Simple transfusion	BCSH, NHLBI
• Acute multiorgan failure syndrome	Simple or exchange transfusion <sup>a</sup>	BCSH, NHLBI
• Acute hepatic sequestration	Simple transfusion	BCSH, NHLBI
• Acute intrahepatic cholestasis	Simple or exchange transfusion <sup>a</sup>	BCSH, NHLBI
• Acute sickle/obstetrical complications during pregnancy	Simple or exchange transfusion <sup>a</sup>	BCSH, ASH <sup>T</sup>
<b>Possible Indications for Transfusion</b>		
• Recurrent Acute Chest Syndrome	Chronic simple or exchange transfusion <sup>c</sup>	BCSH
• Recurrent painful vaso-occlusive episodes	Chronic simple or exchange transfusion <sup>c</sup>	BCSH
• Pulmonary hypertension	Chronic simple or exchange transfusion <sup>c</sup>	BCSH, ATS
• Uncomplicated pregnancy	Chronic simple or exchange transfusion <sup>c</sup>	BCSH, ASH <sup>T</sup>
• Central retinal artery occlusion	Simple or exchange transfusion for arterial infarct	None <sup>d</sup>
<b>Transfusion Generally Not Indicated</b>		
• Acute, uncomplicated painful vaso-occlusive episode	NA	BCSH, NHLBI
• Priapism	NA	BCSH, NHLBI
• Leg ulcers	NA	None <sup>d</sup>
• Nonsurgical avascular necrosis	NA	None <sup>d</sup>

<sup>a</sup> Exchange transfusion should be performed in rapidly deteriorating patients or when there are concerns for post-transfusion hyperviscosity due to high pretransfusion hemoglobin of  $\geq 9$  g/dL.

<sup>b</sup> Chronic transfusion may be utilized to delay splenectomy in children less than two years of age who are at high risk of invasive pneumococcal disease.

<sup>c</sup> Exchange transfusion preferred in patients with iron overload.

<sup>d</sup> There is insufficient evidence for benefit to make recommendations about using transfusion therapy to manage complication. National Heart, Lung, and Blood (NHLBI);<sup>115</sup> British Committee for Standards in Hematology (BCSH);<sup>116</sup> American Society of Hematology ASH<sup>N</sup><sup>118</sup> and ASH<sup>T</sup><sup>117</sup> American Thoracic Society (ATS);<sup>158</sup> NA: not applicable.

## Cerebrovascular disease

**Acute stroke treatment:** Stroke risk for children with SCA is 200 times that of the general pediatric population (see Table 30.4).<sup>119,120</sup> Prior to annual TCD screening, 10% of children suffered from an overt stroke before the age of 20 years.<sup>121</sup> Stroke risk continues at older ages, with hemorrhagic stroke more common between the ages of 20 and 30 years.<sup>122</sup> It is recommended that children and adults with SCD and an acute ischemic stroke should receive an emergent red cell exchange (RCE), preferably by erythrocytapheresis, with a postexchange target HbS <20%; total Hb should not exceed 11 g/dL to avoid hyperviscosity.<sup>118</sup> A simple transfusion may be considered for immediate treatment if there are delays expected in establishing central venous access, procuring and crossmatching multiple RBC units, and mobilizing the apheresis team for erythrocytapheresis. There is a lack of formal studies to guide management of hemorrhagic strokes in individuals with SCD. However, in most cases, RCE is employed as ancillary treatment to standard therapy for acute intracranial hemorrhage for the general population; in this setting, RCE is guided by similar postexchange targets as described for ischemic strokes.<sup>123</sup>

## Primary stroke prevention

Overt strokes have become less common in children and young adults since the institution of TCD screening. TCD measures the flow velocity through the cerebral arterial circulation. Increased flow velocity in the internal carotid or middle cerebral arteries ( $\geq 200$  cm/second) is strongly associated with increased risk of stroke.<sup>124</sup> In the Stroke Prevention Trial in Sickle Cell Anemia (STOP trial), chronic blood transfusions aimed at maintaining HbS concentration to 30% or less decreased the stroke rate from 10% to less than 1% in children who had two consecutive abnormal

**Table 30.4** Stroke Risk in Children with SCA

Condition	Annual Stroke Risk (%)
Healthy children (without SCA or congenital heart disease) <sup>120</sup>	0.003
Children with SCA (HbSS) <sup>121</sup>	0.5–1
Children with SCA and SCI on MRI <sup>130</sup>	2–3
Children with SCA and abnormal TCD <sup>111</sup>	
• Without chronic transfusion therapy	10–13*
• With chronic transfusion therapy	~1
Children with SCA and previous overt stroke <sup>134</sup>	
• Without chronic transfusion therapy	~25–30†
• With chronic transfusion therapy	~6†
Children with SCA, previous overt stroke, and with progressive cerebral vasculopathy despite chronic transfusion therapy <sup>133</sup>	~9‡

SCA sickle cell anemia; SCI, silent cerebral infarction; MRI, magnetic resonance imaging; TCD, transcranial doppler ultrasonography.

\* For the first 3–4 years following the abnormal TCD, and then stroke-free survival plateaus at 60–70%.

† For the first 3–4 years following the overt stroke.

‡ For the first five years following the overt stroke. Source: Fasano et al. (2015).<sup>119</sup> Reprinted with permission of Elsevier.

TCD results.<sup>111</sup> Prior to the publication of the TCD With Transfusions Changing to Hydroxyurea (TWiTCH) study results in 2016, chronic transfusions and chelation therapy were continued indefinitely because the STOP 2 (Optimizing Primary Stroke Prevention in Sickle Cell Anemia) trial demonstrated that discontinuation of chronic transfusions caused reversion to abnormal TCD values and, in some cases, overt stroke.<sup>125</sup> The TWiTCH trial randomized children with abnormal TCDs who had received at least one year of chronic transfusions and who did not have severe cerebral vasculopathy<sup>126</sup> to either continue transfusion therapy and

iron chelation or change to hydroxyurea (HU) and phlebotomy. In the alternate HU + phlebotomy study arm, transfusions were weaned over 4–9 months as HU dose was escalated to maximum tolerated dose (MTD). Phlebotomy commenced once transfusions had stopped, and patients were monitored by TCD every three months to ensure that TCD velocities did not revert to abnormal. The study was terminated by the NHLBI when the first interim analysis showed noninferiority of HU and phlebotomy. This study established HU as an acceptable substitute for primary stroke prevention in children with abnormal TCD without severe vasculopathy after at least one year of chronic transfusion therapy.<sup>127</sup>

Thirty percent of children with SCA will have evidence of silent cerebral infarcts (SCIs; silent strokes) on MRI,<sup>128</sup> with prevalence increasing to as high as 50% in adults.<sup>129</sup> Although individuals with SCIs appear normal on physical examination and by history, they are at increased risk of poor academic achievement, lower IQ, as well as future overt stroke and new SCIs.<sup>130,131</sup> The Silent Cerebral Infarct Multi-Center Clinical Trial (SIT) trial randomized children with SCA who had at least one SCI ( $\geq 3$  mm in two planes on T2-weighted MRI images) to either chronic transfusion therapy or observation (standard of care at the time of the study). Study accrual occurred from 2004 to 2010, when early institution of HU was not the standard of care for children with SCA. The incidence of recurrent cerebral infarcts was reduced in those who received chronic transfusions.<sup>132</sup> However, given a host of factors including a relatively high number needed to treat to prevent recurrent infarcts, the unknown optimum age and frequency for MRI screening, and the widespread prescription of HU in children with SCA with SCI since the initiation of SIT, the role of chronic transfusion therapy for children with SCI remains ill-defined.

### Secondary stroke prevention

In comparison to primary stroke prevention, chronic transfusion therapy is less effective in preventing future strokes and progressive cerebral vasculopathy in patients with a history of overt strokes<sup>133,134</sup> (see Table 30.4). In a retrospective cohort study of 137 children with SCD and history of overt stroke, 22% of patients had a second stroke at a mean follow-up of 10 years.<sup>135</sup> However, this is a significant reduction from a 60% to 90% historical recurrence rate in patients without therapeutic intervention.<sup>136</sup> Although no prospective randomized clinical trial has been completed, a large retrospective observational study demonstrated a significant reduction in the rate of recurrent stroke in patients treated with chronic transfusions to maintain pretransfusion HbS level <30%.<sup>134</sup> As a result, it is recommended that children and adults who have a history of overt stroke should be placed on chronic transfusion therapy.<sup>118,137</sup>

The randomized phase 3 trial, Stroke with Transfusion Changing to Hydroxyurea (SWiTCH), attempted to address whether transition to HU was a safe alternative to chronic transfusions for patients with a history of stroke and iron overload. SWiTCH compared HU + phlebotomy (alternative therapy) to transfusion therapy + chelation (standard therapy) in children with SCD and history of overt stroke. The trial was closed early because of statistical futility on the composite endpoint of iron overload improvement and stroke prevention. At the time of study closure, equivalent liver iron concentrations were seen in both treatment groups, but an increase in recurrent strokes was observed in the HU/phlebotomy arm (7; 10%) compared to the transfusion/chelation arm (0%).<sup>138</sup> Taken together, chronic transfusion with chelation remains the mainstay of secondary stroke prevention in children with SCA.<sup>138</sup> It should be noted that RCE can also eliminate or decrease iron burden in

patients with overt stroke on chronic transfusion<sup>139,140</sup> (see the Erythrocytapheresis section).

When using chronic transfusions for stroke prevention, it is recommended to administer transfusions (simple or exchange) every 3–4 weeks to maintain a pretransfusion HbS level <30% and Hb level >9.0 g/dL.<sup>118</sup> The post-transfusion Hb level target should not exceed 12 g/dL to avoid the potential for increased morbidity due to hyperviscosity.

### Acute chest syndrome

Acute chest syndrome (ACS) is defined as a new infiltrate on chest X-ray in a febrile person with SCD who has at least one respiratory symptom (most commonly cough, chest pain, and shortness of breath).<sup>141</sup> ACS is the second most common cause of hospitalization and the leading cause of death in individuals with SCD.<sup>142</sup> The clinical severity of ACS can range from an incidental finding on chest X-ray, identified after an individual presents with fever and cough, to a rapidly progressive respiratory failure that requires intubation and RCE. The rapidly progressive subtype of ACS has recently been described as a distinct phenotype of the illness that is frequently preceded by thrombocytopenia and often progresses to multisystem organ failure. This rapidly progressive phenotype is more commonly seen in adults compared to children and requires aggressive transfusion support given its high mortality (6.3%).<sup>143</sup>

The management of ACS is primarily supportive and includes respiratory therapy, antibiotics, and often RBC transfusion. Much of the data used to guide transfusion decisions for ACS treatment come from retrospective reports including a large ACS epidemiologic study showing that red cell transfusions improve oxygenation in SCD patients with ACS.<sup>144</sup> There have been no randomized controlled trials comparing either simple transfusion or RCE transfusion versus no transfusion for ACS, nor whether simple transfusion versus RCE results in improved patient outcomes. In clinical practice, patients with milder degrees of hypoxia and/or low Hb often receive simple transfusions, whereas RCE is commonly reserved for more severe cases. Guidelines from the NHLBI, ASH, and BCSH generally recommend simple transfusion for patients with ACS with hypoxia or an Hb concentration significantly below their baseline. RCE is recommended for patients with more severe presentations and/or rapid progression of ACS manifested as increasing respiratory distress or progressive pulmonary infiltrates requiring invasive respiratory support, and/or declining hemoglobin concentration despite simple transfusions.<sup>115,117,141</sup> Patients with milder ACS but an Hb >9 g/dL (i.e., HbSC patients) may require RCE as the initial therapy.

### Aplastic crisis and symptomatic anemia

Parvovirus B-19 can cause transient red cell aplasia (also called aplastic crisis) and symptomatic anemia in individuals with SCD. Reticulocytopenia accompanying a drop in hemoglobin is the hallmark for this complication. Simple red cell transfusion may be necessary depending on the severity of the anemia, the clinical status of the patient, and the degree of reticulocytopenia.<sup>145</sup> Because the anemia is often subacute, patients are typically euvolemic and physiologically compensated. In these cases, RBC transfusion should be administered slowly with serial small aliquots to prevent circulatory overload. Aplastic crisis due to Parvovirus typically does not recur because of sustained protective immune response even in patients on HU.<sup>146</sup>

Patients with severe symptomatic anemia (usually a hemoglobin of 5 g/dL or less, depending on the patient's baseline hemoglobin)

should be transfused to improve oxygen delivery and prevent complications of anemia. The transfusion goal for symptomatic anemia should be the patient's baseline hemoglobin (provided that patient has an adequate reticulocyte count).<sup>147</sup> Transfusion is not indicated in anemic patients who are asymptomatic with a brisk reticulocytosis. Etiologies for the severe anemia should be sought, and the patient should be closely monitored.

### Acute splenic sequestration crisis

Acute splenic sequestration crisis (ASSC) refers to an acute condition of intrasplenic sickling and pooling of large amounts of blood that most commonly occurs in children between ages five months and two years. During ASSC, the blood-filled spleen may enlarge to the point of filling the entire abdomen and the hemoglobin may drop acutely. This requires prompt treatment with volume expanders and simple blood transfusion to remobilize the blood sequestered in the spleen. Care must be taken to avoid overtransfusion that can lead to hyperviscosity-related morbidity when treating sequestration. Overtransfusion can occur when blood is released from the congested organ, increasing the hematocrit (Hct) to dangerously high levels. Recurrent episodes of ASSC occur in 50–75% of patients with the risk highest when the first episode occurs before two years of age.<sup>148</sup> The utility of temporary chronic transfusions in patients with recurrent ASSC is debatable. The NHLBI 2014 Evidence Based Management Guidelines do not recommend initiating chronic transfusions in children with recurrent ASSC, albeit recognizing the low quality of evidence available to guide this recommendation.<sup>115</sup> However, chronic transfusion therapy is often used to delay definitive treatment of splenectomy in very young children with recurrent ASSC until at least two years of age when the pneumococcal polysaccharide vaccine (PPSV23) can be administered.

### Sickle hepatopathy

Hepatobiliary complications are common in individuals with SCD. They can range from asymptomatic cholelithiasis; to acute hepatic sequestration manifesting as painful hepatomegaly, worsening anemia, reticulocytosis, thrombocytopenia, direct hyperbilirubinemia, and mild transaminitis; to sickle cell intrahepatic cholestasis (SCIC), a life-threatening manifestation associated with hepatomegaly, severe direct hyperbilirubinemia, significantly elevated transaminases, coagulopathy, renal insufficiency, and acute liver failure in severe cases.<sup>149,150</sup> Hepatic sequestration is often treated with simple transfusions to resolve the hepatopathy and correct the anemia. Similarly to ASSC, transfusions should be performed carefully to avoid overtransfusion and hyperviscosity.<sup>116</sup> Although SCIC outcome data are limited, transfusions, particularly RCE, appear to provide a benefit for severe cases<sup>151</sup> and are recommended in the management of acute SCIC.<sup>115,116</sup> Correction of coagulopathies with plasma, cryoprecipitate, and platelet transfusions may also be necessary. Chronic RCE has been proposed for patients with recurrent episodes of acute SCIC or chronic progressive hepatopathy.<sup>152</sup>

### Preoperative

When SCD patients undergo procedures, a myriad of perioperative scenarios exist that can increase erythrocyte sickling and place these patients at increased risk for morbidity. These include suboptimal hydration, poor oxygenation, and acidemia. The Transfusion Alternatives Preoperatively in SCD (TAPS) trial compared outcomes of preoperative transfusion to no transfusion

in patients with HbSS/HbS $\beta^0$  undergoing low- or medium-risk surgery and demonstrated that preoperative transfusion was associated with decreased perioperative complications. Specifically, 13 of 33 (39%) individuals who did not receive preoperative transfusion experienced postoperative complications compared to 5 of 34 (15%) patients who received preoperative transfusion. Ten of eleven serious adverse events were ACS: nine in the no-transfusion group and one in the transfusion group. The trial did not include individuals with HbSC or S $\beta^+$ -thalassemia, and low-risk procedures were too low in number to clarify optimal management for this surgical category.<sup>153</sup>

In a previous randomized control trial, preoperative simple transfusion with a post-transfusion Hb target of 10 g/dL was found to be equally effective in preventing postoperative complications compared to RCE which targeted a postexchange HbS  $\leq 30\%$ .<sup>154</sup> Based on this study, it is recommended that individuals with SCD with Hb less than 9 g/dL, and who are undergoing any surgical procedures determined to be medium- or high-risk, or with an anesthesia time of one hour or more, should receive preoperative transfusion. Post-transfusion Hb should not exceed 11 g/dL to avoid hyperviscosity. RCE should be considered in patients undergoing high-risk surgeries (i.e., neurosurgery or cardiac surgery, or general anesthesia time expected to be greater than four hours) or in HbSS/HbS $\beta^0$  patients undergoing medium-to-high risk surgery whose baseline Hb is  $\geq 10$  g/dL.<sup>116,117,147</sup> The utility of preoperative transfusion in patients undergoing low-risk surgeries (i.e., tympanostomy tubes when anesthesia time <1 hour), patients with milder genotypes (HbSC and HbS $\beta^+$ ), or those with a higher hemoglobin ( $\geq 10$  g/dL) or HbF level due to HU treatment remains unclear. Therefore, preoperative transfusion management decisions should be individualized in these circumstances.<sup>147</sup>

### Indeterminate indications for transfusion

#### Pregnancy

Pregnancy carries a high risk for women with SCD and their fetuses. Pregnant women with SCD should receive red cell transfusions for acute SCD complications where transfusions are usually indicated. Further, if the woman was receiving chronic transfusions prior to conception, they should be continued. There is no clear evidence that prophylactic transfusions improve fetal outcome, but they may reduce pain crises and other SCD complications for the mother.<sup>147</sup> There is currently insufficient evidence to recommend universal prophylactic chronic transfusions for pregnancies in patients with SCD. However, transfusions should be considered for women with previous severe SCD-related complications (including during previous pregnancies), women on HU prior to conception due to severe disease, or additional features of high-risk pregnancy (i.e., additional comorbidities or twin pregnancy).<sup>116,117,147</sup> A multicenter feasibility trial comparing standard of care to RCE every 6–10 weeks starting before 18 weeks gestation is ongoing and intends to assess willingness of pregnant women with SCD to participate in a chronic RCE regimen and to evaluate maternal and fetal outcomes.<sup>155</sup>

#### Pulmonary hypertension

Pulmonary arterial hypertension (PAH) is common in adults with SCD; right heart catheterization is the gold standard for diagnosing PAH. However, an elevated tricuspid regurgitant jet velocity (TRV) of 2.5 m/second or higher has been associated with a higher risk of death in adults with SCD.<sup>156</sup> N-terminal-pro B-type natriuretic peptide (NT-proBNP) levels have also been correlated to pulmonary artery pressures. Two large registries identified that adults with

SCD with elevated TRV and NT-proBNP levels had over 10-time increased mortality rates compared to adults with normal values for both markers.<sup>157</sup> Transfusions may prevent chronic regional hypoxia, improve nitric oxide depletion, and improve endothelial dysfunction in the pulmonary vascular bed, all of which would theoretically improve pulmonary arterial pressures.<sup>123</sup> No randomized controlled trials of HU or chronic transfusion therapy for treatment of PAH in adults with SCD have been published. Despite this, based on the benefits of HU in reducing mortality in SCD and the potential of chronic transfusions to mitigate deleterious pulmonary endothelial effects, the American Thoracic Society (ATS) has promoted the use of HU for patients with SCA who have a TRV of 3.0 m/second or greater or a TRV of >2.5 m/second with either an elevated NT-pro-BNP level or RHC-confirmed PAH. The ATS also proposed that chronic transfusions be administered to patients who are not responsive to, or not candidates for, HU.<sup>158</sup> A Phase 3, multicenter, randomized controlled trial of chronic RCE versus standard of care in adults with SCD at high-risk of mortality (based on PAH risk factors or the presence of renal disease) is ongoing.<sup>159</sup> While awaiting the results of this trial, chronic transfusions for adults with PAH may be considered after weighing the risks and benefits of this therapy.

### Ophthalmologic complications

Two common ophthalmological complications of SCD include proliferative sickle retinopathy and acute retinal artery occlusion. Over the age of 30 years, 43% of HbSC patients and 13% of HbSS patients will develop retinopathy, which can lead to significant visual loss from vitreous hemorrhage, and retinal ischemia and detachment.<sup>160</sup> Central retinal artery occlusion (CRAO) is a rare cause of acute blindness almost exclusively in children and young adults with SCD and presents as a sudden, painless loss of vision.<sup>161,162</sup> Because the vision loss is often permanent if macular infarction occurs, individuals with SCD who present with these signs and symptoms often are treated with exchange transfusion despite the lack of randomized controlled trials determining if long-term vision loss is prevented with RCE. Since the central retinal artery is a branch of the ophthalmic artery that originates from the internal carotid artery, some consider CRAO a variation of an acute ischemic stroke and support chronic transfusion therapy in patients with a history of CRAO despite the lack of published evidence. Similarly, chronic transfusions are sometimes considered for patients with severe proliferative sickle retinopathy with vision loss despite a lack of published evidence.

### Contraindications for transfusion

Since SCD is a hematologic disorder, any complication could theoretically be improved by transfusion. However, the benefits of transfusion therapy must be balanced with the risks, which are numerous for individuals with SCD and are highlighted later in this chapter. Transfusions for acute and/or chronic SCD manifestations where there is either insufficient evidence of benefit, or possibly increased risks, are generally not recommended and should be based on a case-by-case assessment after full risk–benefit analysis. Examples where there is little-to-no evidence of benefit of transfusions include treating patients with asymptomatic anemia, uncomplicated painful vaso-occlusive episodes, acute or recurrent priapism, recurrent splenic sequestration (in children >2 years of age), acute kidney injury (in the absence of multisystem organ failure), avascular necrosis (except when preoperative transfusion is needed for surgical intervention), and leg ulcers.<sup>115,116</sup>

### Thalassemia

Thalassemia is the most common monogenetic diseases worldwide.<sup>163</sup> These syndromes are classified according to whether the mutation causing the defect affects the  $\alpha$ -globin gene cluster or the  $\beta$ -globin gene cluster. Duplicated  $\alpha$ -gene clusters occur on chromosome 16 ( $\alpha\alpha/\alpha\alpha$ ). There is one  $\beta$ -globin gene cluster on each chromosome 11 ( $\beta/\beta$ ). Mutations in the globin genes reduce or eliminate the synthesis of the respective globin molecule. The majority of  $\alpha$ -gene mutations are deletional, although structural mutations occur. There are over 120  $\alpha$ -gene mutations reported.  $\beta$ -Globin mutations are primarily point mutations leading to absent  $\beta$ -globin synthesis ( $\beta^0$ -mutations) or decreased  $\beta$ -globin synthesis ( $\beta^+$ -mutations). There are well over 350  $\beta$ -globin gene mutations. Structural variants of the globin genes can lead to thalassemic gene combinations. There are also deletions of  $\beta$ -globin regulatory elements and the other genes in the  $\beta$ -globin cluster that lead to changes in expression of the gamma globin gene (hereditary persistence of fetal hemoglobin; HPFH). The  $\beta$ -globin gene cluster is also regulated by genes on distant chromosomes such as BCL11A, KLF1, and others.<sup>164,165</sup> The Globin Gene Server<sup>166</sup> continues to update the various hemoglobin variants regularly.

### $\beta$ -Thalassemia

The clinical phenotype and severity of  $\beta$ -thalassemia varies considerably. It mainly depends on the type and number of genetic defects of the alpha or beta globin genes and its regulatory regions. The major predictor of severity is the  $\alpha/\beta$ -globin chain imbalance with more severe disease in those genotypes with excess  $\alpha$ -globin and decreased  $\beta$ -globin production. The  $\alpha$ -globin chains produced in excess precipitate within the erythroid precursors resulting in ineffective erythropoiesis and hemolysis.<sup>167</sup> As such, a  $\beta$ -globin gene heterozygote predicted not to require transfusion might in fact require transfusions due to duplicated or triplicated  $\alpha$ -genes leading to a greater  $\alpha/\beta$ -gene imbalance than expected.

The  $\beta$ -thalassemias can be designated into four categories: silent carrier, trait/thalassemia minor, thalassemia intermedia, and thalassemia major (see Table 30.5). A newer disease classification commonly used includes two phenotype groupings: transfusion-dependent thalassemia (TDT) and nontransfusion-dependent thalassemia (NTDT). This newer designation is often used since overlap in disease severity (and therefore transfusion requirements) may exist over a lifetime, notably in individuals with thalassemia intermedia.  $\beta$ -Thalassemia silent carriers have no hematologic abnormalities but do have elevated hemoglobin A2 on hemoglobin electrophoresis. Individuals with  $\beta$ -thalassemia trait ( $\beta$ -thalassemia minor) have elevated A2 as well as mild anemia with microcytosis or microcytosis alone; they do not require RBC transfusions.  $\beta$ -Thalassemia intermedia presents with variable levels of anemia, but often is nontransfusion dependent (NTDT) in childhood. However, some individuals will go on later in life to require chronic transfusions (TDT) due to the increasing severity of anemia or complications of their disease.  $\beta$ -Thalassemia major presents early in life and is transfusion dependent (TDT).

Coinheritance of  $\beta$ -thalassemia and a  $\beta$ -globin structural variant (HbE) results in HbE thalassemia, which accounts for approximately one-half of all cases of severe  $\beta$ -thalassemia worldwide. The mutation responsible for HbE ( $\beta26$  Glu→Lys) results in abnormal RNA processing of the  $\beta$ -globin chain leading to decreased  $\beta$ -hemoglobin production and a structurally abnormal, mildly unstable hemoglobin molecule.<sup>168,169</sup> The clinical presentation of HbE thalassemia can vary widely from a mild asymptomatic anemia

**Table 30.5** Genotype and Classification of Beta Thalassemia and Alpha Thalassemia

Phenotype	Genotype	Clinical Severity
<b>Beta Thalassemia</b>		
• Normal	$\beta/\beta$	None
• Silent carrier	$\beta^0/\beta$ , $\beta^+/beta$	Asymptomatic
• Trait/thalassemia minor	$\beta^0/\beta$ , $\beta^+/beta$	Asymptomatic to mild microcytic anemia
• Intermedia	$\beta^+/beta$ , $\beta^0/\beta^0$ , $\beta^5/\beta^+$ , $\beta^5/\beta^0$ or in combination with gene duplications, deletional forms of $\delta\beta$ -thalassemia, increased $\gamma$ production, or $\delta\beta$ -thalassemia and HPFH and E $\beta^0$ -thalassemia	Late presentation, mild to moderate anemia, occasionally transfusion dependent
• Thalassemia major (Cooley's anemia)	$\beta^0/\beta^0$	Early presentation, severe anemia, transfusion dependent
<b>Alpha Thalassemia</b>		
• Normal	$\alpha\alpha/\alpha\alpha$	None
• Silent carrier	$\alpha^-/\alpha\alpha$	Asymptomatic
• Trait/minor	$\alpha^-/\alpha^-$ , $-\alpha\alpha$	Asymptomatic to mild microcytic anemia
• Hemoglobin H: deletional (H disease)	$-\alpha$	Mild to moderate anemia, Transfusion independent, Variable severity
• Hemoglobin H: non-deletional	$-\alpha^{ND}$ ( $-\alpha^S$ , Hb H-Constant Spring)	Moderate severity, transfusions possible
• Major (Bart's hydrops fetalis)	$-\alpha$	Possibly nonviable, immediate transfusion, chronic transfusion

to life-threatening anemia requiring lifelong chronic transfusions from infancy. The phenotype is influenced by the  $\beta$ -thalassemia mutation, the amount of fetal hemoglobin production, and the coinherited  $\alpha$ -genotype. The severity can evolve with age.<sup>168</sup> HbE homozygotes (HbEE) have prominent microcytosis along with hypochromia and target cells, but only mild anemia.

Individuals with  $\beta$ -thalassemia major present during infancy with progressive anemia, failure to thrive, and signs of extramedullary hematopoiesis (notably hepatosplenomegaly) as the fetal hemoglobin declines. Regularly transfused patients with thalassemia have complications primarily due to the increased absorption of iron from the gastrointestinal tract and iron derived from chronic transfusions.<sup>170</sup> Individuals with  $\beta$ -thalassemia intermedia have very heterogeneous clinical presentations. On one end of the spectrum are those with mild to moderate anemia who rarely or if ever require transfusions. On the other end are patients who are nontransfusion dependent during early childhood but subsequently become transfusion dependent during periods of erythroid stress (e.g., infections and pregnancy) or during adolescence or adulthood. Individuals with  $\beta$ -thalassemia intermedia who are not transfusion dependent still have morbidity associated with ineffective erythropoiesis, splenectomy, or many other complications listed in Table 30.6.<sup>164</sup> Due to increased gastrointestinal absorption of iron, many NTDT patients will require chelation even in the absence of transfusion. Consequently, iron overload remains one of the most significant complications in NTDT and TDT. Decisions about when to pursue chronic transfusions and/or splenectomy in these individuals are complex and must be individualized after careful assessment of their genotypes and clinical manifestations over several months.

### Transfusion goals for $\beta$ -thalassemia

Transfusion in  $\beta$ -thalassemia can alleviate symptomatic anemia, suppress ineffective erythropoiesis, improve growth and development, and reduce iron overload from increased gastrointestinal absorption. Even with identical genotypes, the baseline hemoglobin for the  $\beta$ -thalassemia syndromes is not predictable, but should be established for each child before the initiation of transfusion therapy. If the baseline is consistently below 6 or 7 g/dL, chronic transfusion therapy should be initiated. The goal for transfusion-dependent  $\beta$ -thalassemia is a pretransfusion hemoglobin of 9–10 g/dL (11–12 g/dL in individuals with heart disease), which typically requires transfusion every

**Table 30.6** Complications Seen in Patients with Transfusion-Dependent and Non Transfusion-Dependent Thalassemia<sup>164</sup>

- |   |                                     |
|---|-------------------------------------|
| • Growth and pubertal delay                 | • Thrombophilia and vascular events |
| • Hypoparathyroidism, hypothyroidism        | • Pulmonary hypertension            |
| • Left heart failure: cardiac hemosiderosis | • Hepatic fibrosis and cirrhosis    |
| • Hepatic failure                           | • Hepatocellular carcinoma          |
| • Viral hepatitis                           | • Hepatosplenomegaly                |
| • Diabetes mellitus                         | • Infections                        |
| • Hypogonadism                              | • Extramedullary hematopoiesis      |
| • Osteoporosis                              | • Osteoporosis                      |
| • Infertility, pregnancy                    | • Skin ulcers                       |
| • Hyperbilirubinemia, gall stones           | • Renal abnormalities               |

month in infants and young children, often increasing to every three weeks in adults.<sup>171</sup> If not splenectomized, patients may have hypersplenism or may be overtransfused to hemoglobin levels higher than necessary. Chelation becomes increasingly difficult with intensive transfusion therapy. Patients who have an annual transfusion requirement of greater than 200 mL/kg/year should be evaluated to determine if their transfusion requirement can be reduced.

### Other therapies for $\beta$ -thalassemia

Several pharmacological agents are being considered in thalassemia. While regular transfusion therapy is being considered, it is also essential to check if the patient qualifies for any clinical trials with new therapies or is eligible for hematopoietic stem cell transplant (HSCT). For adults over 18 years of age, luspatercept is a fusion protein that binds to TGF $\beta$  superfamily and augments late stage erythropoiesis. Luspatercept was recently approved for use for transfusion-dependent  $\beta$ -thalassemia based on the phase 3 double-blind, randomized, placebo-controlled trial demonstrating significantly reduced transfusion burden in TDT patients treated with luspatercept at 1.0–1.25 mg/kg every three weeks compared to placebo.<sup>172</sup> Other pharmacotherapies being considered include Mitapivat (activator of pyruvate kinase), VIT-2763 (ferroportin inhibitor), and TMPRSS6-LRx (antisense oligonucleotide targeting TMPRSS6). Gene editing trials are also ongoing.<sup>164</sup>

### **α-Thalassemia**

The clinical phenotype and severity of α-thalassemia also varies based on the number of genetic deletions along with the presence or absence of nondeletional mutations present on the alpha globin gene. In humans, the alpha gene locus is duplicated which results in individuals carrying four functional alpha chain genes (i.e.,  $\alpha\alpha/\alpha\alpha$ ). Alpha thalassemia is most commonly caused by full deletions of one or both α-globin genes. The  $\alpha^+$  thalassemia haplotype results when one of the two alpha globin genes are deleted on chromosome 16, whereas the  $\alpha^0$ -thalassemia haplotype occurs when both pairs of α-globin genes are deleted ( $\alpha^0$ -thalassemia,  $\alpha^-$ ;  $\alpha^0$ -thalassemia,  $-$ ). The two most common  $\alpha^+$ -thalassemia mutations are the 3.7 and 4.2 deletions, with highest prevalence in tropical and subtropical regions. Common  $\alpha^0$ -thalassemia variants include the  $-\text{SEA}$  mutation in Southeast Asia and the  $-\text{MED}$  mutation in the Mediterranean, reaching frequencies of approximately 5% in their respective regions. Less commonly, nondeletional α-thalassemia mutations caused by nucleotide deletions or substitutions exist, which can also ineffectively generate abnormal hemoglobin molecules (e.g., Hb Constant Spring, Hb Paksé). These nondeletional mutations may occur as single mutations or in combination with  $\alpha^+$ - or  $\alpha^0$ -thalassemia haplotypes ( $\alpha\alpha/\alpha^{\text{ND}}\alpha$ ,  $-\alpha/\alpha^{\text{ND}}\alpha$ , and  $-\alpha^{\text{ND}}\alpha$ , respectively).<sup>173</sup>

In the fetus, defective production of α-chains is reflected by the presence of excess γ chains, which form γ4 tetramers, called hemoglobin Barts. In adults, excess β-chains form β4 tetramers, called hemoglobin H (HbH). Because of their very high oxygen affinity, both tetramers cannot transport oxygen, and, in the case of HbH, its instability leads to the production of intraerythrocyte inclusion bodies and a variable degree of hemolytic anemia.<sup>173</sup>

The majority of patients with α-thalassemia syndromes will not require chronic transfusion therapy. Exceptions are those patients with HbH–Constant Spring ( $-\alpha^{\text{CS}}\alpha$ ) and infants with Hb Bart's hydrops fetalis ( $-/-$ ), who survived due to intrauterine transfusions (see Table 30.5). Single gene deletions ( $-\alpha/\alpha\alpha$ ) are not detectable without α-gene mapping. Alpha gene mutations that occur in *trans* ( $-\alpha/\alpha$ ) or *cis* ( $-\alpha/\alpha$ ), referred to as α-thalassemia trait (or α-thalassemia minor), are indistinguishable hematologically and can only be deciphered genetically. Individuals with α-thalassemia trait have hypochromia and microcytosis with or without mild anemia, and do not require transfusion.

Combinations of  $\alpha^0$ -thalassemia and  $\alpha^+$ -thalassemia can lead to a phenotype requiring RBC transfusions on an intermittent basis or chronically. HbH disease ( $-\alpha/-$ ) is the most common  $\alpha^0$ -thalassemia– $\alpha^+$ -thalassemia combination.<sup>174</sup> Patients with HbH disease rarely require transfusions, but may need transfusion during illnesses, pregnancy, or surgery. HbH disease manifests with a mild anemia and splenomegaly. In contrast, HbH–Constant Spring ( $-\alpha^{\text{CS}}\alpha$ ) is a much more severe disease with anemia, requiring intermittent or chronic transfusion, occasionally occurring early in life. Most patients will develop hypersplenism and thrombophilia. Thrombosis following splenectomy is common in this nondeletional form of HbH disease. Later in life, cholelithiasis and skin ulcers can occur. Both HbH disease and HbH–Constant Spring are in the NTDT group of thalassemia; however, some HbH–Constant Spring patients will require frequent or chronic transfusions later in life.<sup>175</sup> The most severe form of α-thalassemia is Bart's hydrops fetalis ( $-/-$ ). The affected embryo develops severe hypoxia in the third trimester and will not survive without intrauterine transfusions or exchange transfusion at birth. Other  $\alpha^0$  thalassemia mutations may cause fetal demise earlier in gestation. These infants require chronic transfusion therapy or HSCT if they survive.<sup>176</sup>

Similarly to β-thalassemia, decisions about when to pursue chronic transfusions and/or splenectomy in individuals with HbH disease and HbH–Constant Spring are complex and must be individualized over time. Monitoring during times of acute illness, infections, pregnancy, surgery, or rapid growth during childhood is required and is the best way to determine when RBC transfusions may be needed to treat severe anemia or to promote growth. For those patients requiring chronic transfusion, the goal should be a pretransfusion Hb of 9–10 g/dL (11–12 g/dL in individuals with heart disease), similarly to patients with transfusion-dependent β-thalassemia.<sup>175</sup>

### **Red cell enzymopathies**

Red cell enzymopathies are caused by alterations to genes that encode RBC enzymes. These congenital nonspherocytic hemolytic anemias are associated with defects in erythrocyte cellular metabolism that affect glucose, glutathione, or nucleotide metabolism.

#### **Glucose-6-phosphate dehydrogenase (G6PD) deficiency**

G6PD deficiency is the most common enzymopathy in humans.<sup>57</sup> G6PD catalyzes the first step of the hexose monophosphate shunt pathway which leads to NADPH production. NADPH provided by this pathway helps protect against cellular oxidative damage by supporting regeneration of reduced glutathione by glutathione reductase. Without maintaining high reduction potential in the RBC, oxidative membrane damage and hemolysis occur when the cells are presented with excessive oxidative stress.

G6PD has classical X-linked inheritance with the deficiency primarily affecting males, although females homozygous for the deletion or heterozygous with lyonization can be symptomatic. There are over 200 mutations leading to inactivation of the G6PD enzyme; most are single base mutations leading to amino acid substitutions and inactivation.<sup>57</sup> Since genetic variants can affect G6PD enzymes quantitatively and qualitatively, five classes of G6PD deficiency are described based on the level of residual enzyme activity and the hemolytic tendency (see Table 30.7). Class I is very rare with very low activity of G6PD and typically manifests as chronic nonspherocytic hemolytic anemia (CNSHA). Other variants (classes II and III) are associated with acute hemolytic episodes when subjected to oxidative stress.<sup>57</sup> Although there are numerous mutations leading to the inactivation of G6PD, two mutations are particularly common: one in areas of the African Diaspora and the other in countries surrounding the Mediterranean, Israel, India, and Indonesia. The African variant is G6PD (A–), which encodes 10–60% of normal G6PD enzyme activity. Individuals may experience intermittent hemolysis associated with significant oxidant stress from infections or exposure to certain

**Table 30.7** Classes of G6PD Deficiency

WHO Classification*	
<b>Class I</b>	Severely deficient (<1%); chronic non-spherocytic hemolytic anemia
<b>Class II</b>	Severely deficient (1–10% activity); mild, acute hemolytic episodes
<b>Class III</b>	Moderately deficient (10–60%); mild, acute hemolytic episodes
<b>Class IV</b>	Normal activity (60–50%); normal
<b>Class V</b>	Increased activity (>150%)

\* WHO classification proposed in 1971 and used universally since. Revision of this classification is currently under consideration which may group class II and class III variants together since their clinical manifestations are largely similar with wide overlap, with the exception that the acute hemolytic episodes tend to be more severe with class II variants than with class III variants.<sup>57</sup>

medications or fava beans.<sup>58</sup> The Mediterranean variant is the second most common G6PD variant allele. It results in <5% activity, and the acute episodes of hemolysis can be more severe. The severity of hemolysis depends on the type of trigger, dose of the exposure (in cases of fava bean or drugs), the level of residual enzyme activity in the patient, and the baseline anemia prior to hemolytic crisis. Numerous drugs have been implicated in the initiation of hemolysis in G6PD-deficient patients and have been risk-defined.<sup>57</sup> In severe hemolysis, transfusion may be urgent. However, a striking feature of G6PD-related acute hemolytic anemia is a rapid recovery due to a swift reticulocyte response.

A small proportion of G6PD mutations result in <1% activity. Individuals with these class I G6PD variants may suffer from severe neonatal jaundice and CNSHA of varying degree. Most individuals with CNSHA will have mild to moderate chronic anemia with reticulocytosis. Like other variants, the hemolysis can be exaggerated by exposure to oxidant stress from drugs, infections, fava beans, etc. Notably, some drugs with relatively mild oxidant potential in patients with class II and III G6PD variants may induce hyperhemolysis in patients with class I variants. It is important to note that blood donors are not routinely screened for G6PD deficiency. However, RBC units from G6PD-deficient donors may be associated with insufficient post-transfusion Hb increments or shorter in vivo survival in neonatal recipients and individuals with SCD, respectively.<sup>177,178</sup> Consequently, because of the potentially negative impact on transfusion efficacy, some have advocated for donor screening for G6PD deficiency to improve transfusion outcomes for vulnerable patient populations (i.e., premature infants, SCD, and/or other chronically transfused populations).<sup>178</sup>

### Pyruvate kinase deficiency

Pyruvate kinase deficiency (PKD) is the second most common inherited RBC enzymopathy after G6PD deficiency, but much less frequently observed. However, it is the most common enzymatic cause of hemolytic anemia due to an abnormality in the glycolytic pathway. There are four isoenzymes ( $M_1$ ,  $M_2$ , L, and R) found on two chromosomes. The gene for the L and R isoenzymes (PK-LR) on chromosome 1 (1q21) uses different promoters to produce the two isoenzymes. The gene for  $M_1$  and  $M_2$  isoenzymes (PK-M) on chromosome 15 (15q22), with alternate RNA splicing, produces these two isoenzymes.<sup>179</sup> The R isoenzyme is expressed exclusively in erythrocytes. PKD is inherited in an autosomal recessive pattern and is either caused by homozygous or compound heterozygous variants in the *PKLR* gene. PK-deficient RBCs cannot catalyze the conversion of phosphoenolpyruvate to pyruvate, impairing glycolysis and adenosine triphosphate (ATP) production. Reduced ATP levels impairs reticulocyte and RBC survival by altering cell rheology, making them susceptible to hemolysis, and increasing splenic uptake.<sup>180,181</sup>

Additionally, PK-deficient RBCs demonstrate increased accumulation of 2,3-bisphosphoglycerate, which shifts the oxygen dissociation curve to the right, thereby decreasing hemoglobin's oxygen affinity.<sup>182,183</sup> PKD has been reported worldwide, but higher frequencies are present in the Pennsylvania Amish and the Roma communities as a result of the founder effect.<sup>184</sup> The majority of *PKLR* mutations are missense substitutions. Among these, R510Q, R486W, and R479H are in patients from northern Europe and the United States, southern Europe, and the Amish communities, respectively.<sup>185</sup> Diagnosis of PKD should include PK enzyme activity and *PKLR* genetic testing as PKD is a heterogeneous disease with some large deletions, and intronic mutations can be difficult to identify.<sup>185</sup>

The clinical features of PKD are highly variable and include prenatal hydrops fetalis, severe neonatal jaundice, transfusion dependency, and asymptomatic anemia. Current treatment modalities are mostly supportive care with transfusions, splenectomy and chelation for those with iron overload. Complications occur irrespective of transfusion status. These include iron overload, bilirubin gallstones, extramedullary hematopoiesis, pulmonary hypertension, and thrombosis. The transfusion requirement can decrease with age (36% of children vs. 11% of adults), and as a result of splenectomy; additionally, fewer hemolytic crises are experienced in adults.<sup>185</sup> Transfusions to maintain an arbitrary Hb value should be avoided but rather should be based on the impact of hemolytic anemia on quality of life and energy level. There are ongoing clinical trials using mitapivat, a PK activator, in patients with PKD. A recent phase 2 study showed mitapivat improved Hb by a mean of 3.4 g/dL (range 1.1–5.8) in 50% of adults with PKD who were not regularly transfused.<sup>186</sup> An open-label clinical trial of mitapivat in transfusion-dependent patients with PKD was recently completed, and results are expected.<sup>187</sup>

### Red cell membrane disorders

The red cell membrane has a unique structure that is deformable without fragmentation and is rapidly responsive to stress changes. These properties are due to the structure of the lipid bilayer membrane and the underlying cytoskeleton. The lipid bilayer has an asymmetric distribution of phospholipids with phosphatidylserine (PS) localized to the inner layer. Both PS and internally located sphingomyelin interact with the spectrin and protein 4.1R anchoring the membrane to the cytoskeleton. Loss of this asymmetry, with PS translocated to the outer membrane, leads to phagocytosis and the membrane destruction seen in sickle cell disease and thalassemia. Key components of the membrane include band 3, glycophorin C, RhAG,  $\alpha$ - and  $\beta$ -spectrin, actin, and ankyrin.

### Hereditary spherocytosis

This is the most common RBC cytoskeletal disorder causing hereditary hemolytic anemia. It can affect all racial groups, but it is particularly common in northern Europeans where the prevalence is one in 1000–2500.<sup>188</sup> The inheritance is dominant in 75% and recessive in 25%. Mutations of ankyrin (ANK1), band 3 (SLC4A1), and  $\beta$ -spectrin (SPTB) genes are most commonly seen in autosomal dominant (AD) HS, while compound heterozygous defects affecting ANK1,  $\alpha$ -spectrin (SPTA1), or protein 4.2 (EPB42) predominate in autosomal recessive (AR) HS.<sup>189,190</sup> The phenotype–genotype correlation is dictated by the causative genetic variants, heterogeneity in the level of expression from splicing variants, and in erythropoiesis<sup>64,190</sup> (see Table 30.8). Current diagnostic options to correctly diagnose RBC membrane disorders include ektacytometry, flow cytometry, quantification of RBC cations and membrane proteins, along with confirmatory genetic testing.

**Table 30.8** Clinical Characteristics of Hereditary Spherocytosis

• Ankyrin	AD, AR, de novo	Mild to severe
• Band 3	AD	Mild to moderate
• $\beta$ -Spectrin	AD, de novo	Mild to moderate
• $\alpha$ -Spectrin	AR	Severe
• Protein 4.2	AR	Mild to moderate

The clinical severity of HS varies from symptom-free carrier status to severe hemolysis. The presence of anemia with reticulocytosis, spherocytes on the blood smear, jaundice, gallstones, splenomegaly, and a positive family medical history for similar findings is highly suggestive for a diagnosis of HS. Mild HS can be challenging to identify because individuals may have normal hemoglobin with no evidence of active hemolysis. Occasionally, mild HS can be exacerbated by illnesses that cause splenomegaly, such as infectious mononucleosis. All types of HS are likely to present with neonatal jaundice within the first 24 hours of life.

Severity of hereditary spherocytosis is sometimes categorized into mild, moderate, moderately severe, and severe depending on clinical and laboratory variables, such as hemoglobin, reticulocytes, bilirubin, transfusion frequency, response to splenectomy, among others (see Table 30.9). It is important to consider other possible diagnoses for chronic hemolysis if the blood smear appearances are not typical, particularly congenital dyserythropoietic anemia. Several cases have been misdiagnosed in the past, with the correct diagnosis being established only when patients fail to respond as expected to splenectomy or using genetic testing.<sup>191</sup>

### Hereditary elliptocytosis

Hereditary elliptocytosis (HE) has a worldwide distribution, but it is more common in malarial areas. HE and hereditary pyropoikilocytosis (HPP) result from mutations in the genes encoding for  $\alpha$ -spectrin,  $\beta$ -spectrin, or protein 4.1R. Defects in these genes reduce horizontal cytoskeletal integrity, thereby affecting the stability of red cell membrane, reduced deformability, and predisposing to hemolysis. HE is frequently asymptomatic, with no or mild hemolytic anemia, and results from monoallelic heterozygous mutations involving one of the above genes with corresponding altered or deficient cytoskeletal proteins. HPP is a more severe form of hemolytic anemia which results from biallelic HE-causing mutations. The most common form of HPP, frequently seen in individuals of African ancestry, results from compound heterozygous mutations involving an *SPTA1* HE-causing mutation along with a low-expression *SPTA1* allele (known as  $\alpha^{LELY}$ ). The  $\alpha^{LELY}$  allele leads to 50%  $\alpha$ -spectrin expression and is clinically silent even in the homozygous state. However, when coinherited with an *SPTA1* HE-causing mutation, neonatal jaundice, anemia, and increased poikilocytosis and fragmented RBCs on peripheral blood smear are frequently observed leading to a diagnosis of HPP in infants.<sup>64,192</sup> Children with HPP typically have moderate to transfusion-dependent anemia early in life, but their hemolytic anemia gradually improves after the first 1–2 years and evolves into an HE phenotype.<sup>64</sup>

### Southeast Asian ovalocytosis

Southeast Asian Ovalocytosis (SAO) is common in malarial regions of Southeast Asia and the Philippines, and has autosomal dominant inheritance. Given that only heterozygotes have been observed, the assumption is that homozygosity is incompatible with life. It is

caused by a 27 base pair deletion of band 3. This membrane disorder is relatively benign with neonatal jaundice with no or minimal transient hemolysis seen in newborns, and resolution in toddler years.<sup>193</sup>

### Hereditary stomatocytosis

These disorders lead to overhydrated hereditary stomatocytosis (OHSt) or dehydrated stomatocytosis (DHSt), otherwise called Hereditary Xerocytosis (HX). OHSt is characterized by an increase in intracellular sodium and water content that is not adequately compensated by reduced potassium. In HX, potassium leaks out of RBCs and is not compensated by an increase in intracellular sodium content.

Autosomal dominant OHSt is caused by a heterozygous missense mutation involving the *RHAG* gene, which codes for the RhAG protein that is part of band 3 complex of the RBC membrane. Patients with OHSt can present with mild to severe hemolytic anemia, reticulocytosis, a low MCH and MCHC, elevated MCV, and abundant stomatocytes along with occasional spherocytes on the blood smear.<sup>64</sup>

HX is also autosomal dominant hemolytic anemia characterized by gain of function mutations in *PIEZ01* or *KCNN4*. The former is a stretch-activated nonselective cation channel and the latter is a calcium-activated potassium channel (also known as Gardos channel). Defects in both these genes result in potassium leak, with no gain in sodium/water. The result is erythrocyte dehydration. This explains why some newborns with HX present with pseudohyperkalemia and perinatal edema. Patients with DHSt typically present with compensated hemolysis or mild anemia, reticulocytosis, a high MCH, MCV, high or high-normal MCHC, and xerocytes (dehydrated erythrocytes that have hemoglobin “puddled” at one end at the periphery) and occasional stomatocytes and target cells on the blood smear. Iron overload is seen even in those without a history of transfusion, especially in *KCNN4*-HX.<sup>64,194</sup>

In both these conditions, RBC transfusions are occasionally required if the anemia is severe, and monitoring for iron overload is required despite low transfusion burden. While splenectomy can partially ameliorate the anemia in OHSt, a high risk of thromboembolic complications postsplenectomy has been reported for OHSt and HX.<sup>64,195,196</sup> Senicapoc, a Gardos channel blocker that was originally designed for an SCD therapy but failed to show measurable clinical benefits, is currently being restudied in *KCNN4*-HX.<sup>197</sup>

### Transfusion protocols

#### Pretransfusion testing and RBC component preparation

Routine transfusion therapy requires ABO and RhD typing. However, extended RBC antigen typing is recommended for patients with SCD who have an increased incidence of RBC alloimmunization when receiving standard ABO/RhD-matched RBC transfusions (pooled prevalence: 35%; 95% CI, 19%–53%).<sup>117</sup> An extended RBC antigen phenotype or genotype (preferred) should be obtained at the earliest opportunity, optimally before first transfusion, for all patients with SCD. An extended RBC antigen profile should include C/c, E/e, K, Jk<sup>a</sup>/Jk<sup>b</sup>, Fy<sup>a</sup>/Fy<sup>b</sup>, M/N, and S/s, at a minimum.<sup>117</sup> This information is critical to identifying absent RBC antigens in the patient, a prerequisite for alloantibody formation, in order to provide antigen-negative RBC units for transfusion and to guide investigations when alloantibodies are suspected. More than two-thirds of the alloantibodies detected in SCD patients target Rh

**Table 30.9** Severity of Hereditary Spherocytosis

	Mild	Moderate to Moderately Severe	Severe
Hemoglobin	>11 g/dL	8–11	6–8 g/dL
Reticulocytosis	3–6%	>6%	>10%
Total Bilirubin	1–2 mg/dL	>2 mg/dL	>3 mg/dL

(primarily C and E) and Kell (typically K) antigens. A recent systematic review demonstrated significantly decreased rates of alloimmunization in SCD patients receiving prophylactic Rh(CcEe)- and K-matched RBCs with pooled alloimmunization incidence rate 0.40 (95% CI: 0.23–0.69) per 100 units phenotypically matched transfusions compared to 1.94 (95% CI: 1.28–2.94) per 100 ABO/Rh(D)-matched units transfused. Consequently, prophylactic Rh (CcEe) and K antigen matching is recommended for all SCD patients needing transfusion.<sup>115,117,198</sup> Extended antigen-matched RBC transfusions (CcEe, K, plus Jk<sup>a</sup>/Jk<sup>b</sup>, Fy<sup>a</sup>/Fy<sup>b</sup>, and S/s) are preferred in patients with history of alloantibodies in order to further minimize sensitization, but finding sufficient RBC units for some patients may be challenging. Although lower alloimmunization rates are seen in thalassemia patients (pooled prevalence: 11.4%; 95% CI: 9.3–13.9%),<sup>199</sup> prophylactic CcEe and K antigen matching is also recommended given that over 75% of the antibodies detected in thalassemia patients are also Rh and Kell antigens.<sup>198,199</sup> Additionally, similarly to SCD, several studies have suggested a reduction in alloimmunization risk in individuals with β-thalassemia when CcEe- and K-matching for RBCs is applied.<sup>198</sup> There are no standardized guidelines recommending prophylactic antigen matching for individuals with other hereditary hemolytic anemias.

Multiple commercially available semiautomated RBC genotyping platforms exist and provide advantages over standard serologic methods for antigen typing. These advantages include determining an extended RBC phenotype in recently transfused patients, simplified testing in patients with interfering allo- or autoantibodies, resolution of discrepant serologic typing results, and testing samples where typing antisera are not readily available.<sup>200</sup> In addition, most commercially available RBC genotyping platforms provide additional RBC phenotype information that may optimize antigen matching protocols particularly for patients with SCD. As an example, a GATA mutation in the DARC gene, which is common in individuals of African descent, only prevents expression of Duffy glycoprotein on erythrocytes while permitting expression in nonerythroid cells. SCD patients found to have a GATA mutation are not at risk for alloimmunization against Fyb despite typing as Fy(b-). These patients can safely receive Fy(b+) RBCs, which markedly expand the pool of compatible donors.<sup>200,201</sup> Another example includes patients identified by genotype with the hybrid *RHD\*DIlla-CE(4-7)-D*, which is also common in individuals of African descent. This hybrid *RHD-CE-D* encodes no RhD antigen but rather a partial C antigen. Patients found to have this hybrid *RHD* allele should receive C-negative RBCs when no conventional *RHCE\*Ce* or *\*CE* trans-allele is present to prevent allo-anti-C development.<sup>201,202</sup>

Fresh RBCs are postulated to have increased in vivo survival and potentially reduce the risk of alloimmunization. For example, higher alloantibody levels have been observed in mice transfused leukoreduced RBCs that were 14 days old compared to fresher units.<sup>203</sup> Although the benefit of providing fresh RBCs has been investigated in other patient populations,<sup>204</sup> it has not been extensively evaluated in patients with SCD, other hemoglobinopathies, or hereditary hemolytic anemias. Currently, there is no standard on RBC unit age restrictions for patients with hemoglobinopathies; however, some centers utilize protocols that recommend “freshest available” units, or units less than 10 days, 14 days, or 21 days.<sup>205</sup> Sickle trait-negative blood products are recommended for SCD patients, although a patient with a rare phenotype may receive sickle trait-positive units when necessary. Leukoreduced products

are indicated for all patients with hemoglobinopathies or hereditary hemolytic anemias. RBCs do not require irradiation for these patient populations unless the patient is undergoing HSCT. Units that have been leukoreduced are widely accepted to be “cytomegalovirus (CMV)-safe,” and there is no demonstrated need to issue units from CMV-seronegative donors if the units are leukoreduced. However, CMV negative units are recommended for the transfusion of patients during pregnancy, if available, to prevent congenital CMV in the fetus. No formal recommendations exist on whether CMV-seronegative patients undergoing HSCT should receive CMV negative units. Citing long-term retrospective studies demonstrating very low to no transfusion-transmitted CMV rates using leukoreduced blood products in these patients, many institutions encourage leukoreduced blood products in this population.<sup>206</sup> Washed RBC units are not indicated unless the recipient has had severe allergic transfusion reactions. Red cell units can be “split” by the blood bank to conserve the blood supply. This practice is more commonly used for pediatric patients whose transfusions are volume based rather than unit based.

### Simple transfusion

SCD patients may receive either simple or exchange transfusions. Simple transfusions are more commonly utilized in SCD patients because they can be performed with a minimal amount of clinical resources and infrastructure. Furthermore, only simple transfusions are used in patients with thalassemia and hereditary hemolytic anemias. The volume for simple transfusion is specifically calculated for pediatric patients rather than transfusion of entire units. A simple method of calculating red cell requirement is

$$\text{RBC milliliters (mL) to transfuse} = (\text{Hb}^d - \text{Hb}^c)(3 / \text{Hct}^u)(\text{Wt})$$

where Hb<sup>d</sup> is the desired hemoglobin, Hb<sup>c</sup> is the current hemoglobin, Hct<sup>u</sup> is the hematocrit of transfused RBC unit(s), and Wt is the weight of the patient in kilograms.<sup>207</sup>

There are some contraindications to simple transfusions. For example, this approach can lead to volume overload with cardiac insufficiency in older patients, those with cardiomyopathy, and/or those receiving rapid transfusion volumes greater than 20 mL/kg. In these instances, slowing the infusion rate (especially if patient is hemodynamically stable), close monitoring, and consideration of diuretics should be considered. Overtransfusion can lead to hyperviscosity and should be avoided in patients with hemoglobinopathies, particularly SCD. Although the Hb concentration that may cause morbidity in thalassemia is higher than in SCD, hyperviscosity can develop in both populations and can produce complications. In normal (HbAA) individuals, arterial oxygen delivery increases as Hb concentration increases to a maximum oxygen delivery at approximately 15 gm/dL (Hb<sub>max</sub>). Beyond this point, any increase in Hb concentration decreases the arterial oxygen delivery due to increased blood viscosity. For patients with SCD, the Hb<sub>max</sub> is shifted downward to 10–11 g/dL.<sup>118,208,209</sup> Consequently, although thalassemia patients tolerate Hb concentrations up to 15 gm/dL following transfusion, an Hb concentration >12 gm/dL in SCD patients following a simple transfusion can lead to hypertension, posterior leukoencephalopathy (PRES), or stroke.<sup>210</sup> These risks are increased during hypoxia if the percent hemoglobin S is greater than 30%. Evidence suggests that cerebral blood flow and oxygen delivery to the brain in SCD is not only dependent on the total Hb concentration but also on the percentage of HbS.<sup>211</sup> When the HbS level is <20% (i.e., post-transfusion

during chronic transfusion therapy), the total Hb concentration can generally be increased up to 12–13 g/dL without concerns for viscosity-related complications.<sup>118</sup> Therefore, the optimal recommended post-transfusion Hb concentration is approximately 12 g/dL for SCD patients undergoing chronic transfusion therapy but not recommended to exceed 10–11 g/dL in the acute setting when HbS is assumed to be >50%.

### **Red cell exchange**

Red cell exchange is often preferred for SCD in emergent situations when rapid HbS reduction is needed in patients with severe sickle manifestations and/or in patients with higher pretransfusion Hb levels to minimize hyperviscosity concerns. It is also utilized in the chronic transfusion setting to prevent iron overload or minimize iron burden in patient with hemosiderosis. Exchange transfusion is preferably performed by automated erythrocytapheresis but can be performed manually by phlebotomy of whole blood immediately before transfusion of RBCs (which can sometimes be diluted to a predetermined Hct), as described in the next section.

### **Manual exchange transfusion**

Manual exchange transfusions are used in both resource-poor countries as well as developed, resource-rich countries when access to apheresis equipment and/or trained personnel is limited. Partial manual exchange (PME) involves phlebotomy of approximately 5–10 mL/kg of whole blood immediately followed by simple transfusion. The volumes of phlebotomy and transfusion depend on patients' baseline Hb and tolerance, and the postexchange target Hb level.<sup>139,212</sup> Alternative approaches involve successive aliquots of whole blood being removed and replaced by reconstituted whole blood (RBCs diluted with either albumin or normal saline) to a desired Hct.<sup>213</sup> There is no universal standard technique for performing manual exchange transfusions for children or adults with SCD, and therefore different protocols exist among treatment centers. PME has been utilized to slow the progression of transfusional iron overload when used for chronic transfusion therapy, but is not as effective as automated red cell exchange (erythrocytapheresis) in this regard.<sup>139</sup>

### **Erythrocytapheresis**

Automated RCE (erythrocytapheresis) is the preferred transfusion method for many SCD patients when it is available. The advantages of erythrocytapheresis include the ability to maintain continuous isovolemia and predictably achieve a target post-transfusion Hb level and percentage of HbS independently of one another. As a result, hyperviscosity can be avoided, notably in patients with higher pretransfusion Hb levels. In addition, the procedure can effectively minimize iron accumulation when optimized in the chronic transfusion setting.<sup>139,140</sup>

Automated RCE can be performed via the conventional method or via a method that incorporates isovolemic hemodilution (IHD), also known as depletion exchange (117). IHD-RCE uses saline (or albumin) replacement to acutely lower the patient's Hct at the beginning of the apheresis procedure while maintaining isovolemia. This decreases the total RBC unit volume needed to attain the target HbS level and allows for the exchange procedure to be more efficient. Current apheresis devices have IHD-RCE capability programmed into the device, allowing it to be fully automated.<sup>214</sup> Although IHD-RCE is not advised in the acute setting when a rapid drop in Hb level may not be tolerated, IHD-RCE can be utilized in the chronic setting in order to potentially decrease the frequency of exchange transfusions by providing an

increased exchange efficiency.<sup>215</sup> However, there is a paucity of studies investigating the long-term safety of IHD-RCE compared to conventional RCE. Young children (<20–25 kg) undergoing automated RCE require a blood (custom) prime when the extracorporeal volume from the circuit exceeds 15% of their total blood volume. For this reason, they also should not receive IHD-RCE.

The major barriers to automated RCE most often include access to apheresis equipment and/or trained personnel and venous access. Although efforts should be made to use peripheral veins for apheresis procedures, central intravenous access is often necessary for both acute procedures and for patients requiring chronic erythrocytapheresis. Percutaneous temporary double-lumen hemodialysis catheters are used and placed in either the internal jugular (preferred), subclavian, or the femoral vein for patients requiring acute RCE. Tunneled and cuffed double-lumen high flow central venous catheters or double-lumen ports have both been effectively used in children and adults who require chronic erythrocytapheresis. These ports require large noncoring needles for apheresis. Ideally, these ports should only be used by the apheresis staff for RCE, and sterile access technique should be employed to prevent infection. High-dose heparin locks (1000 units/mL) and TPA instillations (1 mg/mL) for intraprocedural flow issues are commonly used line care measures. Typically 10 Fr or larger double-lumen catheters/ports are required for adults. The sizes and types of catheter/ports for pediatric patients are based on the weight of the patient; 7 Fr or larger double-lumen catheters are typically required for children between 10 and 20 kg.<sup>216</sup> Alternatively, a single-lumen high flow port may be used (as the access/draw line) with a peripheral intravenous (PIV) angio-catheter (as the return line). Preferred angio-catheter sizes for supporting apheresis include 18 gauge or larger for adults and 22 gauge or larger for children. Larger steel gauge needles are required for the access/draw line when erythrocytapheresis is performed solely via peripheral venous access.

Automated RCE is relatively safe, with few adverse events reported. Hypocalcemia can be avoided with the use of continuous calcium gluconate infusion at approximately 15–20 mg/kg/hour, with subsequent titration based on ionized calcium monitoring.<sup>217</sup> Nausea, vasovagal reactions, and post-RCE transient drops in platelet count below  $100 \times 10^3/\mu\text{L}$  have been reported.<sup>218</sup> Because automated RCE is associated with increased RBC utilization, concerns over alloimmunization have been raised. However, multiple studies have shown that RCE does not result in an increased incidence of alloimmunization compared to simple transfusion or PME despite additional donor exposures.<sup>139,219–221</sup>

### **Transfusion targets and monitoring**

With the exception of acute ischemic stroke in adults and children with SCD where a post-RCE target HbS <20% is recommended,<sup>118</sup> most post-transfusion targets in the acute setting relate to amelioration of sickle manifestations and not exceeding a total Hb of 11 g/dL to avoid hyperviscosity. For SCD patients receiving chronic transfusions for stroke prevention, guidance recommends a pretransfusion Hb level >9.0 g/dL and HbS level <30%;<sup>118</sup> once again, the post-transfusion Hb level target should not exceed 12 g/dL to avoid increased morbidity due to hyperviscosity. Transfusions are usually required every 3–4 weeks to maintain these parameters. The optimal HbS for prevention of other complications is not defined, but a pretransfusion HbS of <40–50% is often used. If the pretransfusion HbS is higher than the target despite simple transfusions at three-week frequency, especially in patients with a

high baseline Hb levels (i.e., >10 g/dL), transitioning to erythrocytapheresis rather than PME may help to maintain better HbS control.<sup>222,223</sup> Alternatively, the addition of hydroxyurea to patients who are chronically transfused has been shown to increase fetal Hb and total Hb, reduce HbS,<sup>224</sup> and potentially decrease transfusion requirements.<sup>225</sup>

The post-transfusion target Hct for patients receiving chronic erythrocytapheresis is dependent on the patient's pre-exchange Hct and HbS, and the degree of iron overload (if present). The goal of chronic erythrocytapheresis for iron overload management is not marrow suppression but rather reducing HbS. Consequently, for optimal iron overload management, the postexchange Hct should be approximately equal to the pre-exchange Hct level. This ensures a net zero iron balance for the RCE procedure.<sup>226,227</sup> In these instances, patients without preceding iron overload will not need iron chelation, and those with iron overload will have efficient chelation due to near-zero cumulative iron burden. Patients with a lower baseline Hct (<25%) probably should have postexchange Hct targeted at 27%, unless under certain circumstances of severe iron overload. Patients who are difficult to maintain at an HbS level under 30% may benefit from a higher postexchange targeted Hct in order to induce transient suppression of erythropoiesis. In patients with higher baseline Hct, IHD-RCE may also be utilized to further increase the exchange efficiency by reducing the postexchange fraction of HbS-containing cells (FCR) remaining.

The Hb target for patients with TDT and hereditary hemolytic anemias is higher than that for SCD patients. For TDT, the pretransfusion hemoglobin should be maintained between 9 and 10 g/dL. This may require monthly transfusion in infants and young children, with transfusions every three weeks in adolescent and adult patients. The post-transfusion Hb should be targeted at 12–13 g/dL. Hypersplenism from splenomegaly can lead to an increase in transfusion requirements. Splenectomy can reduce the annual RBC requirement and should be considered if the annual blood requirement is over 200 mL/kg/year. Prior to splenectomy, patients should be fully immunized against encapsulated organisms (*Streptococcus pneumoniae*, *Haemophilus influenza*, and *Neisseria meningitidis*). Luspatercept, which has been shown to decrease transfusion burden in adult TDT patients, may also be considered in patients with high annual blood requirement.<sup>172,228</sup>

All patients on chronic transfusions should have annual total blood requirement evaluated. Patients with greater than 200 mL/kg/year are difficult or impossible to treat for iron overload with chelation because of the high annual net iron burden from transfusion. Causes for such a high transfusion requirement should be explored, and strategies to decrease transfusion burden should be sought (i.e., in SCD, transition to chronic erythrocytapheresis; in thalassemia, consider splenectomy and/or use of luspatercept).

Baseline testing for HIV, hepatitis B (HBV), and hepatitis C (HCV), and liver function is recommended prior to initiation of transfusions and annually thereafter while maintained on chronic transfusion therapy. HBV immunization and booster vaccination should be administered in all patients who do not have serologic evidence of immunity irrespective of previous or future planned transfusions.<sup>229</sup> Virology testing should be repeated if there is a rise in transaminases, and patients with positive serology for HBV or HCV infections should be referred to a hepatology specialist and followed for hepatocellular carcinoma.<sup>230</sup> All chronically transfused SCD and transfusion-dependent thalassemia patients are candidates for HSCT, so offering HLA-typing for them and their full siblings is advised.<sup>231,232</sup>

## Transfusion complications

### Alloimmunization

Patients with SCD are more susceptible to developing RBC alloantibodies than any other multiply transfused patient population. Although the mechanism underlying increased susceptibility to alloimmunization is unclear, several possibilities have been proposed. Patients with SCD show evidence of increased inflammation at baseline which is accentuated during times of illness. Resultant changes to the innate and adaptive immune systems have been proposed to influence the development of RBC alloantibodies and autoantibodies. The chronic hemolysis in SCD, with resultant constitutively elevated plasma free heme and Hb, has been shown to induce proinflammatory effects on both the innate and adaptive immune systems. This chronic hemolysis and inflammation results in significant immune dysregulation observed in SCD patients and murine models. These effects include abnormal immune cell responses, specifically in regulatory B cells, regulatory T cells, and other T-cell subsets such as follicular T cells and TH17 cells, which have correlated with RBC alloimmunization.<sup>233–237</sup> Furthermore, alloimmunization risk has been shown to be increased when SCD patients are transfused during acute sickle-related events, which are marked by significant inflammation.<sup>238</sup>

A more obvious risk factor relates to greater RBC antigen disparity between donor and SCD recipients. Consistent with this are observations that SCD patients are disproportionately African/African American (or members of other minority groups), while blood donors are historically more often of European descent. Individuals of African descent have different blood group distributions relative to most blood donors, and the most common antibodies formed in SCD patients (against C, E, K, and Jk<sup>b</sup>) are related to the antigenic frequency discordance between donor and recipient (K+: 9% vs. 2%; E+: 35% vs. 24%; C+: 68% vs. 28%; Jk<sup>b</sup>+: 72% vs. 39%, respectively).<sup>239</sup> These findings have prompted the use of transfusion protocols that minimize donor/recipient antigenic disparities by supporting SCD patients' transfusions through RBC units from ethnically matched (i.e., African American) and/or antigen-matched donors.

Because of the high prevalence of RBC alloimmunization in transfused SCD patients as well as the preponderance of alloantibodies to C, E, and K antigens, which comprise almost two-thirds of the antibodies detected, multiple international expert panel guidelines recommend primary serologic matching for Rh (D, C/c, and E/e) and K antigens.<sup>115,117,198,229</sup> Despite this approach, SCD patients remain at high risk of forming alloantibodies to Rh epitopes due to increased prevalence of *RHD* and *RHCE* variants in this population which encode altered or partial Rh antigens.<sup>240,241</sup> These antibodies can be mistaken for autoantibodies, which are common in SCD patients,<sup>242,243</sup> since the patients serologically type as antigen-positive for implicated antibody. Many of these Rh antibodies have been associated with laboratory evidence of delayed hemolytic transfusion reactions or demonstrated decreased survival of transfused RBCs.<sup>240,244</sup> Consequently, in alloimmunized SCD patients, future transfusions should be with antigen-negative or extended genotypic-matched RBCs, which poses significant challenges to transfusion services in providing compatible RBCs for these patients.

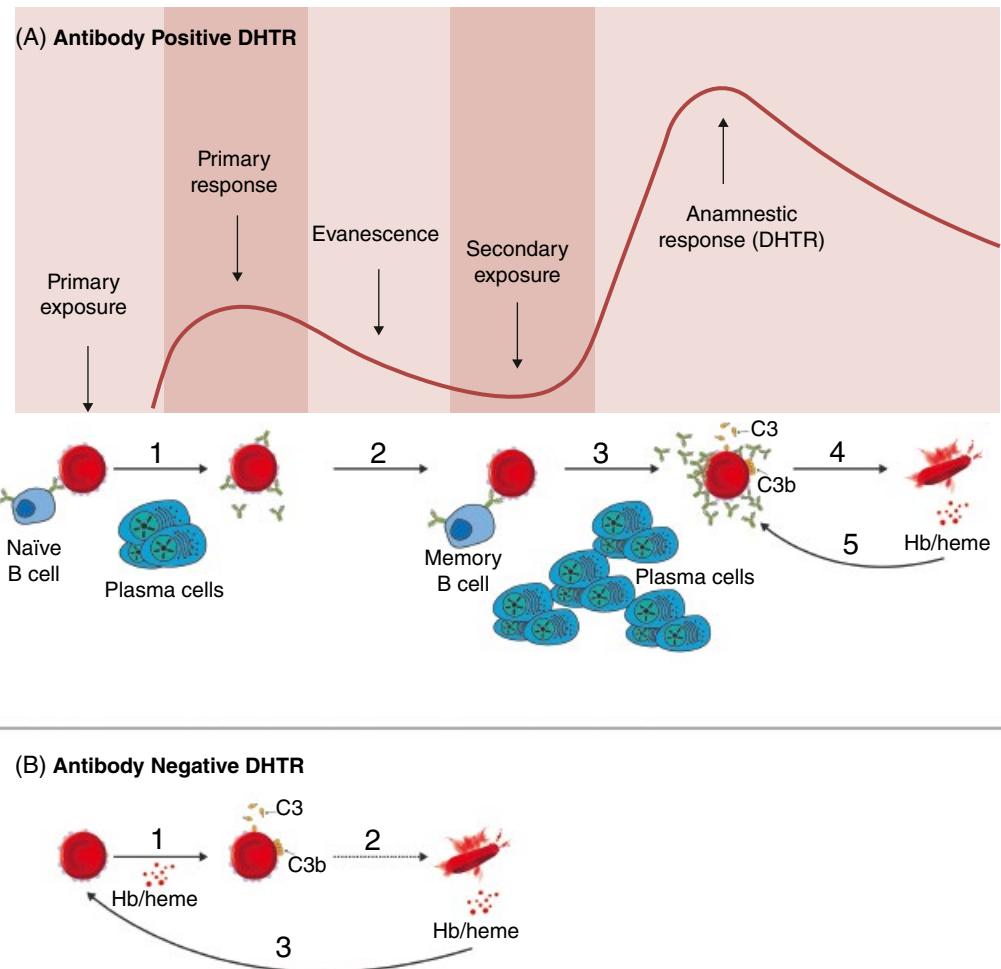
Alloimmunization should be considered, and an investigation for new antibodies should be undertaken, prior to transfusion in any chronically transfused patient if the pretransfusion Hb is significantly lower than baseline. Additionally, in SCD patients, a significant increase in HbS and reticulocyte count from baseline should also alert to a potentially new alloantibody. Autoantibodies

are known to obscure alloantibodies. Therefore, a high index of suspicion is needed to prevent delayed hemolytic transfusion reactions in patients who have an autoantibody because of the risk of an unidentified underlying alloantibody. RBC genotyping can facilitate complex antibody evaluations and guide RBC selection for these patients, who often require extended antigen-matched and/or rare RBC units.<sup>200</sup>

### Delayed hemolytic transfusion reactions and hyperhemolysis

In addition to significantly delaying the procurement of compatible RBCs for future transfusions, RBC alloimmunization increases the risk of potentially developing acute or delayed hemolytic transfusion reactions (DHTRs). DHTRs occur because more than one-third of RBC alloantibodies evanesce over time and fall below the level of detection by traditional blood bank serologic methodologies. When an individual is re-exposed through transfusion to

RBC antigens to which they have previously formed an antibody, immunological memory generated during the primary encounter can facilitate a rapid amnestic response resulting in the destruction of the transfused RBCs (see Figure 30.2).<sup>245</sup> SCD patients experience DHTRs at a significantly higher rate than other heavily transfused populations, with estimates of 4.4–7.7% of episodically transfused adult SCD patients experiencing at least one lifetime DHTR.<sup>246,247</sup> SCD patients transfused for acute complications are particularly more prone to DHTRs compared those receiving chronic transfusions, with reported incidences of 3.5–4.2 DHTRs per 100 transfusions compared to ≤0.1 DHTR per 100 transfusions, respectively.<sup>246–248</sup> However, the true incidence of DHTRs in SCD patients may actually be higher since many DHTRs go undiagnosed because they mimic common complications of SCD, such as vaso-occlusive crisis (VOC). A high index of suspicion is needed because DHTRs in SCD patients are associated with high mortality due to the potential for hyperhemolysis, a life-threatening phenomenon in



**Figure 30.2** Pathophysiology of delayed hemolytic transfusion reactions. (A) Antibody positive DHTR. Naive B cells are activated, proliferate, and differentiate into plasma cells upon initial exposure to RBC alloantigen (1). Antibody evanescence results from the lack of continued RBC antigen stimulation with subsequent diminution of circulating activated B cells but persistence of a memory B cell subset (2). Upon re-exposure to RBC alloantigen, memory B cells activate, proliferate, and differentiate into plasma cells resulting in a more robust antibody response (3). Antibodies engage with RBC alloantigens, with resultant alternative complement pathway activation and intravascular hemolysis (4). Subsequent release of plasma free heme and hemoglobin can contribute to continued complement activation and hyperhemolysis (5). (B) Antibody negative DHTR. Hemolytic transfusion reactions can result in the absence of detectable antibody and may in part be driven by heme-induced alternative complement pathway activation on transfused and autologous RBCs<sup>1</sup> and intravascular hemolysis.<sup>2</sup> Subsequent release of plasma free heme and hemoglobin can contribute to continued complement activation and hyperhemolysis.<sup>3</sup> Source: Based on Thein SL et al. (2020).<sup>245</sup>

which both transfused RBCs and the patient's own sickle erythrocytes are destroyed. Such hyperhemolysis has been shown to progress to ACS, hepatic impairment, renal failure, and death in 50%, 35%, 10%, and 6% of cases of DHTR in adults with SCD, respectively.<sup>249</sup> Although, less commonly seen, hyperhemolysis can occur in other populations, such as patients with thalassemia<sup>250</sup> as well as those without hemoglobinopathies.<sup>251</sup> For currently unknown reasons, select patients with SCD can also develop hyperhemolysis during acute sickle complications in the absence of recent transfusion (Figure 30.2).<sup>252,253</sup>

The most common presenting symptoms of DHTR include hematuria, pain (which is often indistinguishable from a typical VOC), fever, and symptoms of anemia, which occur up to 21 days after a transfusion. Laboratory findings include a significant drop in the total Hb, often below the pretransfusion level, a rapid decline of post-transfusion HbA level with a concomitant increase in the HbS, hemoglobinuria, relative reticulocytopenia or reticulocytosis, and a significant lactate dehydrogenase (LDH) rise from baseline.<sup>248,249</sup> Studies have failed to identify new RBC antibodies or a positive direct antiglobulin test (DAT) in approximately 30% of DHTRs in SCD patients.<sup>245,249</sup> Therefore, clinicians must rely on other laboratory and clinical findings in patients who have been recently transfused in order to make a reliable diagnosis of DHTR. In addition, RBC antibody evaluations should be completed at presentation and repeated at regular intervals for approximately three months from the diagnosis of DHTR in order to detect delayed formation of new antibodies that can inform antigen-negative unit selection for future transfusions. Some experts have recommended the use of a validated risk assessment nomogram for diagnosing DHTRs in adult SCD patients. This nomogram requires total Hb, HbA, and HbS levels to be obtained within 48 hours of an acute transfusion and again at the time a patient represents with symptoms concerning for a DHTR. A DHTR is deemed highly likely when a significant decrease in HbA (>50%) and/or in total Hb levels (>30%) relative to the post-transfusion values are observed, regardless of RBC antibodies being detected.<sup>247,254</sup>

Early recognition of a DHTR is critical to avoid additional transfusions, which may exacerbate the hyperhemolysis, and to initiate salvage treatment. There have been no prospective studies to guide therapy for patient with hyperhemolysis; however, immunosuppressive therapy with IVIG, corticosteroids, rituximab, and eculizumab have been used. High-dose erythropoietin with or without IV iron has also been recommended.<sup>117</sup> Recent studies have implicated alternative complement pathway activation in severe cases of DHTR with hyperhemolysis, and have shown reversal of complement activation, reduction in hemolysis, and rapid clinical improvement in SCD patients treated with an anti-C5 complement-blocking antibody.<sup>255,256</sup> Transfusions with extended antigen-matched RBCs should be reserved for patients experiencing life-threatening anemia.<sup>117</sup> Patients should be monitored for hypertension and fluid overload when they are receiving intensive therapy including transfusions for hyperhemolysis due to an association between transfusion, corticosteroid use, hypertension, intracranial hemorrhage, and stroke.<sup>257</sup>

Although DHTRs are not completely preventable, their incidence can be significantly reduced. Judicious use of transfusions and providing prophylactic antigen-matched RBCs when transfusions are indicated can decrease the incidence of alloimmunization, which poses an increased risk of subsequent DHTRs. Reliable communication within and between institutions, as well as discouraging patients from receiving multisite transfusions, can minimize the

likelihood of missing transient alloantibodies and re-exposure to evanesced alloantibodies. Lastly, identifying patients at high-risk for DHTRs (i.e., heavily alloimmunized patients and/or those with a history of past DHTRs) may inform the use of preventative measures and/or alternatives to transfusions when transfusions are being considered.<sup>245,248</sup>

### Iron overload

RBC units contain approximately 0.8 mg of iron per mL, most of which cannot be excreted from the body.<sup>258</sup> Consequently, a direct link can be observed between transfusion therapy and iron loading. Patients with TDT on a typical chronic transfusion schedule accumulate iron at a rate of 0.3–0.6 mg of iron/kg/day. Because of lower post-transfusion Hb target levels, SCD patients receiving chronic simple transfusions accumulate iron at a slightly lower rate (0.25–0.45 mg of iron/kg/day). The net iron accumulation can be minimized to a near iron-neutral balance in SCD patients by using chronic erythrocyapheresis, rather than simple transfusions.<sup>226</sup> Another key difference in iron loading between patients with SCD and thalassemia relates to the primary site of RBC destruction. In thalassemia, the hemolysis is predominantly within the bone marrow due to ineffective erythropoiesis, whereas in SCD the hemolysis is predominantly intravascular. Many patients with NTDT and some patients with other hereditary hemolytic anemias may also eventually develop iron overload despite low or no transfusion burden because of increased iron absorption due to ineffective erythropoiesis.

Ineffective erythropoiesis is a particular form of anemia which results in increased iron absorption from the gut and release of iron stores from macrophages and hepatocytes into circulation due to downregulation of hepcidin. Hepcidin negatively regulates cellular expression of ferroportin, a membrane transporter for iron entry into the circulation. Elevated erythroferrone secretion by an increased population of erythroblasts within the bone marrow results in the downregulation of hepatic production and increased expression of ferroportin. The resulting iron load is deposited into organs rather than being used to generate more erythrocytes.<sup>259</sup> Ineffective erythropoiesis, resulting in increased iron absorption, is the primary reason why many patients with NTDT eventually develop iron overload despite low transfusion burden. Hepcidin is also increased by inflammation. The proinflammatory state in SCD and its influence on iron overload have also been described and contrasted with that of thalassemia.<sup>226</sup> Hepcidin regulation is partly responsible for the decreased effects of iron loading in SCD compared to thalassemia and other hereditary anemias characterized by ineffective erythropoiesis.<sup>260</sup>

Nontransferrin bound iron (NTBI) and labile plasma iron (LPI), a highly reactive Fe<sup>2+</sup> subspecies of NTBI, rise considerably when the transferrin saturation reaches 60%.<sup>258</sup> Increases in NTBI and LPI are associated with tissue damage from iron overload. In fact, NTBI/LPI deposition in the liver, heart, and endocrine organs results in most of the significant morbidity from iron overload. Iron deposition in endocrine organs can result in growth failure, pubertal delay, diabetes, hypothyroidism, hypoparathyroidism, and hypogonadism. Hepatic fibrosis, cirrhosis, and hepatocellular carcinoma related to cumulative iron exposure can manifest later in life. Left ventricular dysfunction, congestive heart failure, and cardiac arrhythmias are the leading cause of death from iron overload. Due to the chronic inflammatory state and lack of ineffective erythropoiesis, there is less transferrin saturation and less NTBI in SCD compared to thalassemia. However, SCD patients

on chronic or recurrent episodic transfusions are also at risk for iron overload with subsequent liver, endocrine, and/or cardiac dysfunction, and therefore should be monitored and treated similarly to thalassemia patients.

### Monitoring and treatment of iron overload

Monitoring and management of iron overload are an essential part of the treatment of patients with thalassemia, SCD, and hereditary hemolytic anemias requiring chronic or episodic transfusion. Recommendations for monitoring and treating iron overload are largely extrapolated from thalassemia guidelines. Transfusional iron burden should be monitored with serum ferritin levels, as well as liver and cardiac MRI. Serum ferritin is routinely available, but it results in inaccurate determination of iron loading when used alone, especially in SCD patients who are in a chronic proinflammatory state.<sup>261</sup> Therefore, following serial trends in serum ferritin are more informative than single results. Measurement of hepatic iron historically required liver biopsy; however, this has been largely replaced by noninvasive, magnetic resonance imaging (MRI) techniques. Liver iron quantification by R2 and R2\* MRI techniques has been shown to correlate well with liver iron content (LIC) determined by liver biopsy.<sup>262</sup> LIC should be measured by R2-MRI every 1–2 years in patients on chronic transfusions and/or if serum ferritin is consistently greater than 1000 ng/mL.<sup>117,171</sup> Cardiac T2\* MRI represents the gold standard for monitoring cardiac iron accumulation, not only because it allows the estimation of cardiac iron burden, but also because it can reliably evaluate cardiac function.<sup>171,263,264</sup> Cardiac T2\* MRI evaluation is recommended every 6–24 months in TDT patients based on the degree of existing iron overload and the patient's risk for cardiac dysfunction.<sup>171</sup> While less common in SCD patients, iron-induced cardiomyopathy is detectable in about 2.5% of those who are chronically transfused.<sup>265</sup> Consequently, while cardiac iron overload screening is not warranted for all chronically transfused SCD patients, it should be considered for individuals with a high iron burden (LIC >15 mg/g dry weight) for two years or more, history of exceptionally high LIC, evidence of end organ damage resulting from transfusional iron overload, or evidence of cardiac dysfunction.<sup>117</sup> It is advised to assess endocrine function including thyroid, parathyroid, pancreas, adrenal, pituitary glands, and bone density every 1–2 years for TDT.<sup>171</sup>

Iron chelation therapy (ICT) is typically initiated within 1–2 years of starting chronic transfusions, after 10–20 cumulative RBC units (>120 mL/kg), or when the serum ferritin is greater than 1000 ng/mL or the LIC is 7 mg/g dry weight or greater. ICT is typically titrated to maintain serum ferritin less than 1000 ng/mL and LIC less than 7 mg/g dry liver weight.<sup>266</sup> Continued rising iron levels should prompt an evaluation of ICT dose and compliance, the annual transfusion requirement (mL/kg/year), and liver function. Compliance with ICT is probably the most important factor in

reducing the morbidity of chronic transfusion therapy.<sup>267</sup> Frequent evaluation of compliance and counseling is necessary in most patients for effective therapy. The goal of ICT is to provide as much chelation exposure per 24 hour period as possible to reduce the toxic effects of NTBI/LPI.

There are currently three iron chelators approved for use in the United States. These include deferoxamine (DFO), deferasirox (DFX), and deferiprone (DFP). Although the side effects differ for each iron chelator, their adverse effects are all generally more likely when ICT doses are high relative to the level of iron overload.

Deferoxamine (DFO or Desferal®) has a short half-life and therefore only chelates iron during the time infused. DFO is licensed for patients above the age of two years and is administered subcutaneously as an 8–12 hour infusion at least five times a week as monotherapy. Depending on the degree of iron overload, the standard dose range is 20–40 mg/kg/day for children and up to 50–60 mg/kg/day for adults. Dosing should not exceed 40 mg/kg/day until growth has ceased in children.<sup>171</sup> Side effects of DFO are greater with lower levels of iron, which is why therapy is not started until serum ferritin levels reach 1000 ng/mL and why attention is required to avoid over-chelation below 1000 ng/mL. Ascorbic acid (vitamin C) 2–3 mg/kg/day (100–250 mg maximum) is given orally during DFO.

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## CHAPTER 31

# Autoimmune hemolytic anemias and paroxysmal nocturnal hemoglobinuria

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Hemolysis, which is the pathologic destruction of red blood cells (RBCs), can manifest secondary to many underlying disorders including vascular/mechanical problems, microangiopathic processes, congenital disorders of hemoglobin or the RBC membrane, and/or with the absence of critical enzymes. One of the most common means by which RBCs are destroyed is via the immune system, with two of the most important immune-mediated disorders being autoimmune hemolytic anemia (AIHA) and paroxysmal nocturnal hemoglobinuria (PNH).

Although AIHA and PNH share the feature of shortened RBC survival, and both involve immune-mediated cellular clearance, the mechanisms underlying these diseases are distinct as are the tools used for their diagnosis and treatment. Therefore, the aim of this chapter is to provide a comprehensive review of AIHA and PNH, with a particular emphasis on pathophysiology, approach to diagnosis, an overview of how these diseases are medically addressed, and finally challenges these disorders present in the setting of transfusion therapy.

## Autoimmune hemolytic anemias (AIHAs)

### Overall classification

The term AIHA is broad and does not refer to a single disorder but rather reflects a number of unique entities with the common feature of generation of autoantibodies against self-antigens present on the RBC surface. AIHAs are further classified or categorized according to either the reactivity phase (i.e., temperature) of the associated autoantibody or by their association with drug administration. As detailed in Table 31.1, there are three broad subtypes: warm AIHA, cold AIHA, and drug-induced immune hemolytic anemia (DIIHA).

Accordingly, warm AIHA is due to autoantibodies that optimally bind to donor or self RBCs at 37 °C (and typically are of IgG class). On the other hand, in cold AIHA, autoantibodies demonstrate highest reactivity and avidity for donor/self at temperatures below 37 °C. However, it is important to note that because some of these cold antibodies can demonstrate a property called broad thermal amplitude, they can be clinically significant in day-to-day life. As

discussed in detail later in this chapter, cold AIHA is further subdivided into two additional entities, largely dependent on the type of autoantibody mediating hemolysis: cold agglutinin disease (CAD), mediated by IgM class autoantibodies, and paroxysmal cold hemoglobinuria (PCH), mediated by IgG autoantibodies.<sup>1</sup>

The other major category of AIHA is associated with pharmacologic therapy. In DIIHA, autoantibodies (largely IgG in class) are induced by, or against, a drug or its metabolite. As detailed later, induced drug-related autoantibodies can mediate RBC destruction via several pathways.

### Warm autoimmune hemolytic anemia (WAIHA)

#### Epidemiology

The incidence of WAIHA is approximately 1–3 cases in 100,000 individuals per year.<sup>2–4</sup> Therefore, WAIHA is the most common type of immune-mediated clearance, accounting for about 80% of all AIHAs.<sup>5</sup> Individuals of all ages, from the first to the eighth decade of life, can be affected by AIHA (although the median age of onset is the sixth decade of life).<sup>4,6–11</sup> Some genetic factors may be at play in WAIHA, with prior studies demonstrating disease associations with HLA-B loci, as well as some “protection” from WAIHA among individuals expressing HLA-DQ6.<sup>11</sup>

#### Pathophysiology

WAIHA can be considered a primary disorder (that is, idiopathic in nature and not associated with any other disease) or arise as a secondary process in the context of another medical issue. Common diseases giving rise to secondary WAIHA include other autoimmune diseases (e.g., rheumatoid arthritis and systemic lupus erythematosus), lymphoproliferative disorders (e.g., chronic lymphocytic leukemia), infectious processes (e.g., viral illness), and immune dysregulation (e.g., common variable immunodeficiency and stem cell transplant).<sup>2,4,7–11</sup> In pediatric patients in particular, there is a strong association between genes driving primary immunodeficiencies and WAIHA.<sup>11</sup>

**Table 31.1** Classification of the Autoimmune Hemolytic Anemias**Warm Autoimmune Hemolytic Anemia**

- Autoantibodies with optimal reactivity at 37 °C
- Usually an IgG (sometimes due to IgA) autoantibody
- Etiologies
  - Primary: idiopathic
  - Secondary
    - Malignancies: leukemia, lymphoma, ovarian tumors
    - Autoimmune/connective tissue disorders: systemic lupus erythematosus
    - Immune dysregulation disorders, e.g., common variable immunodeficiency
    - Infectious diseases, e.g., HIV, respiratory pathogens

**Cold Autoimmune Hemolytic Anemia****I. Cold Agglutinin Disease**

- Autoantibodies with optimal reactivity at 0 °C, with higher thermal amplitudes in clinically significant disease
- Usually an IgM autoantibody
- Etiologies
  - Primary: idiopathic
  - Secondary: B-cell neoplasms, *Mycoplasma pneumoniae*, and infectious mononucleosis

**II. Paroxysmal Cold Hemoglobinuria**

- Autoantibodies with biphasic reactivity: binding of antibody at cold temperatures and hemolysis at 37 °C
- A particular IgG autoantibody called Donath–Landsteiner or biphasic hemolysin
- Etiologies
  - Primary: idiopathic
  - Secondary: tertiary syphilis, upper respiratory tract infection of viral origin

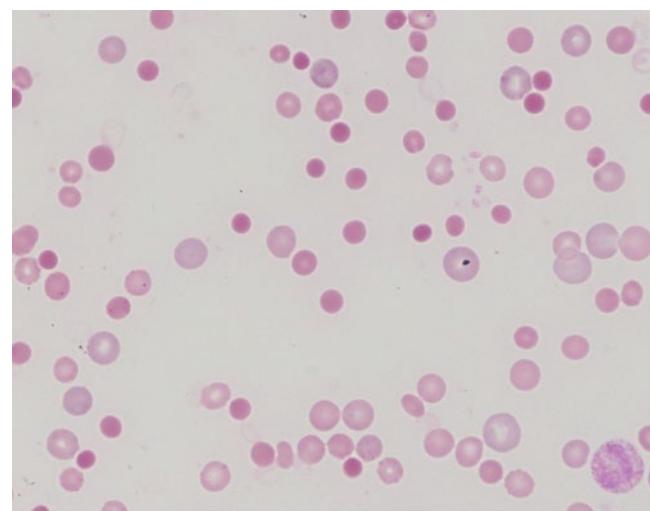
**Drug-Induced Immune Hemolytic Anemia**

- Autoantibodies usually reactive at warm temperatures
- Pathophysiologic mechanism
  - Drug adsorption mechanism
  - Immune complex mechanism
  - Autoimmune induction mechanism

Cases of WAIHA generally are split evenly among primary versus secondary causes in adults.<sup>4</sup> Most patients with primary or secondary disease are female.<sup>2,4,7–11</sup> Warm AIHA may also occur in association with thrombocytopenia, a combination known as Evans syndrome. Platelet destruction in this syndrome is a form of immune thrombocytopenia.<sup>12</sup>

Before delving into the specific pathophysiology of WAIHA, it is worthwhile to briefly review the mechanisms by which RBCs are hemolyzed *in vivo*; these mechanisms are important to understanding aspects of disease associated with WAIHA as well as the other immune-mediated hemolytic entities discussed in this chapter.

Overall, there are two distinct mechanisms of immune-mediated RBC destruction: extravascular hemolysis and intravascular hemolysis.<sup>9</sup> Macrophages located in the spleen and liver are largely responsible for extravascular hemolysis. The spleen is the most common site of extravascular clearance<sup>13</sup> since there is an opportunity for extensive interactions between antibody-coated red cells and macrophages created by the movement of red blood cells from the cords of Billroth through tiny gaps in sinusoidal walls to gain entry to the sinusoids.<sup>14</sup> However, the liver also participates in extravascular hemolysis when large amounts of IgG coat the red cells. This mechanism is important in asplenic patients.<sup>13</sup> On the other hand, intravascular, immune-mediated hemolysis is driven near-exclusively by complement fixation and direct cellular lysis.<sup>9</sup> While this section discusses each of these pathways in detail in the context of warm AIHA, these pathways are active in all potential forms of immune-mediated hemolysis.



**Figure 31.1** Common peripheral smear findings in warm autoimmune hemolytic anemia including microspherocytosis.

The hemolysis of warm AIHA usually proceeds via the extravascular mechanism since warm autoantibodies are typically IgG class and coat the RBC surface at 37 °C.<sup>9</sup> The antibody-coated red cells then circulate to the spleen and liver where macrophages adhere to the Fc portion of the IgG bound to the red cells.<sup>9,13</sup> Once bound to macrophages, the antibody-coated red cells are subjected to either complete or partial phagocytosis by the macrophage. Complete phagocytosis results in the removal of the antibody-coated red cells from circulation. Partial phagocytosis, which is more common,<sup>15</sup> results in the removal of a portion of the red cell membrane and the formation of microspherocytes (Figure 31.1).<sup>9</sup> Microspherocytes are released back into the circulation but have a decreased life span.<sup>15</sup> After a brief period of circulation, they pass back through the spleen where they are destroyed.<sup>16</sup>

The degree of extravascular hemolysis depends on the subclass of IgG, quantity of bound IgG, and antibody specificity. There are four subclasses of IgG: IgG<sub>1</sub>, IgG<sub>2</sub>, IgG<sub>3</sub>, and IgG<sub>4</sub>. Macrophage Fc receptors have the highest affinity for IgG<sub>1</sub> and IgG<sub>3</sub> subclasses.<sup>17,18</sup> Macrophages have variable affinity for IgG<sub>2</sub>, but they have the lowest affinity for IgG<sub>4</sub>. Accordingly, the most common subclasses of IgG found in clinically significant WAIHA are IgG<sub>1</sub> and IgG<sub>3</sub>, followed by IgG<sub>2</sub>. Subjects with IgG<sub>4</sub>-coated RBCs typically do not demonstrate significant hemolysis.<sup>1</sup>

Based on macrophage affinity, one might suspect that the greatest degree of extravascular hemolysis would be observed when red cells are coated by IgG<sub>1</sub> and IgG<sub>3</sub> subtypes. Although this is partly true, in the case of IgG<sub>1</sub>, the quantity of immunoglobulin coating each red cell also plays a role in the degree of hemolysis. Studies suggest that at least 1200 IgG<sub>1</sub> molecules<sup>17,18</sup> must coat each red cell before macrophages can effectively bind and subsequently phagocytose them. Consequently, there is a demonstrated relationship between the quantity of IgG<sub>1</sub> sensitizing the red cells and the severity of AIHA.<sup>19</sup> In contrast, IgG<sub>3</sub> is associated with hemolysis even when the quantity coating the red cells is too low to be detected by a direct antiglobulin test (DAT),<sup>1</sup> which is an average of 330–400 molecules.<sup>20,21</sup> In the case of IgG<sub>2</sub>, the situation is more complex. The gene that encodes the Fc receptor for IgG<sub>2</sub> on macrophages has two alleles, resulting in

differences in affinity. As a result, some people have macrophages with an Fc receptor that has a low-affinity for IgG<sub>2</sub>, and some are endowed with a receptor that has high affinity. Those individuals with high-affinity receptors for IgG<sub>2</sub> have the capacity to destroy IgG<sub>2</sub>-coated red cells, whereas those with low-affinity receptors rarely demonstrate IgG<sub>2</sub>-mediated hemolysis. With IgG<sub>2</sub> antibodies, the destruction of red cells is also influenced by antigen specificity. For example, studies have shown that IgG<sub>2</sub> alloantibodies against blood group A antigen lead to hemolysis, while hemolysis is not seen with IgG<sub>2</sub> antibodies directed against Rh antigens.<sup>22</sup> Despite the findings of differing Fc receptor affinity and IgG<sub>2</sub> alloantibody specificity, the role of IgG<sub>2</sub> autoantibodies in autoimmune hemolysis remains unclear. In summary, IgG<sub>1</sub> and IgG<sub>3</sub> antibodies are the primary mediators of red cell destruction in patients with AIHA, while IgG<sub>2</sub> antibodies are less important.<sup>1</sup>

Although WAIHA usually proceeds via the extravascular hemolysis mechanism, in some individuals presenting with severe hemolysis there may also be evidence of intravascular hemolysis,<sup>9</sup> the second pathway by which RBCs can be destroyed by the immune system. Intravascular hemolysis occurs when antibodies coating RBCs are capable of fixing and activating complement. In this setting, RBC destruction occurs by activation of the classical complement pathway, with ultimate formation of a membrane attack complex (MAC), resulting in pores in the RBC membrane. MAC formation eventually leads to cell swelling and finally lysis.<sup>9</sup>

The ability of antibody-coated RBCs to activate complement depends on many biological factors. In the setting of WAIHA, a largely IgG-driven process, important determinants of complement activation include the subclass and quantity of IgG coating the red cells. As with extravascular hemolysis, subclasses IgG<sub>1</sub> and IgG<sub>3</sub> are most commonly implicated in intravascular hemolysis as they are thought to be more efficient at fixing and activating complement than other IgG subclasses.<sup>23</sup> In addition, studies have demonstrated that a high density of cell-bound IgG is required to produce intravascular hemolysis,<sup>24</sup> likely due to the need for two IgG molecules to be in close proximity to allow for binding and activation of the complement system. Since a high number of IgG molecules are required to coat the RBCs before, by chance, two are in close proximity, the overall density would be expected to correlate with complement activation.<sup>13,25</sup>

Ultimately, despite attempts to divide hemolysis discretely into extravascular and intravascular mechanisms for the purpose of understanding the pathophysiology of AIHA disorders, they do not always occur uniformly in clinical situations. As such, and although extravascular hemolysis predominates in WAIHA, up to one-quarter of cases may demonstrate both forms of hemolysis.<sup>49</sup>

### Clinical features

WAIHA has a spectrum of clinical presentations. Severe anemia as a result of hemolysis can present with varying degrees of fatigue, fever, dizziness, angina, dyspnea, and flank pain, the latter most often correlating with a component of intravascular hemolysis. On the other hand, some patients will have a more slow, indolent course and only present to their physician with mild signs/symptoms. The severity of the onset and disease largely depends on the rate of hemolysis and the rate of decrease in hematocrit and hemoglobin. In chronic, lower grade (primarily extravascular) hemolytic anemia, the pace of the disease may allow for physiologic compensation and, therefore, less severe symptomatology and later presentation for clinical care. Patients may also develop jaundice, pallor, or dark

urine consistent with hemoglobinuria, causing them to seek care. Hepatosplenomegaly is present in approximately half of all WAIHA cases and may be particularly notable in WAIHA secondary to lymphoproliferative disorders.<sup>4,7,26</sup> Patients with idiopathic AIHA have been shown to be at increased risk for venous thromboembolism.<sup>7,27-29</sup> Since about half of WAIHA cases are due to secondary causes, the clinical presentation of the underlying cause may predominate.

### Laboratory findings

Complete blood counts will demonstrate typical features of anemia.<sup>30,31</sup> In a large case series of AIHA (with most reported patients demonstrating WAIHA), the median hematocrit was 24% and often associated with substantial reticulocytosis.<sup>4</sup> In cases of Evans syndrome, the platelet count is also low.<sup>12</sup> Other commonly encountered findings include elevated lactate dehydrogenase (LDH) and total/indirect bilirubin.<sup>4,31</sup> In cases with features of intravascular hemolysis, lab studies may demonstrate hemoglobinemia, hemoglobinuria, and decreased haptoglobin.<sup>4,31</sup> The peripheral blood smear typically shows evidence of extravascular hemolysis in the form of microspherocytes (Figure 31.1).<sup>31</sup> Due to the relatively short lifespan of microspherocytes, their presence in the peripheral blood should be taken as evidence for ongoing hemolysis.<sup>16</sup> Indirect evidence of bone marrow compensation, in the form of increased polychromatophils (morphologic evidence of reticulocytes) and even nucleated red blood cells (when hemolysis is brisk), may also be seen in the peripheral blood smear.

### Immunohematology findings

Serologic evaluation confirms that the hemolytic process is immune mediated. Both direct and indirect antiglobulin tests (DATs and IATs, respectively) may offer contributory findings. DATs use the patient's RBCs as well as polyspecific reagents, including both anti-IgG and anti-C3 (C3b and/or C3d). DATs are typically positive in patients with warm AIHA with findings as follows: +IgG alone is demonstrated in upward of 70–75% of WAIHA, with +IgG in combination with +C3 in about 25% of cases.<sup>32-37</sup> Complement alone is rarely found in pure warm AIHA mediated by IgG (Table 31.2).<sup>32,36,37</sup>

When the DAT demonstrates IgG, eluates can be performed. This laboratory technique essentially strips off the IgG antibody that is coating the patient's RBCs and attempts to reproduce the reactivity with separate donor/reagent commercial RBCs in an IAT-type reaction.<sup>33</sup> The expected IAT findings for an eluate in the setting of WAIHA is panagglutination, defined as reactivity with essentially all donor cells tested.

IATs can also be performed using the patient's serum or plasma. These reactions are performed via tube, gel, or solid phase methods at 37 °C, and typically also demonstrate IgG class autoantibodies in the serum or plasma that are pan-reactive against donor/reagent cells.<sup>33</sup> Reactivity against self is also demonstrable in IAT testing via the autologous control (autocontrol) test (i.e., patient plasma/serum incubated with the patient's own RBCs).<sup>33</sup> However, and notably, IATs are not as sensitive a marker for autoantibodies in the setting of WAIHA as compared with the DAT; IATs may be negative if the autoantibody is not present in high titer and/or the majority of it is adsorbed onto the patient's RBCs. Only when the amount of autoantibody exceeds the binding capacity of the patient's RBCs will the antibody be detectable in the patient's serum or plasma. Although upward of 95–97% of patients with WAIHA will have a positive

**Table 31.2** Typical Serologic Features of Autoimmune Hemolytic Anemia

	Warm Autoimmune Hemolytic Anemia	Cold Agglutinin Disease	Paroxysmal Cold Hemoglobinuria
Direct antiglobulin test	IgG only or IgG and C3; less commonly, C3 only	C3 only	C3 only
Immunoglobulin class	IgG, rarely IgA	IgM	IgG biphasic hemolysis
Eluate	IgG	Nonreactive	Nonreactive
Serum	IgG agglutinating red cells at the antihuman globulin phase, panagglutination	IgM agglutinating antibody, often with titers >1000, reacting at 30 °C in albumin	IgG biphasic hemolysis or Donath–Landsteiner antibody
Antibody specificity	Rh (most commonly)	I, i	P

Source: Based on Parker and Tormey (2017);<sup>32</sup> Petz and Garratty (1980);<sup>36</sup> Sokol et al. (1992).<sup>37</sup>

DAT, only 50–90% of patients will have a positive IAT, depending on the testing methodology.<sup>2,33</sup>

IgG-class autoantibodies in plasma/serum, or those eluted from RBCs, frequently demonstrate panagglutination when tested against commercially available reagent/donor RBCs. But what is the target of this pan-reactivity? Recently, studies have revealed that one highly conserved antigen system predominates as the target of panagglutinins—the external loops of the core RBC membrane protein band 3.<sup>38</sup> This helps to explain why autoantibodies appear reactive against essentially all donor/reagent cells in testing since this antigen/complex is found in >99% of individuals.

Occasionally, rather than demonstrating complete panagglutination, warm autoantibodies may demonstrate apparent blood group antigen specificity; these specificities are more often and more clearly seen in the plasma/serum as compared to the eluate.<sup>33</sup> This can present challenges for blood banks in truly differentiating between alloantibodies and autoantibodies. Clues that one may be dealing with an autoantibody rather than an alloantibody are a positive autocontrol (i.e., patient demonstrating reactivity against self) as well as phenotypic testing that indicates a patient expresses the antigen in question on their RBCs (in other words, if the detected reactivity were due to an alloantibody, one would expect the target antigen to be *lacking* on the patient's own RBCs). The most common specificities seen with autoantibody reactivity are antigens within the Rh blood group system, with autoantibodies described as demonstrating “e-like,” “C-like,” or other Rh-like specificity.<sup>33,39</sup>

Importantly, recent genetic studies have shown that Rh antigenic variants are common, particularly in patients of African descent. Since these Rh genetic variants can be associated with induction of clinically significant alloantibodies, transfusion services could potentially mistake an alloantibody for an autoantibody since patients with genetic Rh variants may demonstrate apparent normal/wild type antigen expression by *serological* methods. Because the presence of alloantibodies to Rh antigenic variants has treatment implications regarding provision of matched RBCs, if there is any doubt about whether reactivity seen is of autoantibody or alloantibody nature, then correlation with genetic studies is prudent.<sup>40</sup>

### DAT-negative warm AIHA

In up to 5% of patients with warm AIHA, the DAT is negative<sup>32–35</sup> indicating either the quantity of bound IgG is too low to be detected by a DAT, the bound autoantibody is not of the IgG immunoglobulin class, the hemolysis is being mediated by some other immune mechanism (e.g., cell-based cytotoxicity), the patient possesses very low affinity autoantibodies, or that the antibody-coated cells get rapidly destroyed and cleared and are therefore not detected by the DAT. Depending on subclass,

relatively few IgG molecules on the RBC surface can result in hemolysis, yet this number may be insufficient to produce a positive DAT.<sup>32</sup> Additionally, and although IgG is the most common immunoglobulin class associated with WAIHA, other immunoglobulin classes have been shown to cause AIHA, including IgA and IgM; these immunoglobulin classes are not detectable by routine DAT reagents (unless they are capable of fixing complement).<sup>9</sup> For example, in 14% of warm AIHA cases, IgA was found coating the red cells.<sup>41</sup> It should be noted that cases solely attributable to IgA autoantibody are rare (less than 1%) and often IgG and/or IgM are found in combination with IgA.<sup>42</sup>

In patients presenting with clinical features highly suggestive of warm AIHA but with negative serological testing, extended investigation should be performed because DAT-negative cases often respond to therapy for WAIHA. One means for assessing whether very low levels of IgG may be present in DAT negative cases is to perform elution studies on the patient's RBCs; as a test that concentrates antibodies, eluates may help to reveal low-level IgG reactivity.<sup>32,33</sup> Polybrene- or polyethylene-glycol-modified direct RBC assays may also be a more sensitive means for detection of low levels of IgG on the RBC surface.<sup>32</sup> Ultimately, if no auto-IgG can be demonstrated by these serological methods, then consideration could be given to using flow cytometry-based assays, or methods to identify surface IgA or IgM; however, these methods are rarely available and typically only offered in limited reference labs in the United States and internationally.<sup>32</sup>

### Positive DAT results in patients without hemolytic anemia

Confirmation of the immune-mediated cause of a suspected WAIHA should be sought and serology performed to determine the type of autoantibody that is coating RBCs. However, it is worth noting that disproportionate weight should not be placed on the results of the DAT. Up to 0.1% of healthy donors and 8% of general hospitalized patients were found to demonstrate a positive DAT in the absence of signs of hemolysis.<sup>43</sup> Although a positive DAT can provide supportive evidence for WAIHA in a patient with hemolysis, it is not independently indicative of the disease. Common causes of false-positive DATs (that is, positive assays in the absence of hemolysis) include polyclonal hypergammaglobulinemia, RBC rouleaux, intravenous immune globulin administration, antiphospholipid syndrome, and technical errors.<sup>32</sup> Therefore, the results of a DAT may be confirmed by eluate studies and, further, should always be interpreted in the context of the total clinical and laboratory picture.

### Treatment

Treatment in WAIHA is aimed at relieving clinical symptoms (e.g., symptomatic anemia) and transfusion dependence; typically, such treatment consists of immunosuppression and, as needed, transfusion support.<sup>44,45</sup> For secondary cases, addressing the

underlying disease also helps mitigate hemolysis. Treatment approaches are provided in more detail below.

### Corticosteroids

The primary initial treatment for warm AIHA is glucocorticoids.<sup>4,44</sup> A standard therapeutic approach for adults includes prednisone 1–2 mg/kg per day orally or an approximately equivalent dose of methylprednisolone via the intravenous route. Per a recent international expert consensus panel,<sup>44</sup> this therapy should generally be trialed for 2–3 weeks, with dose reduction if the patient shows an adequate response, and with treatment ceased within six months if hemolysis is no longer evident.

The efficacy of corticosteroid therapy is likely related to several factors. Steroids have been shown to have an early effect on tissue macrophages, leading to less efficient clearance of IgG- and C3-coated red blood cells within the first few days of therapy.<sup>46</sup> Steroids also affect antibody avidity and ultimately lead to a decrease in antibody production.<sup>47–49</sup>

If clinical relapse does occur, or if only a poor response is attained after the first few weeks of glucocorticoid therapy, then consensus guidance suggests initiation of other forms of immunosuppression, as described in the following sections.

### Monoclonal antibodies—rituximab and alemtuzumab

Rituximab is a genetically engineered monoclonal antibody against CD20. It targets B-cell precursors and mature B-cells. Consensus guidance, as well as other reported experience, recommends implementation of rituximab (1000 mg on Days 1 and 15, or 375 mg/m<sup>2</sup> weekly for four weeks) either in conjunction with corticosteroids as a first-line therapy, or alternatively, as a second-line therapy for cases of WAIHA failing to respond to first-line treatment.<sup>4,44,45,50–54</sup> Use of anti-CD20 as a single-agent therapy has been described in several case studies and retrospective reports, where it has been found to be efficacious in both adults and children with WAIHA resistant to therapy, including some patients with Evans syndrome.<sup>55–64</sup>

Alemtuzumab, a monoclonal anti-CD52 antibody that appears to modulate T-cell activation, has been used both as single-agent therapy and in combination with rituximab. However, its application to WAIHA has only been reported in a handful of papers, with side effects including immunosuppression and infections.<sup>65–67</sup> Current international guidance suggests implementation of anti-CD52 therapy only for refractory cases due to these risks.<sup>44</sup>

### Splenectomy

Splenectomy should be reserved for patients who show no response to corticosteroids, rituximab, or other pharmacological therapies.<sup>44</sup> While this procedure can be nearly as clinically effective as steroids<sup>45</sup> with 60–70% response within two weeks,<sup>50,51</sup> unfortunately many patients who clinically respond to splenectomy still require steroid therapy albeit at lower doses.<sup>52</sup> Despite an initial response, late clinical relapses are also still seen in some patients. These relapses are thought to be due to enhanced antibody production as well as increased clearance of antibody-coated red cells by the liver.<sup>7,49</sup>

It should be remembered that postsplenectomy patients are particularly vulnerable to infection by encapsulated bacteria. When these infections occur, patients can rapidly progress to septic shock due to an inability to efficiently clear the bacteria. Overwhelming sepsis represents a medical emergency with a risk of 3.2% and a

mortality rate of 1.4% in postsplenectomy patients.<sup>53</sup> If spleen removal is undertaken, then pneumococcal and meningococcal vaccines are strongly recommended to prevent infection and mortality in these patients.

### Other immunosuppressive and cytotoxic therapies

Treatment with azathioprine, mycophenolate, cyclophosphamide, antiplasma cell therapies (e.g., bortezomib), and danazol have demonstrated efficacy in reducing autoantibody formation and increasing hemoglobin levels.<sup>4,44,45,68,69–78</sup> However, these approaches are generally reserved for cases of WAIHA that are refractory to the aforementioned therapies.

The role for intravenous immunoglobulin (IVIG) remains unclear and is not recommended as a first- or second-line therapy by recent consensus guidance;<sup>44</sup> only 40% of patients respond to this therapy,<sup>79</sup> and the response is typically only sustained if IVIG infusions are continued on a chronic basis.<sup>80</sup>

Therapeutic plasma exchange may be used as a temporizing measure. However, it is neither effective nor practical for long-term treatment, particularly since IgG is only marginally removed by exchange and therefore should be reserved for severe or refractory cases. Severe warm AIHA is considered a Category III indication for plasma exchange therapy by the American Society for Apheresis (ASFA), suggesting that the optimal role of apheresis has not been established for this disease with only case reports and case series demonstrating (variable) efficacy.<sup>81</sup>

### Transfusion management

Support of patients with WAIHA often requires RBC transfusions for severe and/or life-threatening anemia. Due to the broad reactivity of autoantibodies in this disease, serologic evaluation to select appropriate RBC for transfusion can be complex and time-consuming. Sometimes transfusion is clinically required before the serologic workup is complete. Frequently, even after completion of the serologic workup, RBCs selected for transfusion are still incompatible with the patient's plasma. This finding should not be surprising given that most antibodies found in the patient's plasma/serum are broadly reactive panagglutinins. Transfusion of RBCs to a patient in true clinical need should not be delayed due to inability to find compatible RBC units. In fact, most patients with WAIHA show no adverse response to transfusion of serologically incompatible blood,<sup>82–84</sup> and the survival of transfused incompatible units is comparable to that expected for the patient's own RBCs.

When assessing the need for transfusion, it is helpful to recall that patients with chronic anemia have a long history of physiologic compensation. They may appear hemodynamically stable even with life-threatening anemia. The onset of confusion in a patient with worsening anemia and/or reticulocytopenia should warrant immediate transfusion. Even young adults and children with gradual onset of anemia should be transfused to maintain a hemoglobin level above 4 mg/dL; higher hemoglobin levels are needed for older patients and patients with cardiovascular disease.<sup>85</sup>

### Selection of blood for transfusion

ABO discrepancies and difficulty with Rh typing can arise in association with warm autoantibodies. When ABO discrepancy occurs, accurate ABO typing cannot proceed until the IgG autoantibody coating the red cells is removed. Rh typing may also be problematic,

although the use of monoclonal reagents may improve results in the setting of immunoglobulin-coated RBCs. When transfusion is urgent, group O RBC components can be issued and administered, even if ABO typing is incomplete.

In many patients with autoantibodies, their history of transfusion or pregnancy allows for the possibility of an alloantibody in addition to their autoantibodies. Underlying alloantibodies have been seen in up to 40% of patients with AIHA.<sup>82,86,87</sup> Thus, the identification of alloantibodies that may be obscured by the presence of a panreactive autoantibody is of great importance. The use of adsorption techniques is required for the removal of panagglutinins from the patient's plasma/serum so that any underlying alloantibodies can be tested against commercially available red cells for identification. Unfortunately, recent studies have shown that practices vary widely in the United States with regard to antibody identification in this setting, and there is no standard best practice.<sup>88</sup>

Autologous adsorption, utilizing the patient's own RBCs to draw off autoantibody, is a useful technique in patients who have not been transfused or undergone stem cell transplant in the last three months.<sup>88</sup> If the patient has been recently transfused or transplanted, allogeneic adsorptions may be required.<sup>89,90</sup> Adsorption techniques, particularly allogeneic adsorptions, are complex, labor intensive, and time consuming.<sup>86</sup> Therefore, they may be incomplete when a transfusion is clinically necessary.

Identifying the patient's phenotype aids in the provision of appropriate blood for transfusion by focusing the serological evaluation only on those alloantibody specificities the patient is capable of making. In some cases, it may be necessary or more expedient to infer the patient's phenotype from their genotype. Phenotypically matched RBC units should be safe for transfusion even when the alloantibody workup is unenlightening.<sup>87</sup> In addition, the provision of phenotype-matched blood may prevent the formation of alloantibodies, which may be seen in higher rates in patients with autoimmune disorders.<sup>91,92</sup>

Ultimately, most individuals with warm autoantibodies will not have fully crossmatch compatible RBC units available, and "least crossmatch incompatible" RBC units will be necessarily issued.<sup>93</sup> Certainly, such RBC units should be matched for any alloantibodies identified, either historically or by auto/alloadsorption techniques. As noted previously, some autoantibodies will mimic blood group antigen specificity.<sup>38</sup> While providing RBC units negative for these antigens may be associated with better *in vitro* compatibility by crossmatch techniques,<sup>38</sup> it is generally not necessary to match for these antigens in transfusion practice, particularly for more urgent needs.<sup>82</sup> For cases with severe or florid RBC hemolysis, it may be prudent to attempt what has been sometimes described as an "*in vivo*" crossmatch, wherein incompatible RBCs are infused slowly via a small "test" dose (15–20 mL at a slow rate), with subsequent observation for hemolysis or other symptoms. If said test dose is tolerated without issues, then the remainder of the unit can be infused at a regular rate with close observation.

Finally, and in the absence of symptomatic anemia, there is no distinct threshold at which patients with AIHA should be transfused. Decisions should be made, and products should be selected based on clinical need, comorbid conditions, and degree of compensation at the time of presentation.<sup>4,93</sup>

## Prognosis

Historically, the outcome of warm AIHA was poor, with a 38% mortality rate in the 1950s–1960s.<sup>26</sup> While treatments have dramatically improved over the past 60 years and WAHIA is often considered a mild-to-moderate condition, mortality can be still be

seen from severe disease or complications such as thromboembolic events or infection (secondary to therapy) in up to 5% of adult WAHIA patients.<sup>4,94</sup> The prognosis for children remains excellent,<sup>95–97</sup> with most experiencing self-limited disease.<sup>98,99</sup>

## Cold autoimmune hemolytic anemia

Many individuals will possess cold autoantibodies. However, most of these autoantibodies are benign and not associated with hemolysis. Those antibodies that are responsible for cold AIHA are unique in that, while they are strongly reactive at cold temperatures (i.e., 1–4 °C) they also demonstrate the property of reacting at warmer temperatures closer to core body temperature, a phenomenon known as broad thermal amplitude. Thus, within body parts more likely to be colder than core temperature (e.g., an extremity or tips of fingers, toes, ears, and nose), RBC–antibody interaction occurs and clinically significant hemolysis may ensue.

Unlike WAIHAs, cold AIHA is not a single entity but rather is further subdivided into the CAD and PCH, the unique features of which are described below.

## Cold agglutinin disease

### Epidemiology

CAD is rarer than WAIHA, but is considered overall the second most common immune-mediated hemolytic anemia, accounting for 15–25% of cases of AIHA and showing a slight female predominance.<sup>3,36,100</sup> In a large study of patients with CAD, 61% of patients were female, and the median age at diagnosis was 72 years;<sup>101</sup> this relative female preponderance and older age at the time of onset have been confirmed in other studies.<sup>100</sup>

### Pathophysiology

The primary driver of CAD is cold-reactive, IgM class autoantibodies. Such antibodies have been largely found to be encoded by the *IGH4-34* immunoglobulin heavy chain gene and are often associated with a monoclonal paraprotein of IgM class in serum.<sup>100</sup> Once formed, RBCs are coated with said IgM autoantibodies with binding classically at temperatures <37 °C. Such temperatures can be found in extremities, where autoantibody–RBC interaction occurs in the setting of environmental cold exposure.

Once bound, IgM fixes and activates the classical pathway of complement, leading to the generation and binding of C3 to the RBC surface. If a critical amount of C3 is generated by complement activation, the formation of the MAC may proceed, leading to cell lysis and intravascular hemolysis.<sup>9,100</sup>

If, on the other hand, the IgM has a narrower range of activity (or thermal amplitude), or individuals are relatively complement-deficient (e.g., in the setting of liver disease or recent complement consumption), intravascular hemolysis will be less likely. In these cases, C3 will be formed and bound below the amount needed to form the MAC.<sup>102,103</sup> C3-coated red cells circulate to the spleen and liver where macrophages recognize C3b, an opsonin, and RBCs are then phagocytosed resulting in primarily extravascular hemolysis.<sup>9</sup>

Simultaneously, complement regulatory proteins work to inactivate C3 bound to RBCs. Those RBCs whose bound complement has been inactivated will still circulate through the spleen and liver, although the spleen will not recognize the inactive bound complement.<sup>9</sup> The liver will recognize the inactive bound complement and

sequester these RBCs, frequently allowing them to return to the circulation after a period of time.<sup>100</sup> Thus, when hemolysis in CAD occurs by way of the extravascular system, it is generally at a decreased pace and lesser degree, as compared to intravascular hemolysis. In fact, extravascular hemolysis is the more common pathway in CAD, with only about 15% of patients demonstrating evidence of intravascular lysis.<sup>100</sup> Unlike extravascular RBC clearance in warm AIHA, in CAD, it more commonly occurs in the liver, likely via Kupffer cells.<sup>9,10</sup>

Although the pathways of intravascular and extravascular hemolysis in IgM-mediated CAD are discussed as separate entities for the purpose of understanding, in reality these pathways are often not discrete during disease flares. The percentage of hemolysis accounted for by each mechanism will depend on the titer of IgM, thermal amplitude of reactivity, immune competence of the patient, complement reserve/activity, and resultant number of RBCs with sufficient complement activation for MAC formation.<sup>9,10</sup>

Like WAIHA, CAD can be a primary/idiopathic disorder or can be associated with another disease and therefore be a secondary form of hemolysis. Common disorders that may give rise to, or be associated with, CAD include infectious processes (with a classical association with *Mycoplasma pneumoniae* infection) and lymphoproliferative disorders (e.g., lymphoplasmacytic lymphoma), among other entities.<sup>100</sup>

### Clinical features

As with other types of AIHA, patients with CAD present with symptoms of progressive anemia.<sup>104</sup> Given the pathophysiology, it is not surprising that there is an association between the thermal amplitude of the IgM autoantibody and severity of symptoms. Those patients having autoantibodies with a high thermal amplitude (i.e., immunologically reactive at higher temperatures) are more likely to have severe symptoms of hemolysis.<sup>105</sup> Those with low thermal amplitude, the more common occurrence, typically demonstrate an indolent course with a slower hemolytic rate.<sup>105</sup> Thermal amplitude is further described in Table 31.3. In either case, the anemia can be exacerbated by exposure to cold temperatures, such as during the winter or in a cold operating room, with acute hemolytic crises accompanied by frank hemoglobinuria. In addition, as with warm AIHA, the quantity of IgM, as measured by

titration, is associated with the degree of hemolysis and observable symptoms. A higher titer autoantibody is more likely to be clinically significant.

On physical examination, patients with CAD may demonstrate pallor and jaundice, consistent with hemolytic anemia. Acrocyanosis of the tip of the nose, ears, fingers, and toes is commonly reported with cold exposure<sup>101</sup> and resolves with warming.<sup>104</sup> Unlike warm AIHA, hepatosplenomegaly is not a major feature.<sup>101</sup> In patients with secondary CAD due to an underlying process, whether it be a malignancy or infection, the symptoms associated with the underlying disease may predominate.

Some other complications of CAD may also be seen. For instance, and not unlike WAIHA, there have been reports of increased thromboembolic events in severe CAD, possibly attributable to RBC destruction.<sup>100</sup> However, while such events have been seen in severe cases of CAD, there is insufficient evidence suggesting that thrombotic events are common in chronic or milder forms of this disease.

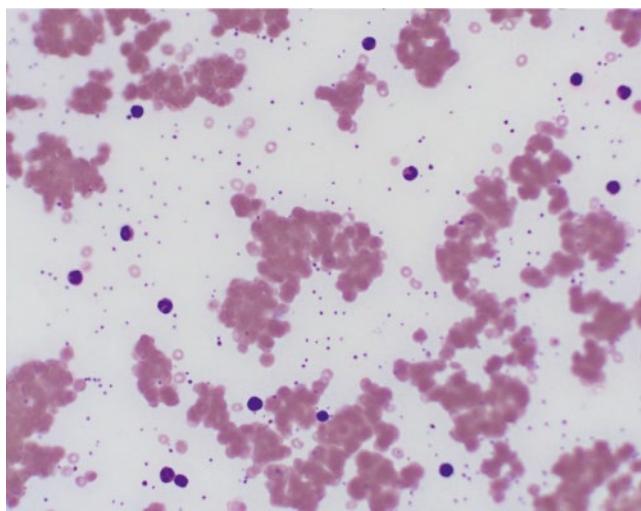
### Laboratory findings

The first noticeable finding may actually be observed at the time of collection of a blood sample, which may demonstrate "clumping" and a gelatinous appearance as it cools from body to room temperature. The agglutination may be reversible by warming the sample. If the specimen cools prior to receipt in the lab or throughout the process of automated testing, spurious results such as macrocytosis or marked elevations in the RBC index mean corpuscular hemoglobin concentration (MCHC) may be encountered.<sup>31,106</sup> These spurious results also provide early suggestion of this diagnosis. In order to avoid preanalytical testing problems, it is often necessary to maintain blood samples at 37 °C from the time of their collection, with immediate delivery to the laboratory for analysis. Otherwise, routine hematologic evaluation will reveal anemia with decreased hemoglobin; studies have shown that individuals with chronic CAD will possess median hemoglobin levels of 8–10 g/dL, although more profound anemia can be encountered.<sup>100</sup> The peripheral blood smear will show agglutination with the formation of irregular aggregates or clumps of RBCs (Figure 31.2). Other commonly encountered findings include elevated LDH and total/indirect bilirubin.<sup>4,31</sup> In cases with predominant features of intravascular hemolysis, lab studies may also demonstrate hemoglobinemia, hemoglobinuria, and decreased haptoglobin.<sup>4,31</sup>

**Table 31.3** Examples of Titer and Thermal Amplitude Studies in Cold Agglutinin Disease

Patient Specimen Dilutions	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512
Adult donor RBCs*	4+	4+	4+	4+	4	4+	3+	2+	1+
"Cord" donor RBCs*	4+	3+	2+	1+	0	0	0	0	0
Incubation Temperature	4 °C	10 °C	15 °C	20 °C	25 °C	30 °C	35 °C	37 °C	40 °C
Adult donor RBCs*	4+	4+	4+	4+	3+	2+	1+	0	0
"Cord" donor RBCs*	4+	1+	0	0	0	0	0	0	0

\* Both adult and "cord" donor cells may be used in thermal amplitude and titer studies, or an adult or cord cell line may be chosen after initial titer studies depending on the strength of reactivity observed and the likely specificity of an autoantibody. In the demonstrated case, both cord and adult cells show similar reactivity at 4 °C, but as temperatures increase, the cold autoantibody in question reacts more strongly with adult cells, implying I-specificity, as can be seen in idiopathic cold agglutinin disease or acquired cold agglutinin disease associated with *Mycoplasma pneumoniae* infection. Given the reactivity to temperatures in excess of 25–30 °C, this cold agglutinin would be considered clinically significant.



**Figure 31.2** A typical example of peripheral blood smear findings in cold agglutinin disease, including large clusters of red cell agglutinates.

### Immunohematologic findings

In CAD, the cold-reactive, IgM autoantibodies (and/or their effects on RBCs) will be demonstrable in the blood bank. The DAT will be characteristically negative with monospecific IgG reagent but will be positive with C3 reagent (Table 31.2).<sup>32</sup> Eluate studies are typically not performed as they are not indicated in the setting of negative IgG DAT results. Instead, the possibility for IgM autoantibodies is further investigated by performing cold agglutinin titers and thermal amplitude measurements (Table 31.3).<sup>31</sup> Notably, thermal amplitude is the single most important determinant of the clinical significance of a cold agglutinin, followed by its titer.<sup>31</sup>

Thermal amplitude measurement is carried out across a range of temperatures up to 37 °C. Once reactivity is shown at temperatures above 28 °C, then said autoantibody should be considered potentially clinically significant. Moreover, while cases of clinically significant CAD are most often associated cold autoantibodies having a titer >>1:1000 (at least one study has shown that the median titer in CAD is 1:2048),<sup>100</sup> hemolysis can occur at titers as low as 1:64.<sup>31,100,107</sup> Because healthy individuals may have low-titer cold autoantibodies, it is important to avoid misidentifying a patient with another cause for anemia as CAD based upon low-titer antibodies, hence the reliance on thermal amplitude as the main indicator of clinical significance.

Cold screens or panels, testing the patient's serum/plasma against cord blood cells (possessing the i antigen) as well as adult RBCs (possessing the I antigen), can be used to determine if the autoantibody demonstrates blood group antigen specificity. The most frequent antibody specificities are anti-i or anti-I, with auto-anti-i most often associated with infectious mononucleosis/Epstein–Barr virus infection and auto-anti-I with *Mycoplasma pneumoniae* infection.<sup>108</sup> Other antigen specificities such as Pr have also been demonstrated, albeit rarely.<sup>31,33,109</sup>

IAT studies are less frequently impacted by cold agglutinins as incubation steps during pretesting can help eliminate the reactivity of many cold autoantibodies, even those that are clinically significant. Therefore, serum/plasma testing for cold autoantibodies is most often negative after incubation steps, allowing for the relatively straightforward identification of underlying/concomitant alloantibodies.<sup>33</sup>

### Positive cold agglutinins in patients without hemolytic anemia

Cold reactive autoantibodies are commonly found in normal individuals. As with warm AIHA, the serologic findings in suspected CAD should always be correlated with clinical presentation so as not to overstate their importance. Cold reactive autoantibodies in the absence of hemolysis are not diagnostic of CAD.<sup>33,100</sup>

### Treatment

Per recent international consensus guidance,<sup>44</sup> therapy for CAD depends upon the subclassification of the disease (i.e., primary vs. secondary) and patient symptoms. Various treatment approaches are briefly outlined below. Treating underlying diseases in secondary forms of CAD also helps to resolve hemolysis.

### Avoidance of cold

For many patients, particularly those with mild or asymptomatic disease, watchful waiting and avoidance of cold exposure are reasonable approaches to therapy. Many patients with mild, chronic anemia are able to use this simple tactic to avoid transfusions or targeted therapy for prolonged periods of time. Should cold exposure be unavoidable (e.g., in the setting of hypothermic surgery), warming techniques should be considered.<sup>110</sup> Notably, while broad thermal amplitude/clinically significant cold autoantibodies can be problematic in the setting of hypothermic surgery, a recent analysis indicated that, in general, clinically *insignificant* cold autoantibodies rarely cause complications in this setting and routine screening for such benign cold autoantibodies is not recommended preoperatively.<sup>111</sup>

### Monoclonal antibody therapy—rituximab and eculizumab

Should patients be more actively symptomatic or transfusion dependent, international consensus guidance, and other published experience, suggests rituximab as a reasonable first-line therapy.<sup>44,111</sup> One recommended dosing regimen is 375 mg/m<sup>2</sup> for four weeks at 7-day intervals, with prospective studies suggesting response rates of 50–80% with this approach.<sup>44,101,111–118</sup>

Consensus guidance also discusses first-line medical approaches for emergent/life-threatening situations in the setting of severe CAD (as anti-CD20 therapy will not result in an immediate reversal of symptoms or hemolysis). In such circumstances, and given the complement dependence of CAD, guidance recommends the use of anticomplement therapies such as the anti-C5 antibody, eculizumab, in conjunction with transfusion and/or apheresis.

### Cytotoxic/chemotherapeutic agents

Some guidance documents and studies recommend the addition of bendamustine (90 mg/m<sup>2</sup> Days 1 and 2 for cycles at 28-day intervals) to rituximab, as either part of first-line therapy or in cases of rituximab-only failures.<sup>44</sup> The addition of fludarabine to rituximab has also been reported as another second-line approach.<sup>44</sup> Antiplasma cell therapies (e.g., bortezomib),<sup>44</sup> which target mature antibody-secreting plasma cells that are not typically susceptible to anti-CD20 therapeutics such as rituximab, as well as chlorambucil have been used with some success.<sup>44,68</sup> If CAD remains refractory to all of the aforementioned approaches, then the consideration of other forms of therapy (e.g., ibrutinib) or enrollment in a clinical trial (e.g., those examining other forms of complement inhibition) may be warranted.<sup>44</sup>

### Other therapies

Therapeutic plasma exchange may be used as a temporizing measure, such as prior to surgery, but body temperature must be

maintained at 37 °C and the use of an inline blood warmer is strongly recommended.<sup>81</sup> The IgM autoantibodies in CAD have a larger intravascular volume and are more efficiently removed by apheresis than the IgG autoantibodies causing WAIHA. Based predominantly on evidence from case reports, ASFA has deemed severe CAD as a Category II indication for plasma exchange, indicating that apheresis is an acceptable second-line therapy.<sup>81</sup> It may also be trialed in cases of emergent/urgent or life-threatening disease.<sup>44</sup>

In contrast to WAIHA, treatments such as IVIG,<sup>39</sup> splenectomy,<sup>44,100</sup> and glucocorticoids<sup>44,100,103</sup> are rarely useful in patients with CAD. Treating underlying diseases in cases of secondary CAD is often helpful in resolving hemolysis.

### Transfusion management

Supportive care of patients with CAD occasionally includes RBC transfusion, particularly for severe, symptomatic anemia. Transfusion may be episodic and routine for those with well-controlled, mild, or chronic disease. Alternatively, it may be urgent for those presenting with an acute exacerbation due to cold exposure or intractable disease.

As with WAIHA, serology evaluation can be challenging, adding a layer of complexity to provision of appropriate blood components. For instance, accurate ABO typing in patients with CAD can be difficult as this analysis relies on RBC agglutination as an end point. Because the blood from patients with this disease agglutinates as the temperature declines and sometimes even at room temperature, cold agglutinins can interfere with ABO interpretation.

Warm washing of red cells to remove the IgM autoantibody may be utilized to facilitate accurate ABO typing. Ultimately, group O RBCs can be transfused if ABO typing cannot be reliably determined. For patients with a positive antibody screen, the distinction between a cold agglutinin reactive at room temperature and possible underlying alloantibodies must be made. As discussed previously, the use of a prewarming technique, where the patient sample is warmed before being tested against an antibody panel of commercially available red cells, may prove useful. If cold reactivity persists even after these steps, then other techniques (e.g., adsorption of the IgM in the patient plasma/serum with rabbit erythrocyte stroma [RESt] or destruction of the IgM with dithiothreitol) may be warranted.

One can attempt to determine the specificity of the autoantibody; often, and as mentioned previously, the antibody demonstrates specificity against the I or i antigen. However, this is mostly an informational undertaking and it is not necessary to transfuse blood that is negative for these specific antigens.

Finally, during actual administration of RBCs, patients should be kept warm. Transfusion with the use of a blood warmer is typically recommended to avoid cooling core body temperature and precipitating hemolysis,<sup>44</sup> although this step has not been definitively proven to be required.

### Prognosis

The prognosis for CAD is better than for WAIHA. Many patients have CAD as a transient problem following an infection and subsequently recover without recurrences. Other patients have a chronic, indolent course. However, there are reports of severe and even fatal CAD in the literature.<sup>120,121</sup>

## Paroxysmal cold hemoglobinuria

### Epidemiology

PCH is among the rarest forms of immune-mediated hemolysis, accounting for about 2% of all AIHAs.<sup>36</sup> It is most commonly seen in children, with a slight male > female predominance.<sup>6</sup> When

syphilis was more common, PCH was also seen in adults in association with tertiary infection. As the incidence of syphilis declined, so too did the incidence of PCH.

### Pathophysiology

In PCH, the responsible autoantibody is an unusual cold-reactive IgG autoantibody, called a biphasic hemolysin, or Donath–Landsteiner antibody. Unlike most IgG antibodies, this antibody binds to RBCs at cold temperatures and in the process irreversibly binds complement. At warmer temperatures, the antibody no longer stays bound. However, complement remains bound and becomes activated, leading to the formation of the MAC and resulting in intravascular hemolysis.

Similar to other AIHAs, PCH can be a primary/idiopathic disorder (extremely rarely) or much more commonly arise secondary to another disease. At present, PCH occurs most often in association with viral infections in children (e.g., upper respiratory illness) or following vaccination in that same population.<sup>6</sup> In adults, and other than the historical association with tertiary syphilis, it can occasionally arise secondary to autoimmune or lymphoproliferative disorders, among other entities.<sup>44</sup>

### Clinical features

The typical presentation is a child with a recent history of upper respiratory tract infection, generally noted a week or two before presentation.<sup>6</sup> Such individuals may demonstrate jaundice, pallor, fatigue, and/or fever, as well as report urine color changes.<sup>6</sup> Hepatosplenomegaly is not generally a prominent feature. Given the cold-dependent nature of the autoantibodies involved, a history of cold exposure triggering signs/symptoms may be reported.<sup>6</sup>

### Laboratory findings

PCH often presents with laboratory evidence of anemia, which may be quite severe due to intravascular hemolysis.<sup>122,123</sup> Reticulocytosis may be present, but there can also be reticulocytopenia. Otherwise, common lab findings include elevated LDH and total/indirect bilirubin as well as undetectable haptoglobin, consistent with a picture of intravascular hemolysis.<sup>4,31</sup> Hemoglobinemia and hemoglobinuria are also characteristically present.<sup>4,31</sup> The peripheral blood smear can demonstrate findings associated with hemolysis, including microspherocytes as well as fragmented forms. Erythrophagocytosis may also be seen (Figure 31.3).<sup>31</sup>

### Immunohematology findings

In patients with PCH, routine DATs are characteristically positive for C3 and negative for IgG; eluate studies are also negative. Although PCH is an IgG-mediated process, because of the exquisitely temperature-dependent nature of the autoantibody routine DAT studies do not demonstrate IgG, but only remnant RBC-bound complement (Table 31.2).<sup>31,33</sup> Similarly, and because of the cold-reactive nature of the IgG autoantibody, IAT studies are also traditionally negative. Like autoantibodies in other forms of AIHA, PCH autoantibodies demonstrate specificity for select RBC antigens, with the P antigen being the target of most Donath–Landsteiner antibodies.

A unique serological assay, called the Donath–Landsteiner test, can be useful to cinch a suspected diagnosis of PCH.<sup>31</sup> This test confirms the biphasic nature of this antibody. As detailed in Table 31.4, the patient's serum is reacted against donor RBCs with and without normal serum (as a fresh source of complement) at various temperatures, including one reaction that takes the specimen from cold to warm.<sup>31,33</sup> Hemolysis occurring in this latter

specimen (Figure 31.4), along with the absence of hemolysis in tubes maintained at only cold or warm temperatures, confirms a Donath–Landsteiner antibody and a diagnosis of PCH. Critically important to this assay is using a freshly collected serum specimen that is maintained at warm temperatures and immediately delivered to the lab for processing such that the reactivity of the cold-dependent antibody is preserved in vitro.<sup>31</sup>

### Treatment

Although the presentation is dramatic, the majority of cases of PCH in children are self-limited and abate as the triggering illness resolves. Therefore, young patients with PCH primarily require supportive care (potentially including RBC transfusion) and cold avoidance during their acute illness. Adults with chronic or more severe disease may benefit from immunosuppression; rituximab has been reported to be successful in such cases.<sup>44</sup> Glucocorticoids and complement inhibition (e.g., eculizumab) may also be an option for more severe or refractory cases,<sup>44</sup> although there is very limited evidence on outcomes with this approach.

### Transfusion management

Since the disease is typically self-limited, transfusion is usually only required in the acute hemolytic phase.<sup>6,44</sup> Selection of RBCs appropriate for transfusion is aided by the fact that the causative

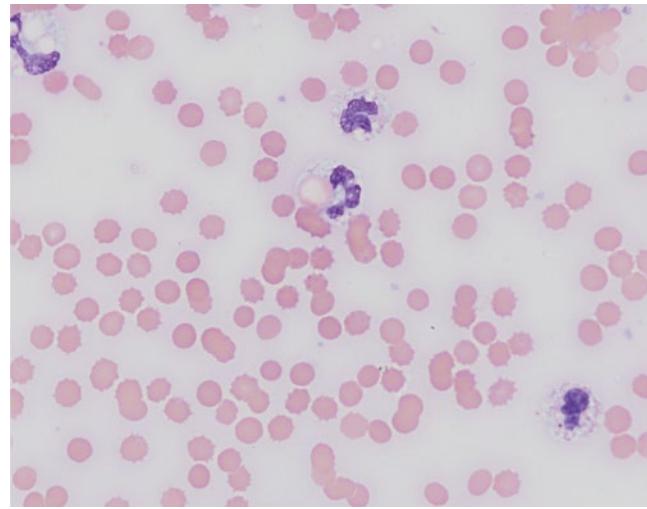
biphasic autoantibody does not often interfere with pretransfusion compatibility testing given its cold-reactive nature. Despite most PCH autoantibodies demonstrating specificity for the P antigen, P-antigen-negative RBC units are scarce. From an empirical perspective, if the patient is maintained in a warm environment and RBC units are infused via an approved blood warmer, then randomly selected donor RBCs should suffice without exacerbating hemolysis. As with other forms of AIHA, small “test” doses of RBCs may be warranted to see if units are well tolerated before increasing infusion rates.

### Prognosis

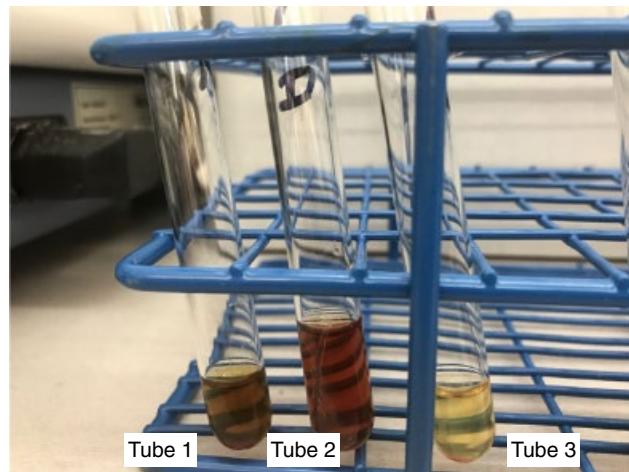
The prognosis is excellent, with the disease in its secondary form most commonly following a self-limited course over a few days to weeks in most cases. The greatest risk to patients is in the acute phase when they may present with severe anemia and require transfusion and supportive treatment.

### Mixed AIHA

In addition to the outlined distinct entities of WAIHA and CAD, some patients present with elements of both forms of these disorders, and therefore this disease process is called mixed AIHA. While there may be a predominance of one type over another, laboratory and clinical findings ultimately overlap and present a picture as outlined in previous sections on WAIHA and CAD.



**Figure 31.3** Erythrophagocytosis in a case of paroxysmal cold hemoglobinuria. This case was associated with a strongly C3+ DAT, intravascular hemolysis, and onset following adenovirus infection.



**Figure 31.4** Positive Donath–Landsteiner test in specimens cooled and then warmed to body temperature; Tube 1 (with supernatant hemolysis) reflects patient's serum + donor RBCs; Tube 2 (also with supernatant hemolysis) reflects patient's serum + donor RBCs + donor serum to supplement complement; and Tube 3 is a control tube, showing no hemolysis in the supernatant.

**Table 31.4** The Donath–Landsteiner Test: Serological Aspects and Expected Outcomes in a Patient with Paroxysmal Cold Hemoglobinuria (PCH)

	Tube 1*	Tube 2*	Tube 3*
<b>Contents</b>	Pt serum + donor RBCs	Pt serum + donor RBCs + fresh donor serum**	Donor RBCs + fresh donor serum [CONTROL]
<b>Specimen</b>	-30 min @ 1–4 °C	-30 min @ 1–4 °C	-30 min @ 1–4 °C
<b>Incubation</b>	-60 min @ 37 °C	-60 min @ 37 °C	-60 min @ 37 °C
<b>Results</b>	Mild–moderate hemolysis in supernatant	Moderate–severe hemolysis in supernatant	No hemolysis in supernatant

\*In parallel, each of the steps is repeated, only with variation in incubation steps. That is, another set of experiments are performed with Tubes 1–3 maintained at 1–4 °C only for 90 minutes and another set of experiments are done with Tubes 1–3 maintained at 37 °C only for 90 minutes. In cases of true PCH, no hemolysis should be observed in these parallel experiments, as the Donath–Landsteiner antibody only causes hemolysis in biphasic testing, that is when the temperature is increased from cold to warm.

\*\*Fresh serum is added in Tube 2 to supplement complement; this is done in case individuals are relatively complement-deficient at the time of testing due to complement consumption in the days leading to their presentation and testing.

Mixed AIHA comprises approximately 7% of all cases of idiopathic AIHA.<sup>124</sup> Like all other forms of immune-mediated hemolysis previously discussed, it can be primary or secondary to another disease process (e.g., autoimmune disease).<sup>125</sup>

While a relatively rare type of AIHA, this entity is a potentially hard-hitting form of disease, with many reported cases demonstrating severe symptom onset including fulminant, and sometimes fatal, hemolysis.<sup>44,126,127</sup> Like other AIHAs, mixed-type hemolysis can also be associated with complications beyond RBC destruction including thromboembolism.<sup>127,128</sup> As such, consensus guidance suggests an aggressive treatment approach combining corticosteroids with rituximab as a first-line therapy.<sup>44</sup> Nonetheless, relapse and/or refractoriness are common. Despite the mixed nature of the autoantibodies involved, splenectomy appears to be ineffective and avoidance of this procedure is recommended.<sup>44</sup> From a transfusion management perspective, guidance as outlined above following a combined approach for WAIHA and CAD is advised with the use of “least incompatible” RBC units frequently required as is the use of a blood warmer for infusion.<sup>127</sup>

## Drug-induced immune hemolytic anemia

### Epidemiology

Drug-induced immune hemolytic anemia (DIIHA) can occur in any age group and may be seen in association with a wide variety of medications, including prescription drugs, over-the-counter medications, herbal formulations, and toxins (Table 31.5).<sup>129–133</sup> The most commonly implicated drug class in current practice is antibiotics and, within this group, cephalosporins are very frequent drivers of DIIHA.<sup>129</sup> Among more recently introduced classes or agents of therapy, there have been increasing reports of immune checkpoint inhibitor medications also driving forms of DIIHA.<sup>131–133</sup> In a large case series of DIIHA, the odds ratio for developing immune hemolytic anemia was significantly increased with cotrimoxazole (trimethoprim/sulfamethoxazole), fludarabine, lorazepam, and diclofenac.<sup>130</sup> Notably, drug-induced/drug-associated RBC antibodies have also been detected in healthy blood donors.<sup>132</sup>

## Pathophysiology

DIIHA can be thought of as drug dependent (i.e., requisite for the drug to interact with the RBC surface in order for hemolysis to ensue) or drug independent (i.e., hemolysis can occur without the drug directly interacting with the RBC surface; autoantibodies are induced by the drug-immune system interaction with RBCs as a bystander).<sup>129–133</sup> Drug-independent hemolysis often closely mimics WAIHA, while drug-dependent hemolysis can be further divided into (1) the drug adsorption mechanism/model and (2) the immune complex mechanism/model.<sup>129</sup> We tackle each of these independently to review their distinct pathophysiologies.

In the drug-dependent/drug adsorption mechanism, thought to be the most common form DIIHA,<sup>129</sup> the drug attaches to the RBC surface. The resultant antibody formed is directed against the drug, with antibody binding to the drug while it is still bound to the red cell membrane. The entire antibody-drug-coated RBC compound is then cleared, typically via extravascular hemolysis. The prototypic drug associated with this mechanism is penicillin.<sup>129</sup>

In the drug-dependent/immune complex mechanism,<sup>9</sup> circulating drug stimulates the immune system to produce antibody directed against the drug. The antibody binds to the circulating drug, and the resulting immune complex can then bind to a red cell. It is unknown whether binding to the red cell is specific or nonspecific. The RBCs, coated by antibody-drug complexes, are typically removed via brisk, intravascular hemolysis. The prototypic drug for this mechanism is quinidine.

In the drug-independent mechanism, the drug induces autoantibody formation.<sup>129,132</sup> The resulting immune-mediated hemolytic anemia is essentially serologically and clinically indistinguishable from WAIHA. The autoantibody may persist even after the offending drug has been discontinued. The prototypic drug demonstrating this mechanism of immune hemolytic anemia is  $\alpha$ -methyldopa, a medication used in the past to treat hypertension.<sup>9,129,132</sup>

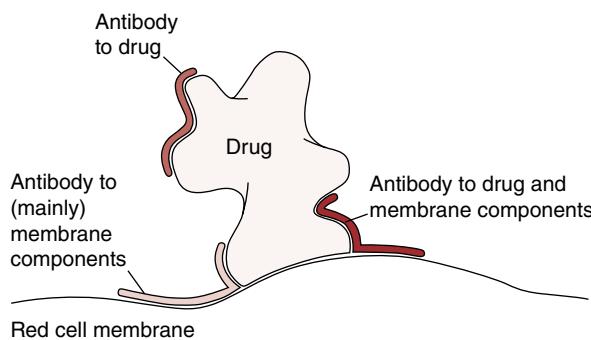
Finally, another relatively new concept in DIIHA is that of nonimmunological protein adsorption or NIPA.<sup>129,132</sup> In brief, drug-RBC interaction does not result in a drug-induced antibody per se, but drug interaction with, and binding to, the RBC

**Table 31.5** Classes of Drugs Associated with Drug-Induced Immune Hemolytic Anemia and Examples of Agents within Those Classes

Drug Classes		Some Reported Examples
Most Common	Antimicrobials, antivirals, and antifungals	Acyclovir, cefazolin, cefotetan, chloramphenicol, cloxacillin, isoniazid, ketoconazole, penicillin, rifampin, tazobactam, tetracycline, trimethoprim/sulfamethoxazole, and quinine
	Antineoplastics	Alemtuzumab, bendamustine, carboplatin, cladribine, fludarabine, imatinib, interferon, melphalan, methotrexate, oxaliplatin, rituximab, and vincristine
	NSAIDs and analgesics	Acetaminophen, apazone, aspirin, diclofenac, dipyrone, ibuprofen, mefenamic acid, methadone, nabumetone, and naproxen
	Antihypertensives and antiarrhythmics	Buthiazide, captopril, furosemide, hydralazine, methyldopa, procainamide, triamterene, and quinidine
	Immunosuppressants	Cyclosporin, hydrocortisone, lenalidomide, and tacrolimus
	Antihistamines	Antazoline, chlorpheniramine, cimedtidine, and ranitidine
	Antidiabetics	Chlorpropamide, insulin, tolbutamide, mesartoin, and phenytoin
	Anticonvulsants	Carbamazepine, cyclofenil, and diethylstilbestrol
	Endocrine system medications	Fluorescein, iomeprol, and metrizoic acid
	Imaging dyes	Carbromal and lorazepam
	Sedative-hypnotics	Nomifensine
	Antidepressants	Streptokinase
Least Common	Thrombolytic	Cianidanol
	Antidiarrheal	

Source: Based on Garratty (2012);<sup>129</sup> Garbe *et al.* (2011);<sup>130</sup> Michot *et al.* (2019).<sup>131</sup>

Data were compiled from several sources.<sup>129–133</sup> Drugs involved may cause hemolysis by one or more of the mechanisms discussed in the text and described in Figure 31.5.



**Figure 31.5** Proposed unifying theory of drug-induced antibody reactions. The thick lines represent antigen-binding sites of the drug-induced antibody. Antibodies may be made to the drug (producing in vitro reactions typical of a drug adsorption [penicillin-type] reaction), the membrane components or mainly membrane components (producing in vitro reactions typical of an autoantibody), or part-drug, part-membrane components (producing an in vitro reaction typical of the immune-complex mechanism).<sup>129</sup> This unifying theory was proposed by George Garratty, Ph.D. (1935–2014), who made significant contributions to the study of drug-induced immune hemolytic anemias. Source: Garratty (2004). Reproduced with permission.

membrane rather results in red cells being targeted for clearance by cellular components of the immune system (e.g., macrophages). These surface modifications may also give rise to changes in the RBC membrane sufficient enough to cause positive DAT results, although no specific antibody is ultimately identifiable.<sup>129,132,133</sup>

Although the explanation of these mechanisms as discrete entities is helpful to understand the many ways in which drugs, antibodies, and RBCs can interact, some patients have laboratory findings that overlap with more than one mechanism. A unifying theory was therefore proposed by Dr. George Garratty, which argues that the antibodies against the drug itself, against the red cell membrane, or components of both could be present simultaneously (Figure 31.5).<sup>123,132,133</sup> The specificity of the formed antibodies and immune reaction depends on the site of interaction between the drug and RBC. The unifying theory explains how patients may present with clinical evidence of multiple simultaneous pathophysiology of DIIHA.<sup>129,132</sup>

### Clinical features

The clinical presentation of DIIHA can be broad, ranging from mild anemia to severe hemolysis with hemoglobinemia and hemoglobinuria, as well as renal failure. As previously described, the hemolysis produced by the drug-independent mechanism is often indistinguishable from WAIHA. Moreover, while it is typically thought that DIIHA is most likely to impact individuals who have only recently started a new medication (i.e., in recent days to weeks), drug-induced hemolysis has also been reported to spontaneously develop in individuals on long-standing, chronic drug regimens.<sup>133</sup> Minimally, a patient should be actively taking a drug, or should have taken a drug within the prior 2–3 weeks, for DIIHA to be considered on the differential diagnosis.<sup>133</sup>

### Laboratory findings

DIIHA presents with laboratory evidence of anemia, which may be severe when there are components of intravascular hemolysis. Like all other AIHAs, common lab findings include elevated LDH and

total/indirect bilirubin.<sup>4,31</sup> Hemoglobinemia and hemoglobinuria also occur when DIIHA is associated with intravascular clearance.<sup>4,31</sup>

### Immunohematology findings

As with pathophysiology, the serologic findings can be divided according to the mechanism of DIIHA.

### Drug-dependent hemolysis—adsorption and immune complex

Starting with drug-dependent/drug absorption models, the DAT is positive in these patients, most commonly revealing IgG-only positivity.<sup>9,31,129,133</sup> Standard eluates and IATs are classically negative. Thus, the patient presenting with signs/symptoms of hemolysis with a positive DAT but negative IAT and eluate should raise suspicion for drug-dependent hemolysis.

When drug-dependent/drug adsorption DIIHA is suspected, further testing can be carried out to confirm this hypothesis. Traditionally, additional testing involves treating donor/reagent commercial RBCs with a solubilized form of the suspected drug.<sup>31</sup> Drug-treated RBCs (as well as untreated, control RBCs) are reacted with the patient's plasma/serum, as well as an eluate from the patient's RBCs. Positive reaction (plasma/serum +/- eluate) with drug-treated reagent RBCs, and negative reactions with untreated RBCs, is strongly suggestive of drug-dependent/drug-adsorption-mediated DIIHA.<sup>31</sup>

In cases of intravascular hemolysis presumed to be due-to-drug-dependent/immune complex mechanisms, the DAT is usually positive for complement. Similar to drug dependent/drug adsorption, the IAT or eluate may be positive if drug is added to the reaction, but this may not always be the case.<sup>31</sup>

Despite its great utility in evaluation for DIIHA, drug-dependent testing has some basic limitations. For instance, the process of drug treating RBCs may induce some false-positive results, not unlike that described in NIPA.<sup>31</sup> False negatives are also a concern; patients are often on multiple drugs and only one or two may be initially suspected to be driving hemolysis. Therefore, if initial candidate drugs fail to yield conclusive results and hemolysis continues, then it is reasonable to consider other drugs the patient is taking or has taken. Finally, hemolysis may not necessarily be attributable to a parent drug but rather one of its metabolites. Such scenarios can be very difficult to assess via in vitro testing and may cause a metabolite-dependent antibody to be missed.

### Drug-independent hemolysis

When patients demonstrate evidence of the drug-independent hemolysis, the serologic findings are essentially indistinguishable from those of WAIHA, with a DAT most commonly positive for IgG only as well as demonstration of panagglutinins in eluates, and panreactivity in IAT. These autoantibodies may persist after the drug is discontinued.<sup>129–135</sup>

In drug-independent cases, laboratory testing to definitively prove drug involvement in hemolysis becomes extremely complex. In fact, many drug-independent cases may be misclassified as primary WAIIHA due to the overlap in clinical and laboratory features. Because there is no drug bound to the RBC surface, drug-treated RBC assays are of no particular use in helping to establish the diagnosis. In these cases, clinical teams must entertain a broad differential and remember to include DIIHA on their list.

### Treatment

Treatment is aimed primarily at discontinuing the causative drug. Corticosteroids are often given, although there are only empirical data to support their use.<sup>133</sup> Intravenous immune globulin may also

be warranted in severe cases.<sup>133</sup> As in essentially all cases of DIIHA, if there is a strong clinical suspicion for this disorder (even with negative testing), then consideration must be given to stopping any drug that may have triggered the reaction.

### Transfusion management

Transfusion support may be necessary for patients presenting with severe anemia and guidance is similar to that discussed earlier for other forms of AIHA. In the case of hemolysis persisting even after discontinuation of the offending drug, as is presumed to be due to the autoimmune induction mechanism, a prolonged period of transfusion support may be required.

### Prognosis

The prognosis is generally excellent for this form of AIHA; full resolution of hemolysis is expected in many cases with drug cessation and/or immunosuppression. Notably, the offending drug or toxin must be avoided indefinitely to avoid provoking future immune stimuli and hemolysis.

### Paroxysmal nocturnal hemoglobinuria (PNH)

PNH is a rare type of hemolytic anemia that can present with thrombosis and bone marrow dysfunction on top of RBC destruction.<sup>136</sup> It is a clonal disorder of hematopoietic stem cells with its biochemical pathogenesis based in a somatic gene mutation.<sup>137</sup> The somatic mutation ultimately decreases the availability of an enzyme required for the synthesis of the anchor molecule, glycosylphosphatidylinositol (GPI), responsible for binding numerous proteins to the external surface of RBCs. Two of the affected proteins, which are responsible for the majority of symptoms seen in PNH, are the complement-regulatory proteins CD55 and CD59. Without GPI binding of CD55 and CD59 to the cell surface, RBCs lack their normal defenses against complement activation (primarily the alternative pathway) and have increased sensitivity to complement-mediated lysis.

### Epidemiology and risk factors

PNH is rare, occurring at a rate of approximately 1–10 cases per million people.<sup>138,139</sup> It has been diagnosed in patients of all ages.<sup>140,141</sup> The median age at diagnosis is early thirties.<sup>141–143</sup> Pediatric cases account for only 10% of reported cases of PNH.<sup>144,145</sup> No apparent gender predilection has been identified.<sup>142</sup>

Although PNH can occur de novo, virtually all patients demonstrate some degree of bone marrow failure.<sup>136</sup> Likewise, RBCs or granulocytes deficient in GPI-linked molecules, such as CD55 and CD59, are identified in a large number of patients with bone marrow disorders such as aplastic anemia and, to a lesser extent, low-risk myelodysplastic syndrome (MDS).<sup>142,146</sup> The leading hypothesis for this close association is that there is a selective growth advantage for PNH clones in these disorders; however, the exact etiology of PNH clones in these patients remains unclear.<sup>146–149</sup> Additionally, the finding of PNH clones may not always be clinically significant. In aplastic anemia and MDS, PNH clones are so commonly found that screening for undetected clones is not routinely recommended in patients with these disorders.<sup>150,151</sup> In most cases, the percentage of abnormal cells is so small that clinical symptoms are not observed and therapy for PNH is not indicated.<sup>146</sup> Furthermore, the stem cell mutations underlying PNH can even be found at a low frequency in healthy controls.<sup>152,153</sup> Therefore, many questions

remain unanswered, including what leads to multipotent stem cell mutations, why the clones sometimes expand to a high enough percentage to cause clinical disease, and why bone marrow failure exists in virtually all cases.

Although paroxysms of hemolysis may occur without preceding risk factors, it is generally agreed that factors leading to complement activation can trigger hemolytic episodes. In a person with underlying mutations giving rise to PNH clones, infection, trauma, pregnancy, and surgery are examples of known triggers of complement activation that can initiate a PNH paroxysm.<sup>154</sup>

### Pathophysiology

PNH is a clonal disorder of multipotent hematopoietic stem cells that ultimately causes increased sensitivity to complement in its progeny cells. In virtually all cases, a somatic mutation in the X-linked phosphatidylinositol glycan class A (PIG-A) gene underlies the disorder. The PIG-A gene encodes for the PIG-A enzyme, which is needed in the first step of biosynthesis of the GPI anchor molecule.<sup>137</sup> GPI anchor molecules are needed to bind numerous proteins to the external surface of hematopoietic cells.<sup>137</sup> The majority of symptoms seen in PNH are due to the lack of binding of two of the affected proteins, CD55 and CD59. These complement-regulatory proteins are normally needed on the external surface in order to neutralize complement activation. CD55 accelerates the destruction of C3 convertase, reducing the activation of complement component C3 which limits extravascular hemolysis by macrophages in the spleen and liver.<sup>155</sup> CD59 prevents the MAC from completing pore production in the lipid bilayer of the cell which, if it were unimpeded, would produce cell lysis through hypertonic swelling.<sup>156</sup> In brief, the PIG-A mutation leads to decreased or absent GPI molecules,<sup>137</sup> which, in turn, results in decreased or absent localization of proteins such as CD55 and CD59<sup>136</sup> to the cell surface and, ultimately, increased cell sensitivity to complement-mediated destruction.

Two conditions must be met in order for the mutation to cause clinical symptoms: it must occur in a multipotent hematopoietic stem cell, and the stem cell carrying the mutation must undergo clonal expansion.<sup>152,157</sup> Evidence for the former requirement is that rare, circulating blood cells with PNH mutations can be seen in healthy blood donors<sup>152,153</sup> and low-frequency PNH clones in MDS rarely, if ever, lead to clinical disease.<sup>136,158</sup> Mutations in these cases appear to arise in hematopoietic cells without self-renewal capacity. If the mutation occurs in a multipotent hematopoietic stem cell, it will result in hematopoietic progeny (e.g., RBCs, white blood cells, and platelets) that also harbor the mutation.<sup>136</sup> Patients with aplastic anemia, unlike those with MDS, more commonly develop PNH because mutations in these patients arise from multipotent hematopoietic stem cells.<sup>136</sup> Yet it is not enough that the mutations occur in a cell capable of creating progeny in all hematopoietic lineages. Low-frequency mutations do not cause clinical disease.<sup>146</sup> On the contrary, patients with greater than 20–25% of their neutrophils and greater than 3–5% of their RBCs lacking CD55 and CD59 surface proteins are more likely to demonstrate clinical signs of hemolysis and require specific treatment than those with lower values.<sup>146</sup> Likewise, patients with a higher proportion of PNH neutrophils (and monocytes) are more likely to demonstrate thrombosis,<sup>159</sup> with neutrophil clone sizes greater than 50% being highly predictive of thrombotic risk.<sup>160</sup> Although there are several purported hypothe-

ses,<sup>153,161–166</sup> the mechanism by which PNH cells can achieve clonal expansion remains unknown.

The ultimate clinical effect of having a sizeable PNH cell population depends on the type of surface protein and cells affected. Affected RBCs show CD59 that is either decreased (called type II PNH RBCs) or absent (called type III PNH RBCs) and, as a result, are exquisitely sensitive to intravascular hemolysis from complement activation. They also show decreased or absent CD55, causing them to be incapable of blocking C3 activation and making them vulnerable to extravascular hemolysis in the spleen and liver.

On the other hand, in platelets, the increased sensitivity to complement resulting from lack of CD55 and CD59 results in their inappropriate activation with a resulting increased risk of thrombosis.<sup>167</sup> In affected leukocytes, the absence of a receptor for urokinase-type plasminogen activator (also GPI-linked) reduces the potential of these cells to convert plasminogen to plasmin.<sup>168</sup> The decreased formation of plasmin, which has a significant role in fibrinolysis, may also lead to increased risk of thrombosis.<sup>169,170</sup> Although all patients with PNH are at risk for thrombosis, there is increased risk with larger PNH clone size.<sup>142</sup> When thrombosis occurs in PNH patients, it often does so in atypical locations such as the portal circulation, although the reason is unclear.<sup>136</sup>

Secondary effects of sizeable PNH clonal populations are also seen. Increased intravascular hemolysis leads to large amounts of free hemoglobin, which overwhelm the normal mechanisms of clearance, such as binding by haptoglobin.<sup>171,172</sup> The circulating free hemoglobin in plasma then scavenges and reacts with nitric oxide.<sup>172,173</sup> The depletion of nitric oxide is thought by some to contribute to arterial constriction, decreased blood flow to organs, kidney damage, and pulmonary hypertension.<sup>174</sup> However, others have speculated that chronic kidney disease in PNH is due to tubular damage caused by microvascular thrombosis<sup>175–177</sup> and iron deposition from hemolysis.<sup>136</sup> Microthrombi may also contribute to pulmonary hypertension.<sup>136</sup> Decreased nitric oxide may also lead to smooth muscle dystonia manifested as chronic dysphagia, abdominal pain, and erectile dysfunction.<sup>178</sup>

In patients with PNH clones and clinical hemolysis, the hemolysis typically occurs at a chronic baseline rate punctuated by episodic periods of an increased hemolytic rate. The pathophysiology of these episodic exacerbations of hemolysis is thought to be due to enhanced complement activation in certain situations; infection, trauma, pregnancy, surgery, strenuous physical activity, and alcohol use are examples of known triggers.<sup>154,179</sup> Additionally, the repletion of iron in a deficient patient is a known trigger of PNH exacerbations; it is thought to cause increased hemolysis by increasing the number of PNH clones.<sup>180</sup> Thus, both enhanced complement activation and increased PNH clone size may form the background upon which a PNH paroxysm occurs.

Finally, although the PIG-A mutation described is prototypical for and present in the vast majority of PNH cases, other mutations have been described.<sup>181,182</sup>

## Clinical findings

PNH classically presents with the triad of hemolytic anemia, thrombosis, and bone marrow dysfunction.<sup>136</sup> Because the clinical findings are variable, three clinical categories of PNH have been described. In classical PNH, patients frequently have >50% PNH granulocytes, and episodic hemolytic anemia accounts for the majority of the clinical picture. These patients may present with fatigue, jaundice, and a history of dark urine, consistent with

hemoglobinuria. The hemoglobinuria may be nocturnal as implied by the disease's moniker; however, it may also occur at other times. These patients are also the most likely to develop thrombosis.<sup>136</sup> The second category of PNH is clinically apparent disease occurring in the context of bone marrow disorders, most frequently aplastic anemia. In the context of an underlying bone marrow disorder, patients with PNH also present with symptoms of anemia. However, the anemia is more likely due to bone marrow failure, and these patients tend to demonstrate fewer symptoms suggestive of hemolysis (jaundice and dark urine). They often have low platelet counts and a lower risk of thrombosis. The third category is subclinical PNH. By definition, these patients are asymptomatic<sup>136</sup> and typically have less than 10% PNH RBCs or granulocytes in peripheral blood.<sup>136,150</sup> The discrete assignment of this disease into three clinical categories may be useful for understanding the variable clinical presentations, but it is not perfect. A particular point of confusion not addressed by these categories is that some element of bone marrow failure underlies virtually all PNH cases.

As mentioned above, patients with PNH clones and clinical hemolysis experience a chronic baseline rate of RBC destruction punctuated by episodes of increased hemolysis in the setting of known triggers.<sup>154,179</sup> Thrombosis is rarely the presenting symptom of PNH,<sup>183</sup> but it is common over the course of the disease and seen in up to 40% of patients.<sup>179</sup> Additionally, thrombi are the most common life-threatening complication in PNH.<sup>159,177</sup> The thromboses in PNH patients are often in atypical locations, most commonly affecting abdominal and cerebral vasculature, with veins more often affected than arteries. Commonly affected abdominal veins include the hepatic, portal, mesenteric, and splenic veins. The sagittal and cavernous sinuses are the most frequently affected cerebral vasculature. Overall, the most common site of thrombosis in these patients is the hepatic veins;<sup>136</sup> thrombi in this location cause a decrease in the normal flow of blood out of the liver, a process termed Budd-Chiari syndrome.<sup>184,185</sup> Despite frequently presenting in these atypical locations, thrombosis may occur in any site, and deep venous thrombosis, pulmonary emboli, and dermal thrombi are relatively common.<sup>136</sup> When thrombosis does occur, it tends to be progressive despite anticoagulant therapy.<sup>159,177</sup>

Patients with PNH may also present with symptoms of increased smooth muscle tone, manifested by chronic dysphagia, abdominal pain, and erectile dysfunction.<sup>178</sup> Over time, these patients may develop renal insufficiency and pulmonary hypertension. There is greater than a sixfold increase in risk of developing chronic kidney disease in PNH patients.<sup>175</sup> Although clinically significant pulmonary hypertension in PNH is rare, testing for terminal probrain natriuretic peptide<sup>186</sup> and transthoracic Doppler echocardiography<sup>187</sup> indicates that pulmonary hypertension is present in approximately half of these patients.

Most patients with PNH demonstrate a chronic illness that persists without therapy. Interestingly, a small subset of patients, up to 15% in a recent study, may achieve spontaneous, long-term remission.<sup>140</sup>

## Laboratory findings

Along with the variable clinical presentation of PNH, the associated laboratory findings vary as well. In classical PNH, which includes patients with intravascular hemolysis and the highest risk of thrombosis, one can expect to find anemia, reticulocytosis, elevated LDH,<sup>137</sup> elevated total bilirubin, and decreased haptoglobin. Bone

marrow evaluation demonstrates an overall hypercellular marrow with erythroid hyperplasia but no karyotypic abnormalities.<sup>150,188</sup> Patients with PNH in the context of an underlying bone marrow disorder also have anemia, but their laboratory results are typically dominated by the effects of bone marrow failure more so than intravascular hemolysis. These patients may have severe thrombocytopenia, normal or only mildly elevated LDH,<sup>136</sup> normal or only mildly elevated bilirubin,<sup>189</sup> and lower levels of reticulocytes than patients with classical PNH.<sup>136</sup> Patients with subclinical PNH have normal or only modestly aberrant blood counts.<sup>136</sup>

Peripheral blood smear findings are also variable and nonspecific, including a broad array of anisocytosis and poikilocytosis.<sup>190</sup>

## Immunohematology findings

### Blood bank testing

In PNH, the DAT and IAT are classically negative.<sup>189</sup> Nonetheless, patients undergoing eculizumab therapy may have a positive DAT for another reason; although this therapy prevents the formation of the MAC and intravascular lysis, it does not prevent C3 coating of RBCs.<sup>191–193</sup>

### Acidified serum test (HAM test)

The earliest confirmatory test for PNH was the acidified serum test or HAM test (named after its developer, Thomas Ham).<sup>194</sup> In this assay, RBCs are combined with acidified serum. PNH RBCs are more sensitive to complement and more likely to undergo lysis in the presence of acid, and spectrophotometry is utilized to quantify the amount of free hemoglobin that is liberated. This test is easy to perform, inexpensive, and reliable, but it cannot quantify the number of PNH cells present.<sup>195</sup> Also, a positive test is not specific for PNH and may be seen in other disorders leading to enhanced sensitivity to complement (such as congenital dyserythropoietic anemia type II [HEMPAS] where hemolysis is produced by an anti-HEMPAS antibody that binds complement).<sup>23</sup> Currently, due to more sensitive and specific results from flow cytometry, the HAM test is not widely used or recommended as a first-line diagnostic strategy.

### Flow cytometry

Because of its sensitivity and specificity for detection of PNH clones, flow cytometric testing has become the gold standard in the diagnosis of this entity. Classically, flow cytometry of the peripheral blood is aimed at detecting a marked reduction, or absence, of the GPI-anchored proteins, CD55 and CD59, on RBC or granulocyte surfaces. Monoclonal antibodies that bind each of these GPI-anchored proteins are tagged with a fluorescent label and combined with a patient sample, allowing the detection of cells capable of binding the antibody. This analysis provides useful information regarding the quantity of cells lacking or deficient in CD55 and CD59 as well as the strength of expression of these proteins on the cell surface.<sup>195</sup> In normal patients, all RBCs and granulocytes demonstrate strong expression of CD55 and CD59. In patients with PNH, by contrast, flow cytometry will demonstrate a variable proportion of red cells and/or granulocytes lacking CD55 and CD59 in addition to a population of cells with strong expression of these surface proteins.<sup>195</sup> Flow cytometry of monocytes and granulocytes may be more accurate, as their numbers are not affected by recent red blood cell transfusions or hemolysis. Lymphocytes can normally show variable expression of GPI-anchored proteins and are therefore undesirable for use in PNH diagnosis.<sup>195</sup> Ideally, PNH should be confirmed with findings of

decreased or absent GPI-anchor proteins in two or more cell lines.<sup>188,196</sup>

In recent years, flow cytometry testing for PNH has evolved to include fluorescent aerolysin (FLAER) in the analysis, which is also aimed at detecting a marked reduction or absence of GPI-anchored proteins. FLAER is based on the finding that GPI-anchored proteins can bind a bacterial toxin called aerolysin. PNH cells lack GPI-anchored proteins and therefore do not bind the aerolysin toxin. By tagging the aerolysin toxin with a fluorescent label, the amount of cells capable of binding the toxin and the strength of binding can be quantified (Figure 31.6).<sup>197</sup> The sensitivity of FLAER is much higher than that achieved with a CD55- and/or CD59-based assay.<sup>198</sup> The detection rate is further improved when the FLAER reagent is combined with fluorescent labeled antibodies for GPI-anchored proteins on granulocytes and monocytes.<sup>195</sup> Specifically, current recommendations use CD24 to specify GPI-anchored proteins on granulocytes and CD14 on monocytes (Figure 31.6).<sup>198</sup> RBCs are not commonly included in analysis with a FLAER assay as they express glycophorin, which is capable of weakly binding aerolysin and thus affecting assay accuracy.<sup>197</sup> Peripheral blood flow cytometric analysis is preferred to that of bone marrow for routine analysis as it represents an analysis of cells that are more homogeneous with respect to stage of maturation and expression of GPI-anchor proteins.<sup>195</sup>

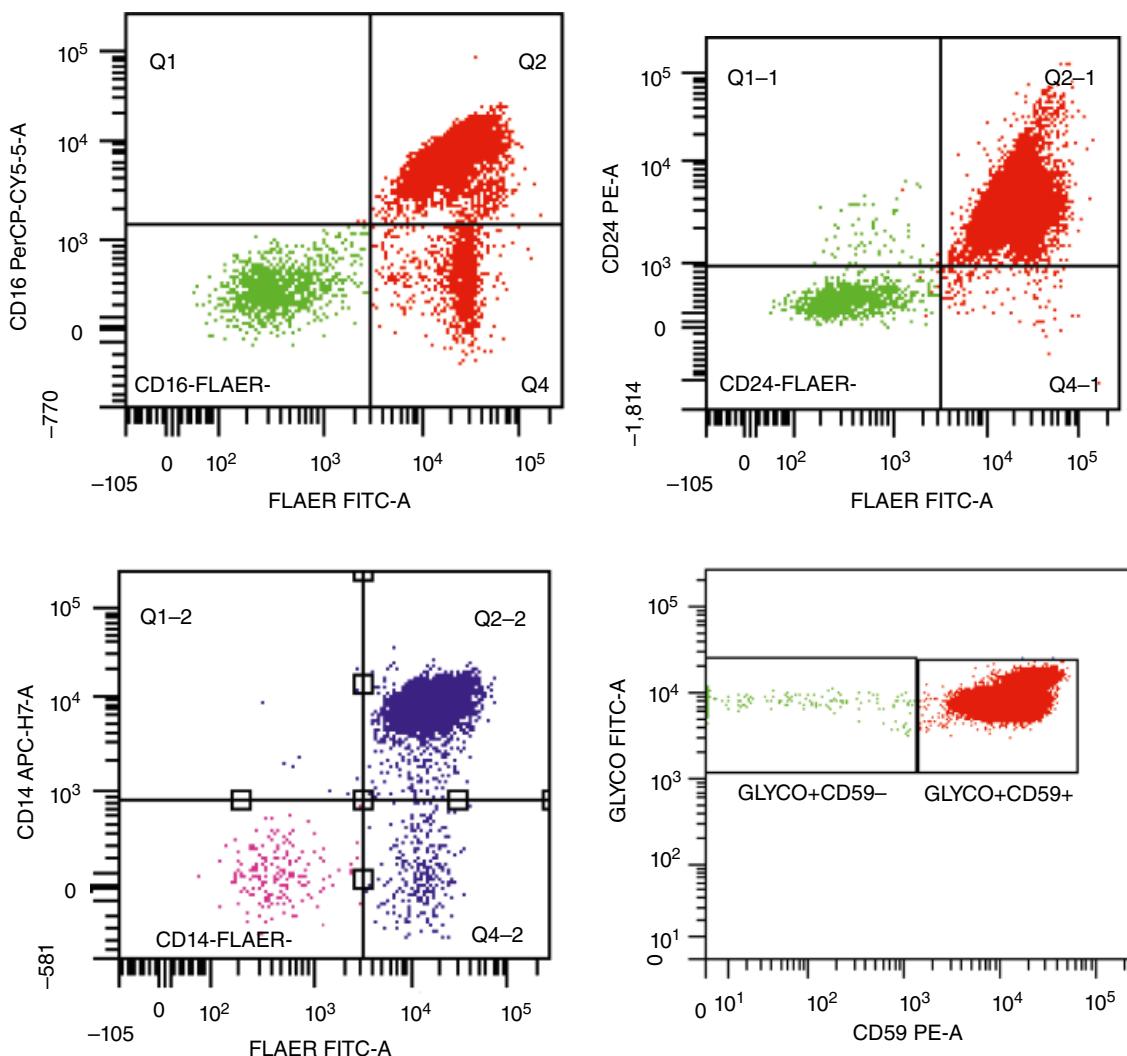
### Treatment

In treating PNH, the choice of therapy (or therapies) depends on the type of symptoms the patient is experiencing (hemolysis vs. thrombosis) as well as the degree of anemia. Indeed, therapy does not need to be initiated in an asymptomatic patient found to have PNH clones; these patients can be actively monitored for the development of symptoms.

### Anticoagulants and thrombolytic agents

Historically, anticoagulants represented one of the mainstays of therapy in PNH. Use of prophylactic anticoagulants has been recommended when >50% of neutrophil clones demonstrate the PNH phenotype.<sup>199–203</sup> Problems with anticoagulant therapy are nonetheless noted in the setting of PNH, including their lack of complete efficacy in preventing thrombotic events—in fact, the risk of thromboembolism in PNH remains high even with prophylaxis.<sup>177,202</sup> Additionally, anticoagulants increase the risk of bleeding, which may be compounded when patients also have thrombocytopenia.<sup>201</sup> Therefore, some experts recommend against routine prophylactic use of anticoagulants.<sup>201</sup> Nonetheless, patients with other underlying risks for thrombosis such as pregnant patients or patients undergoing a recent surgery and with prolonged immobilization should still be considered for prophylactic anticoagulation.<sup>201</sup> Unfortunately, even in patients with a history of thrombosis, anticoagulants are not always successful.<sup>150,188</sup>

Particular attention should be paid to clone size and symptoms of abdominal or chest pain. The risk of thrombotic events has been shown to correspond with clone size. In addition, patients with abdominal or chest pain have a greater risk of thrombosis.<sup>142</sup> Thrombolytic therapy with tissue plasminogen activator may be life-saving in severe thrombus formation, such as Budd-Chiari syndrome.<sup>203</sup>



**Figure 31.6** Examples of flow cytometric dot plots in a patient with paroxysmal nocturnal hemoglobinuria. The upper left and right panels demonstrate loss of GPI-linked proteins and/or anchors on granulocytes (CD16- and FLAER-; CD24- and FLAER-; these populations = PNH clone), while the lower left panel demonstrates loss of GPI-linked proteins and/or anchors on monocytes (CD14- and FLAER-; this population = PNH clone). Finally, the lower right panel demonstrates diminished-to-outright-loss of CD59 on red blood cells (this population = PNH RBC types II and III). Source: Based on Brodsky *et al.* (2000)<sup>197</sup> and Sutherland *et al.* (2018).<sup>198</sup>

### Eculizumab

Eculizumab is a monoclonal IgG antibody that binds to the C5 complement protein, thereby inhibiting the formation of the MAC<sup>204–207</sup> and reducing intravascular hemolysis of RBCs in patients with PNH.<sup>188</sup> Importantly, PNH patients receiving eculizumab also demonstrate thrombotic event rates that decline independent of anticoagulant therapy,<sup>203</sup> making eculizumab the recommended therapy for the majority of patients with PNH and thrombosis.<sup>204–209</sup> Despite these data, the discontinuation of anticoagulants in patients undergoing eculizumab is controversial,<sup>201</sup> and anticoagulants are still used in 70% of patients with PNH and a history of thrombotic event.<sup>142</sup>

Beyond thrombosis prevention, PNH patients on eculizumab show reduced utilization of RBC transfusions,<sup>176,204,207,208</sup> lower LDH levels,<sup>204,207</sup> and less nitric oxide depletion,<sup>191</sup> abdominal pain,<sup>208</sup> pulmonary hypertension,<sup>186</sup> and surgery-triggered hemolysis.<sup>210</sup> Eculizumab also leads to improvement in time-dependent renal function<sup>204</sup> and quality-of-life scores.<sup>186,205,206</sup>

Survival rates are significantly increased both in the short term and long term.<sup>176,204</sup> The reduction of LDH level is sustained over the course of treatment.<sup>204</sup> However, since PNH is a clonal process, this complement-inhibiting drug is not curative,<sup>211</sup> and therapy must be continued for life.<sup>137</sup> Some suggest prescribing eculizumab for patients with symptoms of hemolysis that are not managed by transfusion alone;<sup>212</sup> others use eculizumab in patients with fatigue that affects quality of life, transfusion dependence, thrombosis, frequent symptoms associated with smooth muscle dystonia (dysphagia, abdominal pain, or erectile dysfunction), renal insufficiency, or end-organ disease complications.<sup>188</sup> Its use may be particularly indicated in the perioperative period<sup>210,213</sup> or for pregnant patients.<sup>214–217</sup> See the “Special clinical situations” section later in this chapter for details.

Despite its successes, not all patients respond to eculizumab therapy, and some have persistent symptoms and chronic need for RBC transfusion.<sup>211</sup> Some patients who failed to respond were

found to have specific mutations of C5.<sup>218</sup> Other patients experienced continued extravascular hemolysis due to continued accumulation of C3 on the surface of RBCs.<sup>191,192,219</sup> Further support of this mechanism is the observation of positive DATs with anti-C3 reagent in patients undergoing eculizumab treatment.<sup>211</sup> Eculizumab does not treat underlying bone marrow failure.<sup>211</sup> Overall, eculizumab is well tolerated<sup>171,175,177,186,204–206,220</sup> with the occurrence of adverse events decreasing over time. There is no evidence for cumulative toxicity.<sup>204</sup> Notably, though, its use is associated with an increased risk of infection, particularly serious meningococcal infections. Meningococcal vaccine must be administered two weeks prior to treatment, patients' vaccination status should be kept up to date per current medical guidelines, and they should be followed for signs of infection to expedite prompt antibiotic treatment.<sup>207</sup> Ordering providers should be aware that Eculizumab is very costly, currently averaging over \$400,000 per year of treatment.<sup>211</sup>

### Hematopoietic cell transplantation

The only potentially curative therapy for PNH is allogeneic hematopoietic cell transplantation. However, transplantation using either bone marrow or peripheral blood stem cells as the graft source<sup>221</sup> is associated with substantial morbidity and mortality<sup>222–224</sup> including high rates of rejection, side effects from the preparative regimen, and graft-versus-host disease.<sup>221</sup> Hematopoietic cell transplantation is considered for patients with severe clinical symptoms who are unresponsive to eculizumab therapy or for whom eculizumab therapy is not an option due to cost or lack of availability.<sup>136</sup> Using bone marrow as a source of stem cells may lead to a lower incidence of graft-versus-host disease as compared to using peripheral blood.<sup>224</sup> Nonmyeloablative conditioning regimens show promise for improved outcomes and reduced morbidity and mortality.<sup>221,225–228</sup>

Hematopoietic cell transplantation may also be indicated for the treatment of the patient's underlying bone marrow disorder, independent of PNH.

### Immunosuppressive therapy

Patients with underlying bone marrow disorders, such as aplastic anemia, are more likely to receive immunosuppressive therapy with cyclosporine and/or antithymocyte globulin<sup>142</sup> than other PNH patients. Immunosuppressive, but not bone-marrow-suppressive, therapy is likely the most effective therapy in patients with underlying bone marrow disorders. Corticosteroids may reduce hemolysis and improve anemia in some patients, but they carry an added risk of long-term toxicity.<sup>188</sup> In addition, others have reported little success of corticosteroid therapy in patients with underlying bone marrow disorders,<sup>150</sup> and their use is therefore becoming less common.

### Other therapies

Given that eculizumab is successful in most PNH patients, but that some continue to have extravascular hemolysis due to C3 deposition, novel therapies targeting C3 and other components of the complement activation pathway are being tested in vitro and in animal models, with promising results.<sup>229–231</sup> For instance, ravulizumab is a new C5 inhibitor that is noninferior to eculizumab and may help to decrease breakthrough hemolysis.<sup>232</sup>

In patients with marked hemolysis, the replacement of iron (for iron-deficient patients), vitamin B<sub>12</sub>, and folate to support production of new RBCs may be indicated on a case-by-case basis;

iron replacement, however, should be done judiciously, given some reports of iron replacement triggering hemolysis as discussed earlier in this chapter.<sup>180</sup>

### Special clinical situations

Complement activation is triggered by infection, trauma, pregnancy, and surgery.<sup>154,210</sup> Therefore, the use of eculizumab may be particularly indicated in the perioperative period<sup>210,213</sup> or for pregnant patients.<sup>214–217</sup>

### Pregnancy

As previously discussed, pregnant patients with PNH frequently experience thrombosis. The risk of thrombosis in the setting of PNH is exacerbated by the hypercoagulability of pregnancy. Anticoagulant therapy may be an appropriate strategy to reduce morbidity and mortality during pregnancy. Eculizumab may also be useful and safe in pregnancy. The molecule is a hybrid IgG<sub>2</sub> and IgG<sub>4</sub>, and IgG<sub>2</sub> does not cross the placenta well.<sup>233</sup> However, only a handful of case reports on the use of eculizumab in pregnant PNH patients exist, and there is great heterogeneity in the use of therapy prior to pregnancy, time of discontinuation (either during or following pregnancy), and concomitant use of anticoagulant therapy.

Teratogenic effects of eculizumab have not been observed.<sup>214–216</sup> Fetal outcomes to date have been good.<sup>214–217</sup>

### Elective surgery

A few reports of perioperative eculizumab use suggest it may be helpful in preventing hemolytic paroxysms. One group reported successful cardiopulmonary bypass surgery in a PNH patient on maintenance therapy,<sup>213</sup> whereas another reported prevention of hemolysis using peri-operative induction with eculizumab for distal gastrectomy.<sup>210</sup>

### Underlying bone marrow disorders

Asymptomatic patients with PNH clones do not need to be treated for PNH. Many of these patients have underlying bone marrow disorders, such as aplastic anemia or MDS, and therapy should be targeted toward those conditions instead of PNH.<sup>188,214</sup>

### Children

PNH in children is rare; reports on their treatment are therefore also rare. Studies on the use of eculizumab in children have suggested that its use is safe and effective.<sup>234</sup>

### Transfusion management

Historically, blood transfusions were a mainstay of therapy for PNH patients with moderate-to-severe hemolysis and anemia. RBC administration is still recommended for patients with severe, symptomatic anemia. Unlike AIHAs, however, special testing and selection of specific RBCs for transfusion are not required in PNH unless the patient develops one or more alloantibodies (as can be seen with chronic transfusion needs). RBC components do not typically need special modifications, such as washing or irradiation, unless there are other reasons for these indications (e.g., irradiation for a PNH patient who has undergone stem cell transplantation). Leukoreduced components are recommended to help minimize febrile nonhemolytic transfusion reactions and human leukocyte antigen (HLA) alloimmunization. Previous reports of the need to

wash RBCs have been discredited because the hemolysis reported with unwashed cells was most likely due to minor incompatibilities. Platelet transfusion may also be given for a variety of clinical indications, including the treatment of sequelae in patients with thrombocytopenia undergoing anticoagulant and/or thrombolytic therapy.

### Prognosis

The 10-year survival rate for PNH patients in the past 50 years was poor, at about 50% from the 1940s–1970s.<sup>140,143,176</sup> More recently, the 10-year survival rate for PNH was 75%.<sup>235</sup> Further, one study showed that patients treated with eculizumab had similar survival as compared to age-matched controls.<sup>176</sup>

### Summary

Acquired disorders of hemolysis, such as AIHAs and PNH, can present myriad clinical, diagnostic, and transfusion medicine challenges. Knowledge of these entities, including their patho-

physiology, informs the treatment of these entities. The transfusion medicine community must also keep apprised of major therapeutic developments for these disorders as such treatments can also impact laboratory- and transfusion-related care.

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## CHAPTER 32

# Hemolytic disease of the fetus and newborn

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## Background

In Western countries, in the late 1960s and early 1970s, hemolytic disease of the fetus and newborn (HDFN) was a common disease. Overt fetal or neonatal hemolysis, dominated by D alloimmunization, caused marked anemia. Severely affected neonates were often unstable at birth and required multiple exchange transfusions to avoid kernicterus. Furthermore, HDFN was associated with considerable neonatal morbidity and mortality.

In the affected fetus, anemia is the most important pathological component of HDFN. The anemia causes increased fetal erythropoiesis, which, in severe cases, leads to erythroblastosis with widespread extramedullary hematopoiesis and many nucleated red cells (erythroblasts) in the circulation. If untreated, the most severe stage of erythroblastosis, hydrops fetalis, can lead to stillbirth and is characterized by severe anemia, hypoproteinemia, and massive edema. Bilirubin, by virtue of being lipophilic, crosses the placenta during pregnancy and is removed by the mother's liver. However, after birth, the immaturity of the neonate's liver leads to decreased clearance of bilirubin; plasma levels of unconjugated bilirubin can quickly become high enough to cause serious central nervous system damage. Thus, both anemia and hyperbilirubinemia may cause long-term physiological perturbations in the newborn.

In severe erythroblastosis, the examination of a blood film will show numerous nucleated red cells, but only a few spherocytes due to the rapid destruction of the red cells (e.g., serious anti-D HDFN). In less severe HDFN, spherocytes will dominate the blood film, and only a few nucleated red cells are observed (e.g., ABO HDFN). This explains why the examination of a blood film can be helpful in neonates with pronounced jaundice.<sup>1</sup> Persistent or prolonged jaundice in neonates is a common feature of HDFN and is frequently associated with cholestasis.

Accurate diagnosis of HDFN is important for the current as well as future pregnancies. In the absence of appropriate antepartum screening, monitoring and treatment, the consequences for the fetus or neonate can be severe. However, with appropriate prenatal therapy, red cell mass is maintained, and fetal erythropoiesis is suppressed. For that reason, today most neonates with HDFN who receive appropriate prenatal care present with neither neonatal jaundice nor anemia of clinical importance at birth, and very few

require exchange transfusion postpartum. However, the newborn may present with anemia after the first two weeks of life because of suppression of erythropoiesis caused by intrauterine transfusions (IUT) and the continued presence of hemolyzing maternal antibodies. Additionally, strategies to prevent HDFN, as detailed later in this chapter, have become an important component of prenatal care.

## Immune-mediated HDFN

### HDFN due to ABO incompatibility

The occurrence of O, A, B, and AB blood groups vary among different ethnicities<sup>2–4</sup> (Table 32.1). Accordingly, maternal-newborn ABO incompatibility ranges from 31% in those of European ancestry to 50% in patients of Asian descent. Today, ABO incompatibility is the leading cause of HDFN in high-human development index (HDI) countries.<sup>5</sup> HDFN resulting from ABO incompatibility generally occurs in the offspring of women of blood group O because anti-A, anti-B, and anti-A,B antibodies of the IgG subclass are far more common in mothers who are group O when compared to those who are group A or B.<sup>6</sup> The frequency of HDFN caused by ABO incompatibility has been estimated to be about 1 in 150 births.<sup>1</sup> The higher frequencies of ABO HDFN reported in Africa and Saudi Arabia are likely related to more potent anti-A and anti-B in maternal circulation due to environmental factors, as well as relatively strong expression of A and B antigens on fetal red blood cells.<sup>7</sup>

ABO antibodies are formed independent of antigen exposure during pregnancy or transfusion. Thus, the first ABO-incompatible infant of a mother is potentially at risk. Fortunately, on fetal red cells, A and B antigens are generally not fully developed and remain few and unbranched until the age of 2–4 years.<sup>8</sup> This allows antibody-coated cells to remain in circulation longer, when compared to anti-D mediated HDFN. Additionally, ABO antigens are expressed on virtually all cells in the body, whereas the D antigen is restricted to red cells. Thus, anti-D is “focused” to red cells, whereas anti-A and anti-B are absorbed by tissues and neutralized by soluble A and B substances in plasma. These features may in part explain why ABO HDFN is generally self-limiting and causes mild to

**Table 32.1** ABO Group Occurrence

ABO Group	Occurrence (%) European Descent in the United States (donor) <sup>2</sup>	Occurrence (%) African Descent in the United States (donor) <sup>2</sup>	Occurrence (%) Asian Descent in the United States (donor) <sup>2</sup>	Occurrence (%) in the United States (pregnant women) <sup>3</sup>	Occurrence (%) in Nigeria (pregnant women) <sup>4</sup>
O	45.2	50.2	39.8	46.8	48.0
A	39.7	25.8	27.8	30.2	41.2
B	10.9	19.7	25.4	18.5	7.6
AB	4.1	4.3	7.1	4.5	3.2

Source: Based on Garratty *et al.* (2004);<sup>2</sup> Smith *et al.* (2013);<sup>3</sup> Jeremiah *et al.* (2011).<sup>4</sup>

moderate neonatal jaundice presenting in the first 24 hours of life. In the absence of significant red cell destruction, hemoglobin may be normal or only slightly low.<sup>7</sup> Hemolysis severe enough to cause hydrops is exceedingly rare.<sup>9</sup> However, in ethnic groups that express strong (many and branched) A and B antigen sites, severe anemia, nucleated red cells, and significant neonatal morbidity can be observed.<sup>1</sup>

### HDFN due to unexpected red blood cell antibodies

Nearly all IgG antibodies against red blood cell (RBC) antigens expressed on fetal red cells have been associated with HDFN. HDFN frequency and severity, however, vary. The prevalence of antibody specificities depends on when the study was conducted and the ethnicity of the study population. For instance, the D-negative phenotype is more common in populations of European ancestry (15–17%), less common in those of African descent (8–9%), and rare in those of Asian descent (<1%).<sup>4,8</sup> Where the D-negative phenotype is common, the prevalence of alloanti-D after delivery of D-positive infants was once approximately 16%.<sup>10</sup> Today, routine antepartum and postpartum prophylactic Rh immune globulin (RhIG) administration has reduced the frequency of D alloimmunization to 0.28% to <0.1%.<sup>10–12</sup> Nonetheless, anti-D remains the most common cause of severe HDFN in populations of European ancestry.<sup>1</sup> The next most frequently reported clinically significant alloantibodies associated with HDFN are anti-c, anti-E, and anti-K1.<sup>13</sup> The presence of anti-K1 may be especially concerning because it can cause severe fetal anemia through both direct hemolysis and inhibition of fetal erythropoiesis.<sup>14</sup> Antibodies directed against other antigens in the Rh, Kell, Duffy, Kidd, and MNS blood group systems rarely cause severe HDFN.<sup>13</sup>

The frequency and specific antibody that cause HDFN vary with the population studied (Table 32.2). The clinical severity of HDFN is associated with both antibody specificity and the fetal expression of the RBC antigen (Figure 32.1). For instance, in Chinese women anti-M is the most commonly identified alloantibody. The natural history of anti-M HDFN is generally benign,<sup>13,15</sup> but severe HDFN associated with IgG anti-M has been disproportionately reported in Asian populations in Taiwan,<sup>16</sup> China,<sup>17</sup> Japan,<sup>18</sup> and India,<sup>19</sup> suggesting that racial and genetic factors may impact clinical outcome. Severe HDFN mediated by anti-M has been postulated to be caused by rapid intravascular hemolysis and suppression of erythropoiesis, similar to anti-K1, given that the M antigen is fully expressed in early erythroid precursors.<sup>17,18</sup> Another explanation for severity is concurrent placental disease that allows increased transplacental transport of pentameric anti-M antibodies that are too large to cross healthy placental tissue.<sup>20,21</sup>

### Immunology and mechanism of HDFN

Development of immune-mediated HDFN requires maternal IgG antibodies that can cross the placenta and are directed against paternally derived antigens expressed on fetal red cells. This principle explains why most naturally occurring antibodies to carbohydrate antigens, such as ABO, Hh, and Ii, are unlikely to cause HDFN. These antibodies form without prior exposure to allogeneic red cells as a result of the wide distribution of their cognate antigens in nature. Antibodies to these blood groups are predominantly the IgM isotype, which typically do not cause HDFN as they cannot cross the placenta. Additionally, maternal RBC antibodies directed against paternal antigens that are also expressed on placental tissue are adsorbed by the placenta and do not reach the fetus, reducing the chances of causing HDFN. Furthermore, the I antigen and antigens of the Lewis and P systems are almost entirely lacking on fetal cells; thus, even if corresponding antibodies crossed the placenta, they would not cause HDFN.<sup>23</sup>

Except in the case of ABO, HDFN is primarily mediated by antibodies that are incited by maternal alloimmunization to prior red cell antigen exposure. The precursor event to most forms of clinically significant HDFN is maternal sensitization during previous pregnancies.<sup>24</sup> The immune response to peptide antigens (e.g., D) typically requires T-cell facilitation and is dominated by the IgG class of antibodies. Because the D antigen is extraordinarily immunogenic and induces high-affinity anti-D IgG, it has historically been the major cause of HDFN in Western countries. In alloimmunized pregnancies, maternal IgG antibodies cross the placenta in an active process dependent upon the interaction between maternal IgG and syncytiotrophoblast Fc receptors, which is most active in the third trimester.<sup>7</sup> After transplacental passage, maternal IgG antibodies coat fetal red cells and induce effector cell mediated destruction. Pathogenicity is influenced by many factors including antibody affinity, isotype, subclass, and post-translational modifications. Specifically, the amount of IgG-Fc glycosylation has been associated with the clinical outcomes of anti-D-, anti-c-, anti-E-, and anti-K-mediated HDFN.<sup>25,26</sup>

### Monitoring and management of sensitized pregnancies

In high-HDI countries, the implementation of routine antibody detection and ABO/Rh typing of pregnant women allow for the detection of patients at risk of HDFN. These processes have paved the way for closer surveillance, timely therapy, and improved outcomes. Due to prenatal testing in high-HDI countries, HDFN caused by sensitization to D or other alloantigens is rare; consequently, ABO incompatibility is the leading cause of HDFN and is typically clinically mild. Unfortunately, the implementation of the

**Table 32.2** Frequency of HDFN Associated with Unexpected Antibodies

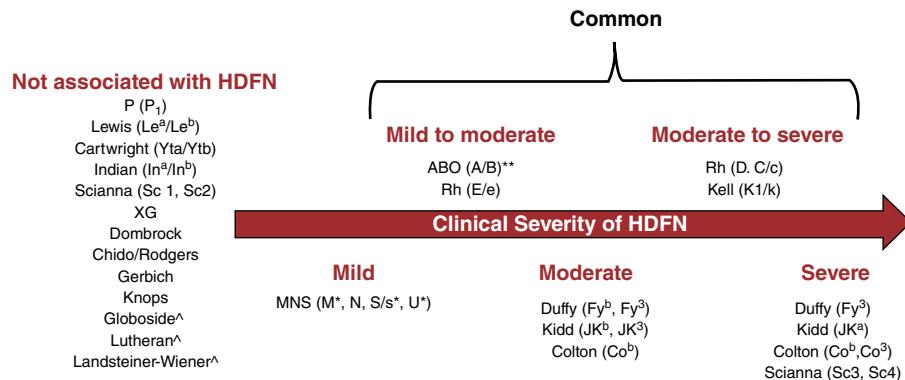
Antibody	Netherlands <sup>13</sup> Number (%)*	United States <sup>3</sup> Number (%)**	Hong Kong <sup>22</sup> Number (%)**	Nigeria <sup>4</sup> Number (%)**
D	NE	20 (0.2)	1 (0.004)	0
E	289 (0.09)	33 (0.4)	13 (0.05)	3 (0.6)
C	21 (0.007)	17 (0.2)	NR	6 (1.2)
C <sup>w</sup>	82 (0.03)	1 (0.01)	NR	NR
c	152 (0.05)	7 (0.08)	5 (0.02)	NR
e	9 (0.003)	2 (0.02)	NR	NR
K1	212 (0.07)	18 (0.2)	NR	5 (1.0)
Js(b)	0 (0)	NR	NR	3 (0.6)
Kp(a)	4 (0.001)	1 (0.01)	NR	NR
Jk(a)	41 (0.01)	4	NR	NR
Jk(b)	2 (0.0007)	1 (0.01)	NR	NR
Fy(a)	68 (0.02)	3 (0.03)	NR	NR
Fy(b)	1 (0.0003)	NR	NR	NR
S	48 (0.02)	6 (0.07)	7 (0.02)	NR
s	5 (0.002)	NR	NR	NR
U	NR	3 (0.03)	NR	NR
M	NR	26 (0.3)	NR	NR
N	NR	3 (0.03)	NR	NR
Mi	NR	NR	38 (0.1)	NR
Total	305,000 pregnancies	8894 pregnant patients	28,303 pregnant patients	500 pregnant patients

NR: none reported.

NE: not evaluated.

\*Percent calculated with the total number of pregnancies.

\*\*Percent calculated with the total number of pregnant patients.

**Figure 32.1** Classification of the clinical severity of HDFN by RBC antibody specificity.

Abbreviations: HDFN, hemolytic disease of the fetus and newborn; RBC, red blood cell.

\*HDFN is typically mild but can be severe.

\*\*Majority of cases do not require clinical intervention.

^None to mild.

same level of routine antenatal antibody detection has not been possible in all lower-HDI countries.

A positive antibody detection test (commonly referred to as the antibody screen) in a pregnant woman warrants additional investigation, and, if indicated, further antepartum monitoring and treatment of the affected fetus. Antibody titration by tube or gel (e.g., column agglutination technology) is the laboratory test typically used for antepartum monitoring. Gel platforms are reported to be more sensitive than the classic tube method, but there is significant interlaboratory variability regardless of testing method.<sup>27</sup> Correlation of antibody titration with clinical severity and outcome are mainly limited to anti-D. Antibody-dependent cellular cytotoxicity (ADCC) has been described as a method to distinguish clinically significant from clinically insignificant antibodies, but is infrequently used in clinical practice<sup>13</sup> as it lacks specificity and is

limited in availability. As such, antibody titration remains the most widely used laboratory test to assess for the risk of potentially significant HDFN and determine the need for additional noninvasive clinical fetal monitoring. Typically, serial antibody titrations are performed until a critical threshold is reached. This threshold may vary depending on the antigen specificity and the facility performing the titration. Ideally, titrations should be performed in parallel with previous samples to ensure the validity of any changes in concentration. Historically, a titer of 1:8 has been considered the critical threshold for anti-K1, but the presence of anti-K1 of any titer may possibly be clinically significant.<sup>28</sup> The critical titer for anti-D ranges from 1:8 to 1:32. For other antibodies, the critical titer may be either a twofold or greater increase in titer during pregnancy and/or a final titer of 1:16 to 1:32; however, these metrics vary, and additional monitoring may be indicated prior to a “critical

titer" being achieved. If there is a history of HDFN, the utility of antibody titers in subsequent pregnancies is less clear; therefore, noninvasive fetal monitoring is recommended rather than performing serial antibody titrations. It should also be noted for anti-M, which is typically, but not always, IgM-only or predominantly IgM, additional testing can be performed to detect and distinguish the titers of IgM versus IgG subtypes.<sup>13</sup>

### **Primary prevention**

The two triggers for maternal sensitization are fetal-maternal hemorrhage (FMH) during pregnancy and RBC transfusion. Both events expose the woman to nonself-RBC antigens and can lead to alloimmunization. As previously noted, the stimulus for maternal RBC sensitization causing severe HDFN has been shown to be mostly due to prior pregnancy (83%). However, up to 3% of cases have been attributed to previous RBC transfusion, while 14% have an unknown cause of sensitization.<sup>24</sup> Protection from D sensitization is the reason for the common practice of reserving D-negative RBC products for females of childbearing age (often an age cutoff of <50 years) when there is not enough time for pretransfusion testing during emergencies or when there is a critical shortage of D-negative RBCs. A more thorough and logical strategy for primary prevention is to provide phenotypically matched or alloantigen negative RBCs to women of childbearing potential for all clinically significant RBC antigens in addition to D. This type of strategy is common for patients with hemoglobinopathies, such as sickle cell disease, where RBCs are prophylactically matched for at least K1, C, c, E, and e to prevent RBC sensitization. The benefits of this practice include decreased risk of delayed hemolytic transfusion reactions and hyperhemolysis, as well as improved availability of compatible blood for future transfusions. In otherwise healthy females of childbearing potential, however, this practice has not demonstrated clear, direct benefit in prevention of severe HDFN when patients seek care from different facilities with different transfusion matching policies. In those circumstances, any potential benefit of providing antigen-matched RBCs to prevent maternal alloimmunization and HDFN appears diluted. However, a study in one European country with a national transfusion policy and a nationalized blood inventory suggests that providing K1 negative blood to female transfusion patients is associated with lower rates of anti-K1 detection in pregnant women, although the number of fathers positive for the K1 antigen was low.<sup>29</sup> Therefore, the prevention of alloimmunization caused by transfusion would require universal adoption of extended matching; however, this practice is not yet established, nor feasible, in most regions and countries.

### **Prevention of D alloimmunization in an exposed pregnancy**

For pregnant women, many countries recommend testing maternal blood type (ABO and D), as well as screening for unexpected RBC IgG antibodies in the first trimester.<sup>30,31</sup> First trimester screening has been shown to be approximately 75% sensitive for clinically significant RBC antibodies; however, sensitivity varies by antibody specificity.<sup>13</sup> In some countries, repeat screening for D-negative mothers is recommended between 28 and 29 weeks gestation, prior to administration of RhIG in potentially affected pregnancies, as late RBC alloimmunization may also occur.<sup>30,32</sup> Repeat screening for D-positive mothers is often performed at this time as well since up to 27% of severe cases of HDFN cases occur in infants of D-positive mothers.<sup>33</sup> Good communication between clinicians and the laboratory performing assessments of maternal specimens is critical to

distinguish between active anti-D alloimmunization and anti-D reactivity resulting from passively administered RhIG as RhIG is not indicated in the former case.<sup>34</sup>

Though the precise mechanism by which RhIG prevents D alloimmunization remains elusive,<sup>35</sup> postnatal administration of RhIG alone to D-negative mothers of D-positive infants has been shown to decrease the rate of D sensitization by 85–95%.<sup>36</sup> National recommendations for antenatal RhIG administration include administration at time of potential FMH, such as amniocentesis or miscarriage, and routine administration in the third trimester due to potentially undiagnosed FMH, though the public health benefits of this latter practice have not been shown to be statistically significant.<sup>37</sup>

Not all D-negative mothers require routine RhIG administration. If paternity is confirmed, paternal D type, fetal RHD genotyping from maternal sera, or paternal RHD molecular zygosity testing when the father is D-positive may guide clinicians in their evaluation of the risk of D sensitization.<sup>38</sup> Non-invasive molecular assays for the RHD gene from cell-free fetal DNA found in maternal peripheral circulation have been implemented in a number of European countries to identify D-positive fetuses early in pregnancy.<sup>39–41</sup> Cell-free fetal RHD assays offer a sensitivity of >99.3% at 10–11 weeks gestation and >99.9% at 24–26 weeks gestation. National programs built around this testing approach have been shown to limit unnecessary RhIG administration to D-negative mothers.<sup>42,43</sup> This technology has also been applied to other RBC antigens implicated in HDFN.<sup>44</sup>

Weak D is the category of D polypeptides that are derived from *RHD* alleles with genetic variations that generally affect trafficking to the cellular surface. Mothers with certain types of weak D mutations may be spared unnecessary RhIG administration as they do not form an anti-D.<sup>45</sup> The most common forms of weak D, types 1–3, fall into this category; molecular weak D genotyping can distinguish weak D types 1–3 from other D variants. Further evidence suggests that weak D types 4.0 and 4.1 may be treated similarly, as these mutations have not been associated with hemolytic transfusion reactions or HDFN due to anti-D.<sup>46</sup> Given the cost savings in avoiding unnecessary RhIG administration, the added expense of targeted molecular testing could be cost neutral, as well as avoid the overuse of a potentially limited supply of RhIG.<sup>47</sup>

Despite RhIG administration, there are some D-negative mothers who still become sensitized and form an anti-D, although the reasons for apparent failures are not clear. One possibility is inadequate RhIG prophylaxis. Two tests are generally used to quantify FMH at parturition and guide RhIG dosing. Initial testing uses the erythrocyte rosette test. If negative, a standard dose of RhIG is given to the at-risk mother within 72 hours of parturition because this dose should cover up to 10 mL of D-positive fetal erythrocytes in maternal circulation. However, when the rosette test is positive, indicating >10 mL of FMH, additional quantitative testing is needed. Typically, the Kleihauer-Betke test is used, although there are other tests that can also quantify FMH.<sup>8</sup> The Kleihauer-Betke acid elution test is a subjective quantitation of hemoglobin F cells present in a maternal sample by microscopic blood smear review. This quantitation is then converted to a predicted volume of fetal RBCs in a standard maternal blood volume. This approach may result in underdosing or overdosing RhIG, as 300 mcg of RhIG is considered appropriate for 15 mL of fetal RBCs in maternal circulation,<sup>8</sup> but vial sizes and dosing standards vary by country. More objective flow cytometric measures can be used to quantify the concentration of D-positive RBCs of fetal origin in a maternal blood sample,<sup>48,49</sup> however, the use of this technique is limited due to

equipment and staffing requirements, as well as established national standards of care.<sup>50</sup> Accurate estimates of FMH are critical for appropriate dosing of RhIG as RhIG “failures” have been associated with conditions that increase the risk of FMH, such as assisted vaginal delivery, caesarian section, and postmaturity (>42 weeks gestational age).<sup>51</sup> Maternal obesity also increases the likelihood of under dosing of RhIG as maternal blood volume is likely greater than the standard 5000 mL maternal blood volume estimates typically used in the Kleihauer-Betke calculations. Significant adiposity may also decrease efficacy of RhIG, which is often administered intramuscularly (IM) and demonstrates poor absorption when administered subcutaneously. Intravenous dosing of RhIG should be considered when IM is not feasible or there are reasons to suspect it may have reduced efficacy.<sup>52,53</sup>

### Care of the sensitized pregnancy

For women who are alloimmunized, early recognition, fetal monitoring, and appropriate interventions may protect the fetus from complications of hydrops fetalis and ensure a safe, near-term delivery. As noted previously, in the absence of a history of affected pregnancies, antibody titers may be helpful to determine when an at-risk fetus requires additional monitoring. However, in women who have had a previously affected pregnancy, early interventions may be required. Prior to 18 weeks gestation, in mothers with previous pregnancies severely affected by HDFN, there are reports of successful treatments with a combination therapeutic plasma exchange and IVIG.<sup>54-59</sup> The intent is to inhibit the antibody enough to allow for the fetus to grow until fetal monitoring and intrauterine transfusions are technically possible.

Once a critical antibody titer threshold is met, or if the mother is alloimmunized against an antigen where titers have not been shown to be predictive, noninvasive fetal monitoring is performed beginning around 18 weeks gestation. Doppler ultrasonography of the fetal middle cerebral arteries (MCA) to measure peak systolic velocity (PSV) has been shown to predict moderate to severe fetal anemia.<sup>60</sup> This approach has replaced invasive, direct measurements of bilirubin from amniotic fluid.<sup>61,62</sup> If a fetus’ PSV is greater than 1.5 multiples of the median for gestational age, then direct measurement of fetal hematocrit via cordocentesis with the potential for immediate IUT is recommended. If the fetus is already at a gestation age compatible with life outside of the womb (typically >35 weeks), delivery may be recommended in lieu of IUT.<sup>38</sup> For affected pregnancies, definitive delivery around 37–38 weeks gestation is generally recommended. At the time of any IUT, the obstetric team should be prepared for urgent delivery of the fetus given potential risks of the transfusion procedure itself.

The blood units utilized for IUT are fresh, type O, D negative, irradiated, CMV negative/safe, sickle cell trait negative, and should lack other antigen(s) to which the mother is sensitized. RBC units are often concentrated to a hematocrit of 75–80%. Additional extended matching to the maternal phenotype has been shown to avoid further maternal sensitization at the time of IUT given the high rate of antibody formation in this population; however, finding multiply antigen negative blood can be difficult.<sup>63-65</sup> Calculations for IUT volume target a fetal hematocrit of 40–50%, but may target a lower post-transfusion hematocrit early in gestation (18–24 weeks) given an increased risk of complications.<sup>66</sup>

Long-term follow-up of infants with HDFN who received IUT has shown an incidence of neurodevelopmental impairment (NDI) of around 4–6%, with cerebral palsy occurring in 2.4% of infants.<sup>67,68</sup> However, milder developmental delays (<1 standard deviation)

have been detected in up to 14.4% of infants who received IUT, with hemoglobin at initial IUT correlating with later measures of IQ.<sup>69</sup> Severe neonatal morbidity, such as sepsis or intraventricular hemorrhage, and severity of HDFN as evidenced by the number of IUTs the infant received were predictive of more severe NDI.<sup>69</sup>

### Care of the affected newborn

#### Initial testing

The direct antiglobulin test (DAT) (see Chapter 12) is one of the cornerstones for the diagnosis of HDFN in a neonatal patient; however, significant practice variation exists in its use.<sup>70</sup> The DAT detects IgG antibodies on the surface of red cells where the number of IgG molecules bound to the erythrocyte surface determines the agglutination strength of the reaction. Using the spin antiglobulin test, the detection limit is 100–150 IgG molecules per red cell. When over 1000 are bound, the DAT is strongly positive (all cells are agglutinated).<sup>7</sup> In sensitized pregnancies, a positive postnatal DAT in the infant has been shown to be 90% sensitive for HDFN, but only 58% specific.<sup>71</sup> Furthermore, significant hemolysis can be observed in patients with a negative or weakly positive DAT. Only 23% of newborns found to be DAT positive have been reported to develop hyperbilirubinemia requiring treatment; however, in infants with clinically significant hemolysis, >80% have a positive DAT.<sup>5</sup> Thus, DAT alone is a poor predictor for identifying neonates who will require perinatal interventions for HDFN and hyperbilirubinemia, and additional clinical measures should be used. It should also be borne in mind that after antepartum treatment of D-negative women with prophylactic RhIG, neonates may demonstrate a positive DAT without signs of hemolysis.<sup>72</sup> Although obtaining a DAT on cord blood samples has been historically considered compulsory in infants of D-negative mothers, newer recommendations suggest that this should no longer be routine.<sup>32</sup> While neither required nor pathognomonic, as discussed previously, blood smear review may also be helpful.

#### Hyperbilirubinemia

Newborns should be screened for hyperbilirubinemia in the early neonatal period. This may be performed using transcutaneous measures of bilirubin. However, for infants who were affected by maternal antibodies during pregnancy, serologic testing and close monitoring of bilirubin levels are recommended to minimize the risk of long-term complications from HDFN.

Infants, even those who were not severely affected in pregnancy, may have early evidence of antibody mediated hemolysis soon after birth. Often within the first week of life, these infants can present with an elevated bilirubin and a normal or high reticulocyte count.<sup>73</sup> Hyperbilirubinemia may be exaggerated due to immature liver function. If inadequately treated, significant unconjugated hyperbilirubinemia can lead to bilirubin-induced neurotoxicity (BIND), which is damage to the brain from free bilirubin that crosses the blood-brain barrier. BIND occurs when total serum bilirubin is ≥30 mg/dL or ≥450 μmol/L, with higher peak levels conveying the greatest risk. Without treatment, BIND can lead to acute bilirubin encephalopathy (ABE), which progresses through three stages: early, intermediate, and advanced. In the early phase, infants may be less responsive, hypotonic, and have a high-pitched cry. In the intermediate phase, the infant may be lethargic with poor suck or irritable with strong suck. They may be difficult to console and exhibit hypertonic arching of the back and neck with stimulation. In the advanced stage, infants may have fevers, apnea, hypertension, inability to feed, and seizures. The condition may progress to a semicomatose or comatose

state. Ultimately, death from ABE can be due to respiratory failure or intractable seizures.<sup>74</sup> Chronic bilirubin encephalopathy, also referred to as kernicterus spectrum disorders, is permanent clinical sequelae due to bilirubin-induced neurotoxicity that manifests after the first year of life. The classic tetrad of kernicterus spectrum disorders includes auditory processing disturbances with or without sensorineural hearing loss, extrapyramidal neuromotor symptoms, oculomotor paresis presenting as an upward vertical gaze palsy, and dental enamel dysplasia.<sup>75</sup> Chronic bilirubin encephalopathy has an incidence of 0.4–2.7 cases per 100,000 births in high HDI countries. However, ABE continues to account for up to 15% of neonatal deaths in countries with low HDI.<sup>76</sup> The primary treatment strategies of phototherapy, IVIG, and exchange transfusion are aimed at preventing the neonatal bilirubin from reaching toxic levels.

### Phototherapy

Since its introduction in the late 1950s, the delivery and understanding of phototherapy have provided major improvements in the clinical treatment of HDFN. The efficacy of phototherapy depends on the following factors:<sup>77</sup>

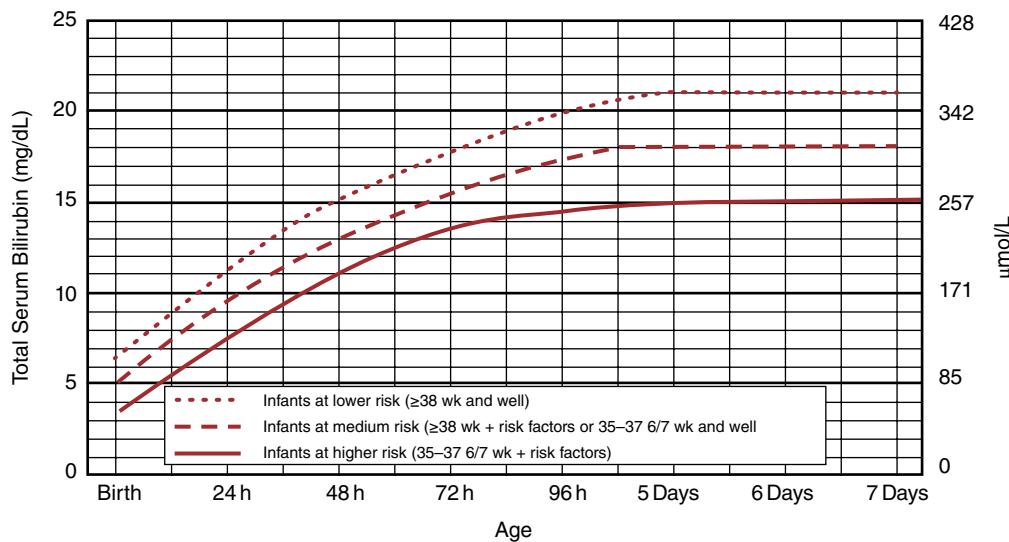
- Spectral qualities of the delivered light (wavelength range 400–520 nm, with peak emissions of 460 nm).
- Intensity of light (irradiance) delivered to the skin surface.
- Skin surface area receiving phototherapy.
- Distance from infant's skin to light source.
- Total serum bilirubin concentration at the start of phototherapy.
- The cause of jaundice.
- Duration of exposure.

Intensive phototherapy is often the first treatment to be initiated when infants present with hyperbilirubinemia due to HDFN and can significantly reduce the total serum bilirubin even in a short period of time. Phototherapy causes photooxidation of the serum bilirubin into water-soluble isomers that are more readily excreted. In addition, phototherapy is thought to decrease the risk of BIND as the conversion of a significant amount (25% or more) of circulating

bilirubin to more water-soluble isomers could theoretically reduce the transfer of bilirubin across the blood–brain barrier and into the brain.<sup>78</sup> Postnatal age and risk-stratified thresholds for initiation of phototherapy are presented in Figure 32.2.<sup>79</sup>

Modern phototherapy devices with high-intensity gallium nitride light-emitting diodes (LEDs) are small, efficient, and easy to use.<sup>77</sup> Compared to earlier versions of these instruments, the current technology can deliver considerably more total radiation to a neonate. The resulting “intensive phototherapy” has become an important alternative to exchange transfusion, even in the treatment of severe HDFN. Intensive phototherapy implies radiation in the blue-green spectrum (wavelengths 430–490 nm) of at least 30 µW/cm<sup>2</sup>/nm (measured at the infant's skin) delivered to as much of the infant's body surface area as possible.<sup>79</sup> Spectral power is the product of the irradiance and size of the irradiated area and is a key metric in phototherapy as high irradiance is of little help if only a small area of skin is exposed.

Phototherapy, when properly performed, is largely considered to be an innocuous procedure, although some concern has recently been raised about increased lethality in tiny, immature infants of <750 g birthweight, as well as future cancer risks in treated infants.<sup>80–82</sup> Additionally, the safety of pathogen inactivated blood products in infants receiving phototherapy has been questioned. Some pathogen inactivation systems work through exposing the blood product to a combination of UV light and a photosensitizing agent. These systems have been shown to improve transfusion safety; however, it is unclear how much of the photosensitizing agent is infused to patients as part of routine transfusions and whether that is enough to increase the risk of injury to infants receiving directed irradiance. Notably, modern phototherapy devices avoid the wavelengths of light that have the potential to cause injury. However, psoralen-treated blood products remain contraindicated in infants treated with phototherapy devices that emit a peak energy wavelength less than 425 nm or have a lower bound of emission bandwidth <375 nm.<sup>83</sup>



**Figure 32.2** The American Academy of Pediatrics (AAP) Guidelines for Phototherapy take into consideration the hours/days since birth as a surrogate for liver maturity along with patient risk factors for BIND to establish total serum bilirubin thresholds above which phototherapy should be initiated. Recommended thresholds are lower in infants <35 weeks' gestation.<sup>79</sup> The threshold to initiate therapies for hyperbilirubinemia may differ slightly between nations due to differences in national health care delivery systems, testing availability, and access to medical follow-up. Refer to local practice guidelines, if available. Image reproduced with permission from the American Academy of Pediatrics (2004).<sup>79</sup>

### High-dose intravenous immunoglobulin (HDIVIG)

Since the beginning of the 1990s, several studies have reported the effects of HDIVIG as an adjuvant to standard therapy for HDFN. Two systematic reviews have concluded that IVIG plus phototherapy reduced the risk of exchange transfusion when compared to phototherapy alone.<sup>84,85</sup> Thus, it may be considered for neonates with HDFN who are near exchange thresholds or in those who fail to respond to intensive phototherapy.<sup>79</sup> HDIVIG is administered as 0.5–1.0 g/kg over two hours and may be repeated. It has been argued that the results of several HDIVIG studies were confounded by the concurrent introduction of newer phototherapy devices with much higher irradiance.<sup>86</sup> Although this cannot be ruled out completely, Huizing *et al.*, in a study with a historical control cohort, found no effect of change of phototherapy devices, while confirming the salutary effect of HDIVIG in both D- and ABO-HDFN.<sup>87</sup> HDIVIG may be inadequate to prevent exchange transfusion in the setting of aggressive HDFN, as was seen in a Dutch cohort of patients with HDFN due to anti-D, the majority of whom required IUT.<sup>88</sup> It is not surprising that with such a high load of circulating D antibodies, a relatively moderate dose of IVIG would not be sufficient to block or reduce the effect. HDIVIG has not been shown to benefit neonates when given prophylactically,<sup>89</sup> rather it has the greatest potential to benefit a targeted population of neonates with HDFN who are close to the bilirubin threshold for exchange transfusion.<sup>90</sup>

It must be remembered that IVIG is a pooled plasma product, which, before the introduction of adequate pathogen inactivation, was involved in several cases of viral transmission. However, since 1994, there has been no transmission of bloodborne infections with pathogen-inactivated IVIG prepared from selected donors<sup>91</sup> (see also Chapter 23).

### Exchange transfusions

The criteria used to assess the need for exchange transfusion include total bilirubin level, trajectory of increase, hemoglobin level, and clinical symptoms. Traditional guidelines suggest exchange transfusion in the following circumstances.

#### Within 12 hours of birth if:

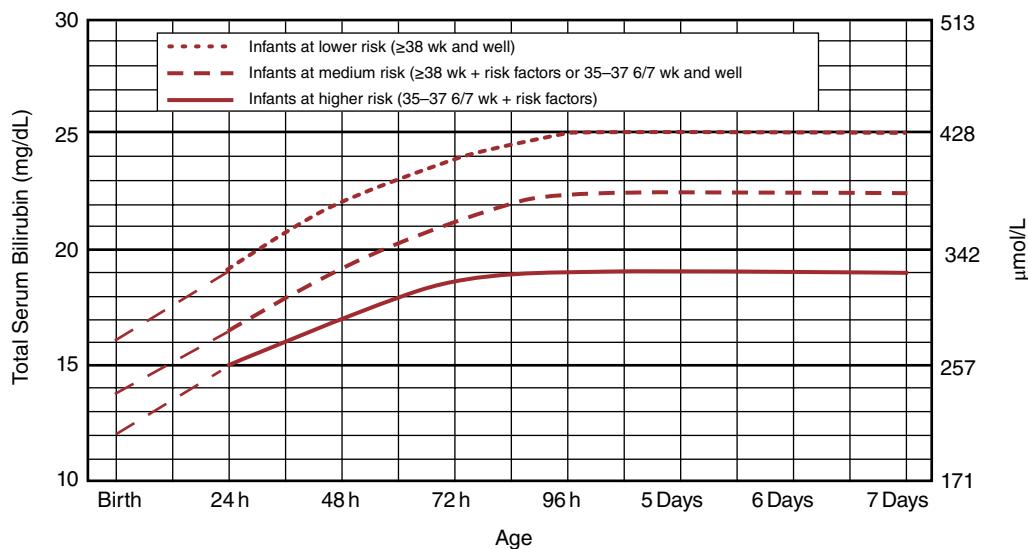
- cord blood bilirubin concentration exceeds 3–5 mg/dL (50–85 µmol/L) for preterm infants and 5–7 mg/dL (85–120 µmol/L) for term infants, or the rate of increase is >0.5 mg/dL/hour (8.5 µmol/L/hour); and
- severe anemia: hemoglobin <10 g/dL combined with hyperbilirubinemia.

#### After 24 hours of birth if:

- the total bilirubin concentration >20 mg/dL (342 µmol/L) or a bilirubin increase of >0.5 mg/dL/hour (8.5 µmol/L/hour), or hemoglobin <10 g/dL combined with hyperbilirubinemia

Today, the need to perform exchange transfusion has been much reduced through the combined use of intensive phototherapy and HDIVIG. In fact, these interventions often significantly decrease bilirubin levels over 2–6 hours needed to set up an exchange transfusion. Exchange transfusions are still considered indicated when intensive phototherapy, with or without IVIG, fails to adequately reduce bilirubin concentration or when the initial serum bilirubin places the infant at risk for bilirubin-induced neurologic damage. Factors potentiating bilirubin toxicity include immune-mediated hemolysis, acid–base disturbances, asphyxia, free heme groups, and other byproducts of hemolysis or drugs that displace bilirubin from albumin and other plasma-binding proteins.<sup>92</sup> Postnatal age and risk-stratified thresholds for initiation of red cell exchange are presented in Figure 32.3.<sup>79</sup>

Exchange transfusions supply the neonate with compatible red cells and fresh plasma, while incompatible red cells, bilirubin, and maternal antibodies in plasma are all removed. A standard exchange



**Figure 32.3** AAP guidelines for exchange transfusion. Similar to the guidelines for initiation of phototherapy, hours/days of age and patient specific risk factors are used to establish total serum bilirubin thresholds above which exchange transfusions may be indicated. Thresholds are lower in infants <35 weeks' gestation.<sup>79</sup> A lower threshold to initiate intensive therapy may be considered in infants with acidosis, septicemia, low serum albumin concentration, or an "ill" appearance. In the presence of one or more such risk factors, clinical judgment may suggest performing an exchange transfusion at TSB levels 50–100 µmol/L lower than those indicated by graph lines. Immediate exchange transfusion is recommended if the infant shows signs of acute bilirubin encephalopathy. Image reproduced with permission from the American Academy of Pediatrics (2004).<sup>79</sup>

transfusion of twice the infant's blood volume reduces incompatible fetal red cells by about 85%, while bilirubin and maternal antibody concentrations are reduced by 25–45%.<sup>23</sup> The amount of blood needed for an exchange transfusion is 170 mL/kg (200 mL/kg in preterm infants), with 25–50 mL added to compensate for dead space in the tubing and blood warmer. An exchange transfusion consists of hemoglobin S-negative red cells that are compatible with maternal antibodies and are reconstituted with fresh frozen group AB plasma to achieve a hematocrit of 45–60%, depending on the desired target hematocrit in the neonate. The blood should be CMV seronegative/safe (leukocyte reduced), irradiated, and preferably <5 days old. Platelet transfusion may be considered after an exchange transfusion if an infant's platelet count is reduced, which often occurs after multiple exchange transfusions.

Exchange transfusions are associated with a mortality of 2% and serious complications in 12% of patients. Because the frequency of exchange transfusions is decreasing and practitioners have fewer opportunities to attain and maintain the necessary procedural skills, the complication rate is unlikely to improve. It is important that maternity and neonatal services develop, maintain, and teach appropriate written guidelines for the use of exchange transfusion in HDFN and the performance of the procedure itself.

### Simple transfusions

While the clinical indications for exchange transfusion are becoming less common, the need for simple transfusion has increased.<sup>1</sup> Antibody mediated hemolysis due to HDFN may present or persist beyond two weeks of life. This may be exaggerated by the natural expansion of the infant's intravascular volume and progression toward physiologic nadir. For these infants, the bilirubin remains elevated, with a normal or high reticulocyte count. Hyporegenerative anemia has also been described in infants affected by HDFN. These infants may be older (>2 weeks old) and do not have signs of acute hemolysis. Instead, this hyporegenerative state is thought to be due to antibody mediated destruction of RBC precursors, low erythropoietin production, and bone marrow suppression due to IUT or early postnatal transfusions in a dose-dependent manner.<sup>73,93</sup> Antibody specificity and antigen density on early RBC precursors may underlie which infants have a greater likelihood of suffering from hyporegenerative anemia, such as those affected by anti-K1 or possibly anti-M, as discussed earlier.

Simple red cell transfusions are required to maintain an affected infant's hemoglobin through the early neonatal period, especially if infants receive treatment with phototherapy and HDIIVIG alone or together. In an observational cohort of almost 300 infants affected by HDFN, more than half of those who did not require IUT required simple red cell transfusion, most often in the first week of life. A higher reticulocyte count at birth and higher bilirubin were associated with the need for simple transfusion(s). In contrast, exchange transfusion reduced the need for simple transfusion.<sup>94</sup> For infants who received IUT, there may be a period where the infants' hemoglobin remains adequate due to the persistence of antigen negative transfused RBCs in circulation. However, close monitoring is warranted as up to 88% of these infants required transfusion as the previously transfused cells senesced. In this situation, the transfusion is needed typically during the first two weeks of life but can be delayed beyond three weeks of life.<sup>94</sup> Similar to infants who did not receive IUT, those who received an early exchange transfusion had a decreased likelihood of needing future simple transfusions; however, lower reticulocyte counts were associated with an increased likelihood of future anemia, highlighting the sustained effect of IUT on

erythropoiesis.<sup>94</sup> The likelihood of needing additional transfusions decreases as infants age; however, close clinical and laboratory monitoring is necessary through physiologic nadir and recovery.

### Breast milk

Several case reports have implicated breast milk as an ongoing source for maternal antibody exposure as evidenced by prolonged hemolysis and persistent antibody detection in affected neonates.<sup>95–97</sup> Elegant murine models of anti-KEL have demonstrated positive DATs in KEL positive pups who were only exposed to the antibody through breast feeding from sensitized mothers.<sup>98</sup> Several maternal IgG class antibodies have been detected in breast milk;<sup>99</sup> however, the direct link to enteric absorption has yet to be identified in humans. Currently, maternal RBC alloimmunization is not a contraindication for breast feeding, but further study is warranted.

### HDFN, anemia, transfusion, and NEC

Necrotizing enterocolitis (NEC), defined as ischemic necrosis of the intestines, is a common emergency in preterm and low birthweight infants. Several retrospective studies have associated NEC in term or near-term babies with HDFN. In one retrospective cohort study of infants between 30 and 38 weeks' gestational age, the incidence of NEC was significantly higher among infants with HDFN compared to those without HDFN (1.3% vs. 0.4%, relative risk 3.40, 95% CI 1.09–10.63).<sup>100</sup> Blood transfusions, a common intervention for infants with HDFN, have also been associated with the occurrence of NEC. Authors hypothesized that this was due to changes in intestinal perfusion, especially when infants are concurrently receiving enteral feeds; however, much of the data are retrospective and results are mixed.<sup>101</sup> In one prospective cohort study, exposure to severe anemia, and not blood transfusion, was associated with NEC.<sup>102</sup> HDIIVIG has also been associated with increased risk of NEC development.<sup>103</sup> Given the confounding interactions, additional studies are warranted to segregate the effects of HDFN itself, the anemia it causes, and the interventions used to treat HDFN including transfusions and IVIG. Given the late, hyporegenerative anemia that can occur in infants affected by HDFN, case reports have suggested that the administration of exogenous erythropoietin may minimize the need for transfusion support and avoid potential deleterious effects of RBCs.<sup>104,105</sup> This is currently an area of active research in the EPO-4-Rhesus Study (NCT03104426).

### Future therapies

Metalloporphyrins, which decrease bilirubin production through inhibition of heme oxygenase, may represent a novel mechanism to limit bilirubin accumulation. In preclinical models of BIND, minocycline has been shown to be neuroprotective, but this must be balanced against the known adverse effects on dentition and bone. Phenobarbital has also been shown to increase bilirubin clearance through activation of enzymes that enhance conjugation.<sup>76</sup> However, with all of these therapies, further research into efficacy and safety is needed.

### HDFN in low HDI nations

Although the mortality and morbidity of HDFN have been greatly reduced in countries with high HDI, HDFN still poses a serious challenge in countries with low HDI. In these countries, the introduction of maternal antibody screening for blood group antibodies and D blood type, routine prophylactic RhIG treatment, and development of affordable and effective phototherapy options should be encouraged. Filtered sunlight phototherapy (FSPT), which uses

sheets of film to remove most ultraviolet and infrared light from sunlight while transmitting effective levels of therapeutic blue light, has been shown to be as effective as electric phototherapy in low-resource settings.<sup>106</sup> Unfortunately, given reliance upon sunlight, delays in care may occur if infants present at night or on cloudy days; thus, lower bilirubin thresholds to initiate phototherapy may be warranted.<sup>107</sup> Testing of serum bilirubin may be limited in low HDI areas due to suboptimal laboratory services, as well as cost of the testing; however, recent studies have validated the use of transcutaneous measures of bilirubin for screening and intervention in these settings.<sup>108–110</sup> The adoption of novel technologies, such as LED phototherapy machines, has the potential to further improve outcomes.<sup>111,112</sup> Because HDIVIG is an expensive therapy, the use of other medications to reduce the postpartum formation of bilirubin could be of interest.<sup>113</sup>

## Conclusion

In immune-mediated HDFN, maternal IgG antibodies—usually related to the ABO, Rh, or Kell blood groups—cross the placental barrier, causing hemolysis of fetal red cells. Although HDFN can be fatal, advances in diagnosis, treatment, and prevention have made immune-mediated HDFN increasingly a preventable and treatable condition. Improvements in diagnostic modalities, including maternal blood typing and screening for antibodies, antepartum treatment, and routine prophylactic RhIG given to D-negative women at risk, have improved outcomes for affected pregnancies. Postpartum management includes treatment of hyperbilirubinemia and anemia. Hyperbilirubinemia is usually treated by means of intensive phototherapy, with HDIVIG serving an adjuvant role to further reduce hemolysis. In addition, anemia is corrected by simple transfusion of red cells. These approaches have reduced the indications for exchange transfusion to very severe HDFN or when

intensive phototherapy combined with HDIVIG fails to reduce hyperbilirubinemia to levels that are considered safe. Novel therapies that convey additional neuroprotection or work to inhibit bilirubin production require further study. Additional approaches for treatment and prevention of HDFN and neonatal hyperbilirubinemia are needed to improve neurodevelopmental outcomes, especially in low HDI areas.

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## CHAPTER 33

# Obstetric transfusion practice

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## Introduction

Blood loss during childbirth of up to about 1000 mL is usually well tolerated because healthy mothers are in a state of physiological hypervolemia.<sup>1</sup> However, postpartum hemorrhage (PPH) of more than 1000 mL in 24 hours can cause significant morbidity and mortality. This amount of blood loss is not rare, occurring in about 5% of the deliveries in well-resourced countries and in up to 20% in under-resourced countries.<sup>1</sup> Furthermore, lesser blood loss can also cause significant problems in mothers with anemia, preeclampsia, or other obstetrical complications. Maternal blood loss is associated with nearly one-quarter of all maternal deaths globally and is the leading cause of maternal mortality in most low-income countries.<sup>2</sup> Obstetric hemorrhage and preeclampsia together account for 50% of maternal deaths in poor as well as well-resourced countries.<sup>2</sup> In addition to effects on the mother, blood loss during pregnancy and the concomitant reduction in oxygen-carrying capacity also pose threats to the fetus.

## Blood loss in pregnancy

### Obstetrical causes

Among the several factors that can contribute to maternal blood loss, parturition remains the most significant. For example, in a Dutch national survey on severe morbidity among 358,874 pregnancies from August 2004 through August 2006, major obstetric hemorrhage (a bleed during delivery that required transfusion of at least four units of red blood cells) occurred in 4.5 per 1000 births.<sup>3</sup> Thus, hemorrhage accounted for 70% of all severe obstetrical complications (which occurred at a rate of 7.1 per 1000 births) in this dataset. Table 33.1 shows the most frequent etiologies.<sup>3</sup> The risks of major obstetrical bleeding increased dramatically as pregnancies progressed from the early pregnancy phase (51 bleeds; 3.2% of total), through the antepartum period (135; 8.5%), and into postpartum phase (1480; 93%). In some cases, women experienced both antepartum and postpartum hemorrhages. In the latter two categories, bleeding was most commonly related to physical/structural issues with the placenta and/or uterus such as abruptio placentae and *placenta praevia* (antepartum) as well as retained placenta or uterine

atonia (postpartum). Only a small percentage of the hemorrhages were assigned to nonobstetrical causes, such as clotting disorders (see below). Nonetheless, given that we are focused on obstetrical transfusion practices in this chapter, we now turn our attention to conditions that can cause coagulopathies that complicate pregnancies, as well as transfusion therapies to address these problems.

### Hematological causes

Among the few maternal hematologic diseases that can cause blood loss, maternal thrombocytopenia of various causes and inherited bleeding disorders are of most significance.

#### Maternal thrombocytopenia

##### *Gestational thrombocytopenia, HELLP, and ITP*

A platelet count below  $150 \times 10^9/L$  is not infrequent during pregnancy. Once laboratory artifacts (such as EDTA agglutination) are ruled out, about 6% of third trimester pregnancies demonstrate thrombocytopenia of  $80\text{--}150 \times 10^9/L$ . Typically, this represents an incidental finding and is termed *gestational thrombocytopenia* (GT).<sup>5,6</sup> While GT is a diagnosis of exclusion, of unknown cause and significance, patients should be carefully monitored. If the blood film is normal and the mother has no history of immune thrombocytopenia (ITP) or antiphospholipid antibodies, no further testing is indicated. However, if the platelet count drops below  $70\text{--}80 \times 10^9/L$ , other causes of thrombocytopenia should be sought.<sup>7</sup> In particular, GT has been considered as subclinical idiopathic thrombocytopenic purpura (ITP) or as a precursor of HELLP (hemolysis, elevated liver enzymes, and low platelets) syndrome.<sup>8,9</sup> Women who develop GT may be at higher risk for postpartum HELLP syndrome.<sup>8</sup>

HELLP syndrome is a member of a class of microangiopathic hemolytic anemias referred to as *thrombotic microangiopathic anemias* (TMAs). HELLP is less common than GT, occurring in 0.2–0.8% of pregnancies.<sup>10,11</sup> Among these patients, 70–80% develop preeclampsia.<sup>12</sup> The peak incidence of HELLP is at 36 weeks of gestation, although 10% of cases can occur prior to the 27th week while one-third present up to seven days after a normal delivery not complicated by preeclampsia.<sup>13</sup> As with GT, platelet levels are reduced in HELLP, with a median of  $57 \times 10^9$  platelets/L as shown in a large survey of 442 affected patients

**Table 33.1** Primary Diagnosis in Major Obstetric Hemorrhage Defined as the Need for At Least four Units of Blood

Timing	Diagnosis <sup>†</sup>	n	(%)
Early pregnancy (n = 51)	Ectopic pregnancy	29	(56.9%)
	Spontaneous abortion	10	(19.6%)
	Termination of pregnancy	10	(19.6%)
	Miscellaneous <sup>‡</sup>	2	(3.9%)
Antepartum (n = 135)*	Abruptio placetae	61	(45.5%)
	Placenta praevia	54	(40.3%)
	Miscellaneous <sup>§</sup>	7	(5.2%)
	Unknown diagnosis	12	(9.0%)
Postpartum (n = 1480)*	Retained placenta or placental rests	703	(47.8%)
	Uterine atonia	567	(38.5%)
	Hemorrhage following CS	183	(12.4%)
	Perineal tears/episiotomy	148	(10.1%)
	Clotting disorders	116	(7.9%)
	Placenta acreta/increta/percreta	109	(7.4%)
	Rupture of cervix	58	(3.9%)
	Uterine rupture	44	(3.0%)
	Uterine inversion	13	(0.9%)
	Miscellaneous	65	(4.4%)
	Unknown diagnosis	10	(0.7%)

Source: Zwart et al. (2008).<sup>4</sup> Reproduced with permission of Wiley.

\* In 76 cases, both antepartum and postpartum diagnoses were coded.

<sup>†</sup> Up to three diagnoses could be coded postpartum.

<sup>‡</sup> Molar pregnancy and placenta percreta.

<sup>§</sup> Rupture of uterine/ovarian artery, rupture of ovarian cyst, placenta percreta, vasa praevia, retroplacental hematoma, and rupture of uterine vein.

**Table 33.2** Laboratory Values in 442 Cases of HELLP

	Median	Range
Platelets 10 <sup>9</sup> /L	57	7–99
ALAT U/L	249	70–6193
LDH U/L	853	564–23,584
Bilirubin µmol/L/mg/dL	26//1.53	8.6–436//0.51–25.65
Creatinine µmol/L/mg/dL	97//1.10	53–1414//0.6–16.1
Urine acid µmol/L/mg/dL	462//7.7	174–900//2.9–15

Source: Sibai (1990).<sup>36</sup>

(Table 33.2).<sup>10</sup> In addition to platelet counts being lower than those typically seen in GT, patients with HELLP also present with right-upper abdominal pain, nausea, and vomiting, which can suggest the diagnosis. Fortunately, severe bleeding rarely occurs despite low platelets because preeclampsia is associated with vasoconstriction. Nonetheless, if not treated, severe cases of HELLP can result in DIC, pulmonary edema, acute renal failure, abruptio placentae, and liver hematoma, with maternal mortality as high as 20%. The definitive therapy for HELLP is ending the pregnancy, which leads to resolution of the disease within 10 days. Thus, in mothers with HELLP, elective birth should be considered as soon as it is clinically feasible. Clinical judgement is required for scheduling childbirth. On the one hand, attention should be paid to the child's ability to survive premature birth. On the other hand, extending the pregnancy to unnecessary lengths may also be deleterious for the child; neonatal death in mothers with HELLP ranges from 10% to 60%, depending on intervention, while elective preterm deliveries can reduce the fetus' risk of death (as well as the mother's risk). While mild thrombocytopenia and schistocytes are observed in up to 50% of these neonates, these findings typically resolve without treatment.

ITP is a less common cause of pregnancy-associated thrombocytopenia than either GT or HELLP, with an estimated incidence of 1–5 cases per 10,000 pregnancies.<sup>14</sup> Often, the mother has a

history of ITP, although ITP can also present initially during pregnancy. In either case, the decrease in platelets is most pronounced during the third trimester.<sup>15,16</sup> Treatment is driven by maternal indications. During the pregnancy, corticosteroids are first-line therapies, with the goal of maintaining platelets above  $20 \times 10^9/\text{L}$  or higher in case of bleeding symptoms.<sup>16,17</sup> Intravenous immunoglobulin (IVIG) is often reserved as second-line treatment. IVIG may also be used in preparation for delivery and can be combined with platelet transfusions at that time. A platelet count  $>50 \times 10^9/\text{L}$  is considered safe for delivery. Specific aspects of delivery procedures should be based on obstetric indications, with the understanding that procedures should be avoided which increase the fetus' hemorrhagic risk.<sup>18</sup> Since ITP is not associated with severe maternal bleeding during delivery, by itself it is not an indication for caesarean section (CS).<sup>19</sup> Should CS be required, British guidelines recommend a platelet count  $>80 \times 10^9/\text{L}$  for this procedure and for epidural anesthesia.<sup>18</sup>

In pregnancies complicated by ITP, neonatal platelet counts of less than  $50 \times 10^9/\text{L}$  are seen in only a fraction of the cases, and related complications are fortunately rare. The risk for neonatal thrombocytopenia is 4.9% directly after birth but increases to 38% in the first two weeks after birth.<sup>20–22</sup> Platelet levels typically reach their nadir at four days after delivery (range of 1–7 days after parturition),<sup>14–16</sup> and thrombocytopenia can persist for months.<sup>23</sup> Predicting which children are most likely to experience significant thrombocytopenia is difficult. A platelet count less than  $50 \times 10^9/\text{L}$  is most likely when maternal ITP is refractory to corticosteroids, other immunosuppressive treatments, and splenectomy.<sup>15,16,24</sup> In contrast, if the mother is asymptomatic, severe fetal thrombocytopenia is close to the background rate of 1% in term newborns. Nonetheless, there is only a weak overall correlation between maternal platelet count and neonatal thrombocytopenia.<sup>16,17</sup> Furthermore, maternal treatments for ITP do not appear to affect neonatal platelet counts.<sup>5,10,25</sup> The occurrence of maternal ITP does not predict significant fetal complications. Intracranial hemorrhage (ICH) or other severe neonatal bleeding is not increased when the mother has ITP, with a rate of 0–1.5%.<sup>16,17,26,27</sup> Nevertheless, brain imaging of the child is recommended with platelet counts  $<50 \times 10^9/\text{L}$ .<sup>16</sup> Perinatal mortality associated with maternal ITP is about 0.6%.<sup>20</sup>

### TTP and HUS

TTP, which results from autoantibodies directed against the ADAMTS-13 protease that cleaves large molecular weight von Willebrand factor (vWF) multimers, can present in the second and third trimesters. TTP must be differentiated from other TMAs, especially HELLP, since TTP is not associated with preeclampsia and does not require termination of pregnancy. Rather, TTP in pregnancy can be effectively treated with plasma exchange as in nonpregnant patients. Accordingly, a history of TTP in remission is not a contraindication for pregnancy.<sup>28</sup>

HUS and TTP are often included in the same differential. In pregnant patients, however, there are key differences. HUS primarily affects children and young adults, whereas TTP can be seen in older women.<sup>18</sup> Furthermore, atypical HUS (aHUS), the most common form in pregnancy, typically presents from two days postdelivery onward (mean 26 days). While 5–10% of HUS cases occur in the antepartum period, TTP on the other hand most commonly presents prior to parturition. At presentation, 50% of females with aHUS have thrombocytopenia below  $100 \times 10^9/\text{L}$ .<sup>6</sup> aHUS is typically associated with complement dysregulation due to mutations

in one or more complement genes,<sup>18</sup> although 6–10% of aHUS cases are caused by acquired antibodies against complement factor H.

Early diagnosis of TTP or HUS is essential to start treatment because most fatal events occur in the first few days. Plasma exchange is the first-line effective therapy for TTP; plasma exchange is also effective in patients with aHUS due to antibodies against factor H.<sup>18,29</sup> For this reason, it is acceptable to start exchange treatments and then modify accordingly based on ADAMTS-13 levels (<10% of normal levels are associated with TTP) and/or low complement levels (in aHUS). If testing supports the diagnosis of aHUS, then plasma exchange should be discontinued and Eculizumab therapy initiated.<sup>30</sup> Platelet transfusions for thrombocytopenia in TTP and aHUS are only indicated in cases of severe bleeding and not for prophylaxis. If possible, delivery can be postponed until the patient's condition has stabilized or resolved.

## DIC

While DIC can be seen in severe TMAA, the occurrence of DIC in pregnancy is usually caused by primary obstetric (abruptio placentae) or septic pathology. While 20–40% of obstetric patients with critical illness have symptoms of DIC, the strict reliance on a DIC score to identify DIC in pregnancy (e.g., based on decreased platelet count, increased D-dimers, decreased fibrinogen, and prolonged prothrombin time) is not reliable primarily because D-dimers start to increase in the first trimester and are always increased after the 35th week. Nonetheless, it is appropriate to have a high index of suspicion for DIC in critically ill pregnant patients because fibrin deposition from DIC can contribute to multiple organ failure, depletion of platelets and coagulation factors, and severe bleeding. While the definitive treatment focuses on addressing the underlying etiology, supportive care may also be necessary. Platelet transfusions are recommended in bleeding thrombocytopenic patients (<50 × 10<sup>9</sup>/L), while plasma and cryoprecipitate should be provided in patients with low fibrinogen, coagulopathy, and bleeding.<sup>31</sup> Of note, in contrast to TTP and aHUS, HELLP is not associated with overt DIC, although 70% of these patients demonstrate reduced antithrombin III (ATIII) levels.<sup>32</sup>

## APS

Antiphospholipid antibody syndrome (APS) is an acquired condition of hypercoagulability, often associated with systemic lupus erythematosus (SLE). APS is characterized by arterial and venous thrombosis, gestational vascular complications, recurrent fetal loss, growth retardation, and prematurity. Patients with APS demonstrate antibodies against β2-GPI (Glycoprotein 1), anticardiolipin autoantibodies, and/or prolongation of plasma clotting time (lupus anticoagulant/LA).<sup>33</sup> Based on randomized studies, women with recurrent abortion that may be due to APS are treated with a combination of low-dose aspirin and low-molecular-weight heparin.<sup>26,33,34</sup> There is no risk for fetal bleeding, but the mother has a slightly increased risk for bleeding during delivery.

## Inherited bleeding disorders

There is a growing list of rare inherited thrombocytopenic disorders typically resulting from mutations in genes involved in megakaryocyte and platelet production. Many of these diseases also manifest with platelet dysfunction. It follows that women with these disorders are at particular risk of bleeding during pregnancy as are their children if they inherit the same mutations. A retrospective survey from the European Haematology Association

identified an inherited thrombocytopenia prevalence of 2–3/100,000, with most of the cases from Italian patients. In this survey, 339 pregnancies were complicated by 13 different forms of inherited thrombocytopenia (50% with MYH9-related diseases). Surprisingly, however, there was no dramatic increase in bleeding during pregnancy, although 5% were transfused with platelets and/or red cells, which is higher than in the general population. None of the 156 children who inherited their mother's disorder showed intrauterine bleeding, but the authors mentioned that such bleeding has been reported in the literature. Five newborns had petechiae, and two suffered cerebral hemorrhage.<sup>35</sup> Planned delivery and an individualized treatment plan should be considered for these mothers. Such a policy also applies to woman with inherited platelet function disorders such as Glanzmann thrombasthenia or Bernard Soulier syndrome, for which tranexamic acid with or without rVIIa is recommended.<sup>36</sup>

Mothers with von Willebrand disease (vWD) are at increased risk for postpartum hemorrhage and have up to fivefold higher risk of transfusions.<sup>37</sup> As bleeding in vWD or more rare inherited coagulation factor deficiencies can persist or reappear up to two weeks or longer after delivery, appropriate therapies (desmopressin, vWF concentrates, or specific clotting factor concentrates) are recommended for at least 3–5 days after delivery.<sup>38</sup>

## Chronic maternal anemia

Physiological anemia due to plasma volume expansion peaks around the 25th week of pregnancy. *Anemia in pregnancy* is defined by WHO as an Hb <6.8 mmol/L (11 g/dL), although significant fetal problems such as growth retardation are usually not noticed until Hb drops to 5.5–6.2 mmol/L (9–10 g/dL).<sup>39,40</sup>

In otherwise healthy pregnant women, anemia is most often caused by nutritional deficiencies. However, anemia can certainly complicate the pregnancies of mothers with hemolytic diseases, such as thalassemia, SCD, and paroxysmal nocturnal hemoglobinuria (PNH). In addition to anemia, pregnant women with PNH are also at increased risk for thrombosis requiring antithrombotic treatment. Randomized studies in pregnant women with SCD have shown that maintenance of a hematocrit above 33% with less than 35% sickle cells significantly reduced painful crises, although pregnancy outcomes were not improved.<sup>41</sup> Since patients with chronic hemolytic anemias are often high antibody responders, they usually receive phenotypically matched red blood cell transfusions (for C, E, and Kell).

## Transfusions in pregnancy

### Maternal transfusions

#### Major obstetric bleeding

Clotting problems that most often accompany preeclampsia, placental abruption, and amniotic fluid embolism increase the risks of bleeding in pregnancy and thus the need for transfusions. Among these groups, women with preeclampsia are hypovolemic before hemorrhage starts and require special attention. Urinary output should be carefully monitored as it is the best indicator of renal perfusion in a bleeding or preeclamptic woman and should be maintained at least 30 mL/hour.

Life-threatening massive bleeding after delivery is most commonly caused by uterine atony (Table 33.3). Treatment of PPH includes transfusion along with oxytocin, misoprostol, prostaglandin, vascular embolization, surgical interventions, and hysterectomy.

**Table 33.3** Risk Factors for Postpartum Hemorrhage

- Overdistended uterus (multiple pregnancy, large fetus, and polyhydramnios)
- Prolonged labor
- Induction of augmentation of labor
- Postpartum hemorrhage in previous pregnancy
- Chorioamnionitis
- High parity
- Coagulation disorders (e.g., HELLP syndrome or placental abruption)

### Hemorrhage protocols

To support transfusion needs, postpartum hemorrhage protocols have developed to more closely resemble massive transfusion protocols (MTP) such as those used for trauma.<sup>42</sup> These involve earlier transfusion interventions, without waiting for consumption and dilutional coagulopathies.

### California maternal quality care collaborative (CMQCC)

To help prepare for rapid bleeding events, specific guidance for all sections involved in obstetric emergencies is recommended. In addition to identifying risk categories and collecting data, the CMQCC established tiered protocols to follow in the event of obstetric hemorrhage. Established in 2006 as a partnership between Stanford University and the state of California to reduce maternal mortality in the state, the CMQCC achieved a reduction in maternal mortality of more than 50% while the rest of the United States observed an increase.<sup>43,44</sup> Well-planned protocols using online tool kits to address all aspects of obstetric hemorrhage helped achieve this outcome. Recognizing factors that include all teams involved in the hospital setting along with clear-cut roles to play in these events improves the outcomes in obstetric hemorrhage.<sup>44,45</sup> An example of an emergency management plan is shown in the figure that follows. More tools and resources are available at CMQCC.org.

#### CMQCC Obstetric Hemorrhage Emergency Management Plan: Table Chart Format Version 2.0

	Assessments	Meds/Procedures	Blood Bank
Stage 0	Every woman in labor/giving birth		
Stage 0 focuses on risk assessment and active management of the third stage.	<ul style="list-style-type: none"> <li>• Assess every woman for risk factors for hemorrhage</li> <li>• Measure cumulative quantitative blood loss on every birth</li> </ul>	<b>Active Management Third Stage:</b> <ul style="list-style-type: none"> <li>• Oxytocin IV infusion or 10u IM</li> <li>• Fundal Massage-vigorous, 15 seconds min.</li> </ul>	<ul style="list-style-type: none"> <li>• If Medium Risk: T &amp; Scr</li> <li>• If High Risk: T&amp;C 2 U</li> <li>• If Positive Antibody screen (prenatal or current, exclude low level anti-D from RhoGam):T&amp;C 2 U</li> </ul>
Stage 1	Blood loss: > 500mL vaginal or >1000 mL Cesarean, or VS changes (by >15% or HR ≥ 110, BP ≤ 85/45, O <sub>2</sub> sat < 95%)		
Stage 1 is short: activate hemorrhage protocol, initiate preparations and give Methergine IM.	<ul style="list-style-type: none"> <li>• Activate OB Hemorrhage Protocol and Checklist</li> <li>• Notify Charge nurse, OB/CNM, Anesthesia</li> <li>• VS, O<sub>2</sub> Sat q5'</li> <li>• Record cumulative blood loss q5-15'</li> <li>• Weigh bloody materials</li> <li>• Careful inspection with good exposure of vaginal walls, cervix, uterine cavity, placenta</li> </ul>	<ul style="list-style-type: none"> <li>• <b>IV Access:</b> at least 18 gauge</li> <li>• Increase IV fluid (LR) and Oxytocin rate and repeat fundal massage</li> <li>• <b>Methergine</b> 0.2 mg IM (if not hypertensive) May repeat if good response to first dose, BUT otherwise move on to second level uterotonic drug (see below)</li> <li>• Empty bladder: straight cath or place foley with urimeter</li> </ul>	<ul style="list-style-type: none"> <li>• T &amp; C 2 Unit PRBCs (if not already done)</li> </ul>
Stage 2	Continued bleeding with total blood loss under 1500 mL		
Stage 2 is focused on sequentially advancing through medications and procedures, mobilizing help and Blood Bank support, and keeping ahead with volume and blood products.	<ul style="list-style-type: none"> <li>• OB back to bedside (if not already there)</li> <li>• <b>Extra help:</b> second OB, Rapid Response Team (per hospital), assign roles</li> <li>• VS &amp; cumulative blood loss q 5-10 min</li> <li>• Weigh bloody materials</li> <li>• <b>Complete evaluation</b> of vaginal wall, cervix, placenta, uterine cavity</li> <li>• Send additional labs, including DIC panel</li> <li>• If in Postpartum: Move to L&amp;D/OR</li> <li>• Evaluate for special cases: -Uterine Inversion -Amn. Fluid Embolism</li> </ul>	<b>Second level Uterotonic Drugs:</b> <ul style="list-style-type: none"> <li>• Hemabate 250 mcg IM or</li> <li>• Misoprostol 800 mcg SL</li> </ul> <b>Second IV Access</b> (at least 18 gauge) Bimanual massage <b>Vaginal Birth:</b> (typical order) <ul style="list-style-type: none"> <li>• Move to OR</li> <li>• Repair any tears</li> <li>• D&amp;C: r/o retained placenta</li> <li>• Place intrauterine balloon</li> <li>• Selective Embolization (Interventional Radiology)</li> </ul> <b>Cesarean Birth:</b> (still intraop) (typical order) <ul style="list-style-type: none"> <li>• Inspect broad lig. posterior uterus and retained placenta</li> <li>• B-Lynch Suture</li> <li>• Place intrauterine balloon</li> </ul>	<ul style="list-style-type: none"> <li>• Notify Blood Bank of OB Hemorrhage</li> <li>• Bring 2 Unit PRBCs to bedside, transfuse per clinical signs—do not wait for lab values</li> <li>• Use blood warmer for transfusion</li> <li>• Consider thawing 2 FFP (takes 35 + min), use if transfusing &gt;2u PRBCs</li> <li>• Determine availability of additional RBCs and other Coag products</li> </ul>
Stage 3	Total blood loss over 1500 mL, or >2 units PRBCs given or VS unstable or suspicion of DIC		
Stage 3 is focused on the Massive Transfusion protocol and invasive surgical approaches for control of bleeding.	<ul style="list-style-type: none"> <li>• Mobilize team</li> <li>-Advanced GYN surgeon</li> <li>-Second Anesthesia Provider</li> <li>-OR staff</li> <li>-Adult Intensivist</li> <li>• <b>Repeat labs</b> including coags and ABGs</li> <li>• Central line</li> <li>• Social Worker/family support</li> </ul>	<ul style="list-style-type: none"> <li>• Activate Massive Hemorrhage Protocol</li> <li>• Laparotomy:               <ul style="list-style-type: none"> <li>-B-Lynch Suture</li> <li>-Uterine Artery ligation</li> <li>-Hysterectomy</li> </ul> </li> <li>• Patient support               <ul style="list-style-type: none"> <li>- Fluid warmer</li> <li>- Upper body warming device</li> <li>- Sequential compression stockings</li> </ul> </li> </ul>	<b>Transfuse Aggressively</b> Massive Hemorrhage Pack <ul style="list-style-type: none"> <li>• Near 1:1 PRBC:FFP</li> <li>• 1 PLT apheresis pack per 4–6 units PRBCs</li> </ul> <b>Unresponsive Coagulopathy:</b> After 8–10 units PRBCs and full coagulation factor replacement: may consult re rFactor VIIa risk/benefit

### **Massive transfusion protocol**

Extreme cases of obstetric hemorrhage may require transfusion support as robust as the MTP. The proactive transfusion of plasma and platelets in predefined ratios with red blood cells has been shown to reduce total blood use and mortality in trauma.<sup>46,47</sup> Although the evolution of the MTP has been dictated by military and trauma data, an increasing amount of research has been devoted to assessing whether MTP has a positive effect in the setting of obstetric hemorrhage. The use of cryoprecipitate or other fibrinogen products is an especially important component of MTP in obstetrics because hypofibrinogenemia develops early during major obstetric hemorrhage.<sup>38,48–52</sup> As is the case with the MTP in trauma, there are many differing opinions on the correct ratios of blood products to supply. Regardless of the ratios used, the importance of using the MTP as one possible tool to combat severe obstetric hemorrhage is supported by major societies in obstetrics and gynecology.<sup>45,52,53</sup>

### **Cell salvage**

Cell salvage involves the collection of shed blood to be transfused into a bleeding patient. While the use of cell salvage requires thorough planning and preparation and may not be feasible in an emergency obstetrical setting, it should be considered for high-risk patients and those that do not accept allogeneic blood. Current filtration systems in use have removed the risk of causing amniotic fluid embolism. Additionally, limiting the use of cell salvage during cesarean section deliveries has reduced fears of introducing infections.<sup>45,54–58</sup> It is important to remember that fetal blood can be transfused to the mother during cell salvage; if necessary, Rh immunoglobulin can be administered to prevent alloimmunization.

### **Laboratory testing**

Although transfusion ratios are predetermined in MTP, coagulation testing is also encouraged in order to guide customization of blood product usage when necessary.<sup>38</sup> Laboratory evaluation of coagulation indices is an important part of guiding the use of blood products or pharmacologic therapy. As the coagulation status of a patient changes with resuscitation, more frequent laboratory information is needed. Thromboelastography (TEG [Haemonetics, Braintree, MA, USA]) and thromboelastometry (ROTEM [Tem International, Munich, Germany]) have emerged as technologies to monitor coagulation parameters in such situations. Furthermore, the results compare favorably to traditional testing despite usually having a faster turnaround time.<sup>45,59,60</sup>

### **Pharmacologic therapies**

Because the most common cause of PPH is uterine atony, drugs used to increase tone (oxytocin) are first-line therapy. Additionally, pharmacologics that aid coagulation may also be used to control bleeding.

### **Antifibrinolytics**

After uterine atony, one of the main causes of PPH is the degradation of fibrin. The use of antifibrinolytics like tranexamic acid (TXA) to prevent fibrin breakdown and aid in hemorrhage control has been used in cardiac surgery and trauma successfully for years.<sup>45,61,62</sup> More recently, large studies have focused on TXA for use in PPH.<sup>63–68</sup> The World Maternal Antifibrinolytic (WOMAN) trial found in 2017 that 1 g of intravenous TXA given within three hours significantly reduced mortality associated with PPH. The WOMAN trial found that a small window existed for intervention with TXA, as a delay of 15 minutes could lead to a decrease in the

benefits seen by as much as 10%. Furthermore, at three hours there was no benefit observed with administering TXA. Much of the initial hesitation with use of TXA in PPH came from the concern of causing emboli or renal dysfunction; however, the investigators found no increase in such complications with the dose studied.<sup>69</sup> Even though the majority of subjects included in this study were from developing countries, the evidence was strong enough for the WHO, ACOG, and CMQCC to update the OB guidelines to include TXA as a second-line drug for PPH.<sup>70,71</sup>

### **Fibrinogen**

Fibrinogen is the precursor to fibrin, which is important in management of hemorrhage. Although there are differences in the recommended level of fibrinogen for optimal treatment of PPH (200 mg/dL internationally or 125 mg/dL in the United States),<sup>72</sup> the need to maintain adequate levels for hemostasis is universal. As the guidance for adequate levels in PPH vary worldwide, so does the product primarily used to replace fibrinogen. In North America, cryoprecipitate is the product of choice for replacing fibrinogen, while purified fibrinogen concentrates (FC) are used in other countries. In the United States and Canada, FC is only approved for the treatment of congenital afibrinogenemia, although off-label use in acquired hypofibrinogenemia does occur. The advantages of FC over cryoprecipitate include pathogen inactivation, precise dosing, and immediate availability (does not need to be thawed prior to use). However, recent studies investigating the use of FC versus placebo in PPH showed no difference in the groups when used as preemptive treatment. Importantly, the studies found no increase in thromboembolic events associated with FC administration. The largest of the studies did however uncover findings to suggest that replacing fibrinogen at levels greater than 200 mg/dL in obstetric hemorrhage does not improve outcomes.<sup>73–76</sup>

### **Coagulation factor replacements**

Targeted coagulation factor replacement has been successful in treating and preventing hemorrhage in specific patients and situations. Four-factor prothrombin complex concentrates (PCC) contain proteins C and S in addition to factors II, VII, IX. In the United States, this product is FDA approved for reversal of coagulation deficiency caused by vitamin K antagonist therapy (warfarin) when Vitamin K administration is not timely enough to replenish cofactor levels (active bleeding or urgent surgery/invasive procedure).<sup>77</sup> Currently, evidence is lacking for the use of PCC in PPH.<sup>73</sup> Recombinant factor VIIa (rFVIIa) is FDA approved in the United States for hemophilia A/B that is complicated by inhibitors, acquired hemophilia, and Glanzmann's thrombasthenia.<sup>78</sup> Instances of thromboembolic events urge the careful use of rFVIIa.<sup>79–81</sup> The use of rFVIIa is considered a last choice in PPH when all other interventions have failed.

### **Postpartum anemia**

The need for transfusion to treat anemia is reduced in the postpartum period as compared to preparturition. At 3–6 weeks after delivery, healthy women with a postpartum median Hb level of 6.5 mmol/L (10.5 g/dL; range 7–15 g/dL) demonstrated no relationship between the Hb level and the quality of life.<sup>82</sup> Similar findings were seen in women who experienced lower Hb nadirs (3–4.9 mmol/L; 4.8–7.9 g/dL) at 12–24 hours after PPH. When these patients were randomized to receive transfusion of two RBC units ( $n = 259$ ), or not ( $n = 262$ ), physical fatigue was slightly better in the transfused group at 3–7 days postpartum, but the quality of life and physical complications were no different up to six weeks

after delivery.<sup>83</sup> These data suggest that Hb concentration of 4–5 mmol/L (7.0–8.0 g/dL), or lower (3 mmol/L; 5.0 g/dL), is usually well tolerated if isovolemia is maintained. Furthermore, even in the case of PPH, transfusion is usually only required for symptomatic anemia. There are no good studies of pregnant women with lower Hb concentrations; however, studies on Jehovah's Witness patients and others who declined blood transfusion found that morbidity and mortality rates start to accelerate below the level of 5.0 g/dL.<sup>84,85</sup>

### **Platelet transfusions during pregnancy and for delivery**

Platelet transfusion may be required under several different obstetrical situations. In otherwise uncomplicated pregnancies, most obstetrical guidelines recommend considering platelet transfusion prior to vaginal delivery when the mother's platelet count is below  $50 \times 10^9/\text{L}$ .<sup>18,86</sup> If local anesthesia is needed, most guidelines advise a platelet count of 50 or  $80 \times 10^9/\text{L}$ .

The situation becomes more complicated if the patient is suffering from thrombotic disorders. Platelet transfusions should be considered for patients who are thrombocytopenic secondary to TMAAs, ITP, or DIC; TTP, however, is an exception since it is considered a relative (but not absolute) contraindication for platelet transfusions. In these thrombocytopenic conditions, characterized by enhanced platelet phagocytosis or consumption, platelet transfusions are typically used therapeutically when the patient is bleeding rather than prophylactically. Because of different pathophysiology and bleeding history, an individual treatment plan for every pregnant patient with thrombocytopenia is required.<sup>87</sup>

### **Blood products used and special precautions**

#### *Red cell transfusions*

In Western countries, women are routinely screened early in pregnancy for RhD expression as well as for red cell alloantibodies. The objective is to identify potential causes or risks for hemolytic disease of the fetus and newborn (HDN), which can significantly complicate pregnancy. Women who are RhD-negative are candidates for RhIg administration, while those with preexisting red cell alloantibodies are brought to the attention of their obstetricians for evaluation and closer management. Of note, in addition to anti-RhD antibodies, antibodies against Rhc and Kel1 can also cause severe HDFN.<sup>88</sup> In more than 40% of cases, mothers with these latter antibodies had received prior transfusions indicating the significance of transfusion exposure.<sup>89</sup> Many national guidelines recommend the use of Kel1-negative (often also Rhc-compatible) donors for woman up to the age of 45–50 years, but this practice is not yet uniform.

#### *Platelet transfusions*

Although platelet units contain minimal amounts of red cells, that quantity is still sufficient to immunize RhD-negative recipients against the RhD antigen. Thus, when blood inventory challenges necessitate that RhD-negative women of childbearing potential receive RhD-positive platelet units, prophylaxis with RhIg is indicated.<sup>90</sup>

#### *Immunoglobulin*

To prevent HDN due to anti-RhD antibodies, all RhD-negative mothers should receive RhIg between the 28th and 34th weeks of gestation. Furthermore, these RhD-negative mothers should also

receive additional RhIg within 72 hours after delivery if the child is confirmed to be RhD-positive.<sup>91</sup> This approach reduces the incidence of RhD HDN in RhD-negative mothers to less than 1%. Nevertheless, HDN due to RhD antibodies remains the most frequent cause of severe HDN. This typically occurs due to medical, laboratory, or clerical errors, for example if RhIg is given in a dose that is too low to prevent alloimmunization following a large fetomaternal hemorrhage.<sup>92,93</sup>

In recent years, tests have been developed to perform fetal RhD typing on the maternal plasma; a negative RhD result means the mother will not be exposed to RhIg treatment unnecessarily.<sup>94</sup> This approach can also be used to type for other red cell antigens, including C, c, E, and Kel1.

High-dose intravenously immunoglobulin (IVIg) is another immunoglobulin preparation that may be administered during pregnancy. For example, IVIg may be used to increase the maternal platelet count in ITP and to reduce severe fetal bleeding in fetal or neonatal thrombocytopenia (FNAIT).

#### *Special safety aspects of transfusion products*

Blood products have been shown to transmit infectious agents that may harm the fetus, including CMV and parvo-B19. For this reason, there has been considerable debate over the years regarding the safest RBC and platelet products for pregnant CMV-seronegative women who require transfusion. Data show that units from donors who are CMV-seronegative have a similar safety profile to leukoreduced units from CMV-untested donors.<sup>95,96</sup> While the question of superiority of one method over the other has not been resolved, a large prospective observational trial showed that the transfusion of leukoreduced RBC and platelet units obtained from CMV-seronegative donors (the “belt and suspenders” approach) completely prevented CMV transmission to seronegative pregnant women.<sup>97</sup> While some countries test for parvovirus B19, this is not a widely available approach to providing B19-safe blood for transfusion.

#### *Low-income countries*

The blood transfusion services in some low-income countries have difficulty providing the quality or quantity of blood products taken for granted in the United States and other developed countries. This issue can have significant ramifications for pregnant women when considering that approximately 25% of maternal deaths can be traced to inadequate access to blood transfusion.<sup>98</sup> Furthermore, pregnant women in low-income countries are more likely to have underlying anemias due to malaria, iron and folic acid deficiency, SCD, and infectious diseases, suggesting that a relatively small hemorrhage can be deadly. To address these challenges, bed nets, nutritional supplements, antihelmintics, presumptive treatment of malaria, folic acid, and ferrous medication should be utilized as part of antenatal care to mitigate causes of anemia.<sup>99</sup>

Prevention of postpartum hemorrhage is another critical component of obstetrical care. While effective approaches include the use of oxytocic drugs, early cord clamping, and active efforts to deliver the placenta, actual practices differ between countries.<sup>100</sup>

It is also important to note that in some countries emergent blood donation by relatives is a common source of blood for maternal transfusion. However, the transmission of hepatitis and HIV is a serious risk with this practice and has been ascribed as the cause for 8–16 million hepatitis B, 2.3–4.7 million hepatitis C, and 80,000–160,000 HIV infections annually.<sup>101</sup>

## Fetal transfusions

### Alloimmune hemolytic disease

The state of the art for fetal transfusions has progressed significantly since 1963 when Liley transfused red cells into the fetal intraperitoneal cavity under X-ray guidance.<sup>102</sup> Survival rates for the procedure were approximately 50%,<sup>103</sup> and those fetuses had variable red cell uptake into the circulation that was negatively affected by conditions such as severe hydrops. Success improved significantly with the introduction of real-time ultrasound in the 1980s, which allowed transfusion directly into the umbilical vein of fetuses as young as 16 weeks.<sup>104</sup> The most frequent indication for fetal transfusion is anemia secondary to maternal alloimmunization to red cell alloantigens; other causes of fetal anemia that can be treated by in utero transfusion include parvovirus B19 infection,<sup>105,106</sup> severe fetomaternal hemorrhage,<sup>107</sup> placental chorioangiomas,<sup>108</sup> and homozygous α-thalassemia.<sup>109</sup>

Under ultrasound guidance, a 20–22 gauge needle is used to both aspirate blood for diagnosis and deliver red cells, platelets, or drugs into the fetal circulation. The umbilical vein is the preferred target for several reasons when the placenta is inserted anteriorly: the vein has a larger diameter than the umbilical arteries; because of its location, missing or overshooting the vein is most likely to leak blood into the amniotic cavity rather than Wharton's jelly, while the opposite is true when targeting the artery that can produce arterial spasm and fetal bradycardia; and flow of the transfused blood can be monitored by ultrasound during the transfusion. Typically, the mother is premedicated to reduce anxiety and sedate the fetus; muscle relaxants such as atracurium or pancuronium are also used to produce fetal paralysis that reduces the chances of needle displacement.<sup>110</sup>

After the needle is inserted, and prior to transfusion, 1–2 mL fetal blood is aspirated to quantify hemoglobin, hematocrit, and mean corpuscular volume. These values are used to calculate transfusion volume based on pretransfusion fetal hematocrit, estimated fetal placental blood volume, and hematocrit of the donor blood.<sup>111</sup>

Blood components to be transfused are extensively processed beforehand. Units are typically group O, RhD-negative, lack antigens against which the mother has produced alloantibodies, and crossmatch-compatible with maternal serum. To reduce the risks of CMV transmission, units are leukoreduced and at many centers also selected from the CMV seronegative inventory to further mitigate risk. To prevent transfusion-associated graft-versus-host disease, the components are also irradiated. At the time of this edition, the use of pathogen-reduced products in this population is being widely studied with a consensus still pending. Prior to transfusion, the unit is centrifuged, the supernatant removed (containing plasma, anticoagulant, and preservation solution), and 0.9% saline is added to a final packed red cell concentration of 80%.<sup>112</sup> The blood is transfused at 5–10 mL per minute while ultrasound is used for continuous monitoring of blood flow and the fetal heart rate.<sup>111</sup>

In the setting of alloimmune fetal anemia, due to maternal anti-RhD antibodies for example, the goal of fetal transfusion is not only to replace the volume of destroyed red cells but also to suppress ongoing production of incompatible fetal red cells. To this end, a second transfusion is given 1–2 weeks later, and additional transfusions are performed every 3–4 weeks afterward until the fetus can be safely delivered. It is important that prior to each transfusion the maternal antibody screen is repeated and the units to be transfused are crossmatched against the newest maternal sample. More than 70% of women with HDN requiring intrauterine transfusions

develop multiple erythrocyte antibodies,<sup>112</sup> most often caused by ongoing exposure to fetal red cells.<sup>112</sup>

The outcomes of fetal transfusion have improved significantly over the last half century. For example, at Leiden University Medical Centre, 593 ultrasound-guided intravascular transfusions of fetuses between 17 and 35 gestational weeks between 1988 and 1999 resulted in an 86% overall survival rate;<sup>88</sup> survival further improved to 98% between 1999 and 2009.<sup>113</sup> Although techniques have doubtless improved over this time, better pretransfusion management has also led to fewer mothers presenting with fetuses suffering from severe irreversible hydrops.<sup>88</sup> A follow-up study of 291 infants at a median age of eight years after fetal transfusion showed neurodevelopmental impairment in 4.8%, which is not statistically significant different from the general population. Hydrops and prematurity were identified as risk factors for the children with impairment.<sup>114</sup>

After birth, additional transfusion management including exchange transfusions or top-up transfusions may be required despite efforts to reduce the need for transfusion through IVIg administration.<sup>115,116</sup>

### Fetal or neonatal alloimmune thrombocytopenia

Immune-mediated thrombocytopenias, including fetal/neonatal alloimmune thrombocytopenia (FNAIT) or ITP, are identified in 0.3% of newborns.<sup>117–119</sup> Red blood cell alloimmunization can also cause thrombocytopenia in 10–30% of severely hydropic fetuses presumably through inhibition of thrombopoiesis due to expanded erythropoiesis.<sup>120</sup> With the exception of FNAIT, in utero bleeding such as intracerebral hemorrhage (ICH) is rare in these conditions and intrauterine platelet transfusions are not indicated.<sup>16,17,19,26</sup>

In contrast, FNAIT carries a significant risk for ICH. About 20% of fetuses affected by FNAIT will develop ICH, which can occur as early as the 20th week of gestation.<sup>121</sup> FNAIT is caused by maternal alloantibodies against polymorphic antigens on fetal platelets. In Caucasians, anti-HPA-1a antibodies cause 85% of FNAIT cases despite the fact that only about 2% of mothers are capable of producing these antibodies (HPA-1a negative).<sup>122</sup> Most of the remaining cases of FNAIT are caused by HPA-5b, HPA-3a, and HPA-15 antibodies,<sup>123</sup> although in some cases no antibodies can be detected suggesting that the target platelet antigen has not yet been discovered.<sup>124</sup> When considered from the perspective of pregnant women alloimmunized to platelet antigens, thrombocytopenia occurs in up to 85% of fetuses who express the cognate antigen. Despite frequent thrombocytopenia, the development of ICH is variable in FNAIT and appears to correlate with ICH in previous pregnancies; the risk ranges from 7%, typically when there was a sibling without ICH, to 80% when a sibling had ICH.<sup>125</sup>

While parental HPA genotypes can be used to predict the risk of FNAIT in a child, such screening is not routine. Accordingly, FNAIT is usually only diagnosed when a child presents with thrombocytopenia and/or ICH. While this index child must be managed reactively, the diagnosis of FNAIT allows physicians to manage the risks of FNAIT in later siblings. Parental genotyping can be used to assess the risk that a subsequent fetus may suffer FNAIT. A homozygous father will invariably pass the gene for the offending antigen to the fetus; a heterozygous father has a 50–50 chance of transmitting that gene, indicating the need for fetal genotyping.

If the fetus is determined to be at risk for FNAIT, antenatal management protocols are directed at preventing ICH. Unfortunately, the utility of quantifying maternal anti-HPA antibodies is unclear in this regard, although studies suggest it may predict the severity of

fetal thrombocytopenia.<sup>126–128</sup> The most direct way to assess the risk of ICH is by quantifying fetal thrombocytopenia through fetal blood sampling (FBS) beginning as early as 20 weeks of gestation.<sup>129,130</sup> However, the cumulative risk of fetal loss by repetitive FBS is approximately 6% per pregnancy,<sup>131,132</sup> while the cumulative risk for emergency delivery due to FBS is 13–17%.<sup>133,134</sup>

Given these significant risks, antenatal management is gradually transitioning from routine use of repetitive FBS, to a less invasive approach (fewer FBS procedures combined with IVIG and intrauterine platelet transfusions before delivery), to a completely noninvasive approach (IVIG only). Many centers have adopted this last approach and initiate weekly high-dose maternal IVIG when the fetus is judged to be at risk for FNAIT. As compared to FBS-guided treatment protocols, empiric IVIg administration without FBS guidance has been shown to be a cost-effective strategy.<sup>135</sup> In some cases, corticosteroids are administered as well.

Weekly maternal administration of IVIg (1.0 g/kg maternal weight) has been shown to increase platelet counts in fetuses with FNAIT. While an increase in platelet counts is only seen in 30% and 85% of cases, even fetal nonresponders whose platelet count remains depressed show reduced risks of ICH.<sup>134,136,137</sup> The mechanisms of IVIg action in FNAIT remain unclear, with several leading candidates. First, IVIg may downregulate maternal B cells including those producing anti-HPA antibodies. Second, IVIg may increase maternal catabolism of IgG as well as dilute out anti-HPA antibodies, essentially reducing the maternal titer of the offending antibody. Third, IVIG may block the placenta receptor that shuttles maternal antibodies, including anti-HPA antibodies, across the placenta. Fourth, in the fetus, IVIg may block Fc receptors on macrophages and prolong the lifespan of fetal platelets coated with anti-HPA antibodies. Fifth, IVIg may downregulate endothelial activation by platelet antibodies that crossreact with endothelial cells, accounting for the reduction in ICH when no impact on platelet count is observed.<sup>138</sup> In a mouse model of FNAIT, maternal IVIG administration demonstrated many of these hypothesized mechanisms as well as reduced bleeding and increased fetal survival.<sup>139</sup>

### Fetal transfusions for other indications

Parvovirus B19 infection during pregnancy can produce fetal anemia and thrombocytopenia by causing maturation arrest in hematopoietic stem and precursor cells. Parvo B19V is a frequent cause of nonimmune hydrops fetalis, and 46% of these hydropic fetuses have platelet counts below  $50 \times 10^9/L$ , which can further decrease after transfusion of red blood cells.<sup>140</sup> Intrauterine transfusions are indicated in Parvovirus B19 infected fetuses, although a single red cell transfusion is usually sufficient.<sup>106</sup>

Massive fetomaternal (transplacental) hemorrhage is a rare and serious complication of pregnancy that can produce severe fetal anemia, hydrops, and death. Reduced fetal movement may be the presenting condition, and the diagnosis can be confirmed by

increased flow in the middle cerebral artery measured by ultrasound<sup>141</sup> and fetal red cells in the maternal circulation in the Kleihauer-Betke test.<sup>135</sup> In the preterm period, fetal red cell transfusion is a realistic option.

After the introduction of laser coagulation of placental anastomosis as treatment for severe twin-to-twin transfusion syndrome (TTTS), TAPS (twin anemia–polycythemia sequence) is described as a new syndrome.<sup>142</sup> After laser coagulation, very small residual anastomosis may lead to chronic anemia and polycythemia in one of the twins.<sup>143</sup> Robyr *et al.* described 13 cases of TAPS (13% of their group of TTTS cases).<sup>144</sup> In 12 of the 13 cases, 1–5 fetal red cell transfusions were performed. Slaghekke described that, when the Solomon laser technique was used, the incidence of TAPS decreased from 16% to 3%.<sup>145</sup>

Very rare indications for fetal transfusions are placental chorioangioma and alpha-thalassemia.

### Conclusion

Postpartum hemorrhage is a serious risk factor in obstetrics. Most maternal mortality can be prevented using oxytocics, embolization of uterine vessels, and blood transfusion. In some cases, fetal transfusion of red blood cells and/or platelets is required.

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## CHAPTER 34

# Transfusion in infants and children

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Physiologically speaking, infants and children are not simply “small adults” when considering causes and treatments of anemia, thrombocytopenia, and coagulopathy. Several factors such as gestational age, congenital diseases, immature liver, maternal factors, and transplacental antibody transfer must all be considered when evaluating and treating neonates with anemia, thrombocytopenia, bleeding, or coagulopathy. Bleeding tendencies and diseases prevalent in childhood must be understood for optimal transfusion support. Additionally, special consideration must be given to the product(s) transfused to infants and children, whether the transfusion be for prophylactic reasons or otherwise. These considerations include ABO compatibility, total blood volume, immaturity, immunosuppression, immunodeficiencies, and blood donor exposure. This chapter first briefly reviews the etiology of anemia, thrombocytopenia, and coagulopathy in infants and children, and then provides a general overview of transfusion support.

## Red blood cell transfusion

### Pathophysiology of anemia in infants

Anemia in infants may be due to prematurity, blood loss, decreased RBC production (including rare instances of bone marrow failure), or increased RBC destruction (hemolytic disease of the fetus and newborn, mediated by maternal RBC alloantibodies, is covered in Chapter 32).

Anemia noted at birth in preterm infants is typically a result of incomplete iron transfer from the mother to the fetus via the placenta, in combination with incomplete erythropoietic activity of the fetal marrow.<sup>1</sup> After birth, this anemia is significantly exacerbated by phlebotomy associated blood loss; it has been estimated that this blood loss in preterm infants may approach as much as 11–22 mL/kg per week.<sup>2,3</sup> During the first weeks of life, all infants experience a decline in the number of circulating RBCs caused by physiologic factors. In healthy term infants, the nadir blood hemoglobin value rarely decreases to less than 9 g/dL (mean = 11–12 g/dL) at an age of approximately 10–12 weeks. Because this postnatal decrease in the hemoglobin level is universal and is well tolerated by term infants, it is referred to as “physiologic anemia” of infancy. Among preterm infants, this decline occurs at an

earlier age and is more pronounced in severity.<sup>4</sup> Mean hemoglobin concentration decreases to approximately 8 g/dL in infants of 1.0–1.5 kg birth weight and to 7 g/dL in infants weighing less than 1.0 kg. Anemia of infancy occurs as the production of hemoglobin F (with high oxygen affinity) transitions to the production of adult hemoglobin, hemoglobin A, with lower oxygen affinity; simultaneously there is a transient reduction in erythropoietin production.<sup>5</sup> This physiologic anemia improves when receptors in the kidneys and liver sense the hypoxia, leading to increased erythropoietin production.

As compared to infants born at term, preterm infants have a relatively diminished erythropoietin response to anemia.<sup>6</sup> However, since erythroid progenitor cells of preterm infants are responsive to erythropoietin in vitro, inadequate erythropoietin production is likely more of a factor than marrow unresponsiveness.<sup>7</sup> The use of erythropoietic-stimulating agents has been extensively studied in preterm infants over the past few decades. Whereas erythropoietin has been shown to stimulate erythropoiesis, conflicting data exist regarding the impact on neurologic outcomes. Ohls *et al.* demonstrated that erythropoietin improved neurologic outcome at 18–22 months in preterm infants and decreased transfusion burden.<sup>8,9</sup> In contrast, a multicenter randomized controlled trial (Preterm Erythropoietin Neuroprotection Trial [PENUT]), published in 2020, found no decrease in severe neurodevelopmental impairment or death by two years of age in infants treated with erythropoietin.<sup>10</sup> However, a subanalysis found that the infants randomized to erythropoietin required a lower volume of transfused red blood cells after 10 days of life.<sup>11</sup>

Another variable to consider in anemic preterm infants is the relative deficiency of micronutrients such as iron, folate, and other vitamins required for erythropoiesis,<sup>1</sup> in combination with shortened endogenous RBC survival compared to adults.<sup>5</sup> A 2012 review found that infants who receive iron supplementation have a slightly higher hemoglobin level, improved iron stores, and a lower risk of developing iron deficiency anemia compared with those who are not supplemented.<sup>12</sup>

Multiple additional strategies have been studied in preterm infants to decrease anemia and thus RBC transfusion burden, including delayed cord clamping, decreased phlebotomy blood loss, and pharmacologic interventions.<sup>5</sup> Delayed cord clamping ( $\geq 30$  seconds) has

been shown in a systematic review and meta-analysis of preterm infants to decrease mortality, increase peak Hct, and decrease the need for RBC transfusion.<sup>13</sup> As such, the American College of Obstetricians and Gynecologists (ACOG) recommends that cord clamping in vigorous term and preterm infants should be delayed until 30–60 seconds after birth.<sup>14</sup> Simple and novel strategies are also in use to decrease therapeutic phlebotomy loss.<sup>2</sup>

### **Red blood cell transfusion in infants**

Red blood cell transfusions in term and preterm infants are administered to increase oxygen-carrying capacity, and studies involving near infrared spectroscopy (NIRS) have confirmed increased cerebral-mesenteric oxygenation following transfusion.<sup>15,16</sup> Transfusions are more common in extremely low birth weight infants (ELBW, <1000 g) than in most other patient populations, with approximately 90% of such infants requiring some sort of transfusion support.<sup>17,18</sup> Among very low birth weight infants (VLBW, <1500 g), approximately 40–50% require transfusion. Despite the prevalence of anemia in these low birth weight populations, optimal transfusion protocols remain unclear.<sup>19–21</sup>

To help address this issue, two large randomized control trials investigating restrictive versus liberal transfusion thresholds in premature infants have recently been completed. A multicenter study (transfusion of prematures [TOP]) evaluated the effects of liberal (Hct 32–38% in critical and 29–35% in noncritical infants) versus conservative transfusion thresholds (Hct 25–32% in critical and 21–29% in noncritical infants) in 1824 randomized infants <1000 g and <29 weeks, with primary outcomes of death or neurodevelopmental impairment at 22–26 months corrected age.<sup>22</sup> A similar number of infants (423 or 50.1%) in the higher-threshold group died or survived with neurodevelopmental impairment, compared with 422 (49.8%) in the lower-threshold group. The neurodevelopmental data from TOP, with no evidence of a difference at 22–26 months corrected age in infants randomized to liberal versus conservative transfusion thresholds, are similar to those from the recently published ETTNO trial (the Effect of Transfusion Thresholds on Neurocognitive Outcomes of extremely low birth weight infants). ETTNO included 1013 infants in Europe with birth weight <1000 g and reported similar primary outcomes of death or major neurodevelopmental impairment at 24 months corrected age regardless of liberal (Hct 28–41%) or restrictive (Hct 21–34%) transfusion strategy assignment.<sup>23</sup> The results of TOP and ETTNO contrast with those from a prior smaller study (Premature Infants in Need of Transfusion [PINT]).<sup>24,25</sup> PINT included 456 preterm infants under 1000 g and reported a potential cognitive benefit in the liberal transfusion group. One important consideration for both the TOP and ETTNO studies is that neonates were not randomized to the most restrictive Hct values until they were older than 21 days of life. Prior to the TOP and ETTNO results being published, general Hct thresholds for preterm infants were developed using a compilation of existing British, American, Australian, and Canadian practice guidelines;<sup>26</sup> more detailed Hct thresholds by postnatal age have also been published.<sup>21</sup>

In term infants, red blood cell transfusions are most likely to be administered during surgery or while receiving extracorporeal membrane oxygenation (ECMO). In infants facing major surgery, definitive studies are not available to establish the optimal hematocrit. However, it seems reasonable to maintain the hematocrit >30% for major surgery because of the limited ability of the neonate's heart, lungs, and vasculature to compensate for anemia. Additional

factors include the inferior off-loading of oxygen to tissues by the infant's own red cells because of the diminished interaction between fetal hemoglobin and 2,3-DPG plus the developmental impairment of neonatal pulmonary, renal, hepatic, and neurologic function. The amount of anticipated blood loss must be strongly considered in preoperative transfusion decisions. With a likelihood of large blood loss, some physicians might prefer the preoperative hematocrit to be relatively high.

Some studies have documented RBC transfusion volumes as high as 30–40 mL/kg/day in infants and children on ECMO, but it is not clear that these transfusions increase tissue oxygenation.<sup>27</sup> Further, red blood cell and platelet transfusion volume in infants receiving ECMO have been shown to correlate with in-hospital mortality, although this correlation could be confounded by disease severity.<sup>28</sup> One facility that changed their Hct threshold from 40% to 35% for infants receiving ECMO did not find a detrimental impact on patient outcomes.

In infants with severe respiratory disease, such as those requiring high volumes of oxygen with ventilator support, it has historically been customary to maintain the hematocrit at approximately 35–40%—particularly when blood is being drawn frequently for testing. This practice is based on the belief that transfused donor RBCs, containing adult hemoglobin (with increased O<sub>2</sub> availability for a given PaO<sub>2</sub>), will provide optimal oxygen delivery throughout the period of diminished pulmonary function. Consistent with this rationale for ensuring optimal oxygen delivery in neonates with pulmonary failure, a hematocrit near 40% may be maintained in infants with congenital heart disease that is severe enough to cause either cyanosis or congestive heart failure. Following similar logic, it seems reasonable to maintain the hematocrit above 30–35% for moderate cardiopulmonary disease. Overall, however, data on transfusion support for term infants are quite limited, and additional studies would be beneficial to help inform practice.

### **Special considerations regarding RBC transfusion in preterm infants**

Red blood cell transfusion burden has been associated with multiple comorbidities in preterm infants, including necrotizing enterocolitis (NEC), bronchopulmonary dysplasia (BPD), retinopathy of prematurity (ROP), or death.<sup>17,29</sup> This is not without controversy, however, as a large meta-analysis failed to find any such relationship<sup>30</sup> and most recently the TOP trial reported similar secondary outcomes in the higher and lower hemoglobin threshold groups.<sup>24</sup> NEC is among the best studied of these complications, with many studies describing a temporal association between RBC transfusion and NEC<sup>31–38</sup> and with one meta-analysis identifying RBC transfusion as a significant risk factor for NEC.<sup>39</sup> However, other studies, including a prospective study and another meta-analysis, failed to find such an association,<sup>40,41</sup> and a recent GRADE review<sup>42</sup> has questioned the generalizability of previous study results. A multicenter birth cohort study of very low birth weight infants (<1500 g) identified the severity of anemia prior to RBC transfusion, specifically hemoglobin ≤8 g/dL per week, as being an independent risk factor for NEC.<sup>43</sup> These findings are consistent with an animal study.<sup>44</sup> Further, a subgroup analysis of a case-crossover study found that infants with anemia were at greater risk of NEC than infants who were not anemic.<sup>45</sup>

Beyond NEC, one study found early RBC transfusion (in the first 10 days of life) to be an independent risk factor for severe ROP

in preterm infants, independent of gestational age;<sup>46</sup> another found mechanical ventilation and transfusion burden to be associated with ROP.<sup>47</sup> It has been hypothesized that the increase in hemoglobin A (and the decrease in hemoglobin F) associated with RBC transfusion increases oxygen to the retina and plays a contributory role in ROP development.<sup>48</sup> Early anemia and RBC transfusion have also been associated with BPD in some studies.<sup>49</sup>

### Pathophysiology of anemia in children

Anemia in children is most frequently a result of decreased RBC production or ineffective erythropoiesis, secondary to antineoplastic chemotherapy, iron deficiency, chronic kidney disease,<sup>50</sup> thalassemia, and other causes. Blood loss, through trauma or surgery, may also lead to anemia in children. Less frequently, antibody or nonantibody-mediated RBC destruction are the causes of anemia.

### Red blood cell transfusion in children

Transfusions in children are broadly given for the same reasons as in infants, to increase oxygen-carrying capacity. Historically, studies of RBC transfusion thresholds in the pediatric population have lagged behind those in adults. A landmark study published in 2007, the Transfusion Strategies for Patients in Pediatric Intensive Care Units (TRIPICU) study,<sup>51</sup> randomized children in the ICU to receive RBCs at a restrictive (Hgb 7 g/dL) or liberal (>9.5 g/dL) threshold. The restrictive arm was found to have similar rates of new or progressive multisystem organ dysfunction. Thus, the TRIPICU investigators concluded that in stable critically ill children, a hemoglobin threshold of 7 g/dL for red-cell transfusion can decrease transfusion requirements without increasing adverse outcomes. This study, other published guidelines,<sup>52,53</sup> and the recommendations described below support the general pediatric transfusion thresholds shown in Table 34.1.

The Pediatric Critical Care Transfusion and Anemia EXpertise Initiative (TAXI) was formed through the Pediatric Critical Care Blood Research Network (BloodNet) and the Pediatric Acute Lung Injury and Investigators (PALISI) Network to develop evidence-based guidelines for transfusions in children. In 2018, after evaluating the existing evidence, this initiative published a series of guidelines for transfusion in children.<sup>54</sup> General good practice statements included evaluating laboratory values in the setting of the patient's condition and checking hemoglobin values prior to each additional RBC transfusion when feasible. Additional recommendations were separated by types of patient populations and conditions as follows:

- For critically ill children that do not otherwise meet the conditions described below,<sup>55</sup> the TAXI group recommended RBC transfu-

sion if the hemoglobin concentration is <5 g/dL. If the patients were hemodynamically stable, the group recommended not administering an RBC transfusion if the hemoglobin is ≥7 g/dL. The group determined that there was insufficient evidence to make a recommendation when the hemoglobin was between 5 and 7 g/dL.

- For critically ill children with respiratory failure,<sup>56</sup> the group recommended RBC transfusion if the hemoglobin is <5 g/dL. However, if the children with respiratory failure did not have severe acute hypoxemia or other qualifications, the TAXI group recommended not administering RBC transfusions when the hemoglobin is ≥7 g/dL. The appropriateness of transfusions at hemoglobin concentrations between 5 and 7 g/dL was left up to clinical judgement.
- For critically ill children with nonhemorrhagic shock,<sup>57</sup> the group could not recommend a specific RBC transfusion threshold. However, they made a weak recommendation not to administer RBCs for a hemoglobin ≥7 g/dL in children with severe sepsis.
- For critically ill children with non-life-threatening bleeding,<sup>58</sup> TAXI recommended that RBC transfusion should be given for a hemoglobin <5 g/dL and should be considered for a hemoglobin between 5 and 7 g/dL. They also recommended an RBC:plasma:platelet ratio between 2:1:1 and 1:1:1 for critically ill children with hemorrhagic shock.
- For critically ill children with acute brain injury,<sup>59</sup> the group recommended RBC transfusion be considered for hemoglobin values between 7 and 10 g/dL.
- For children with cardiac disease,<sup>60</sup> the TAXI group recommendations were broken down based on patient subgroups. For all such patients, the components contributing to oxygen delivery should be optimized before initiation of RBC transfusion except in cases of hemorrhagic shock. In children undergoing cardiac surgery (repair or palliation) or heart transplants, the group recommended considering not just the hemoglobin but also the overall clinical context. In infants and children with congenital heart disease, TAXI recommended investigating and treating preoperative anemia. In hemodynamically stable children with CHD and adequate oxygenation (for their cardiac lesion) and normal end organ function who are awaiting cardiac surgery, they recommended that the risk/benefit ratio be carefully considered before RBC transfusion was given. The group also commented that there is no evidence that a hemoglobin >10 g/dL is beneficial in children with ventricular dysfunction or in children with a normal heart and idiopathic or acquired pulmonary hypertension. For children with uncorrected heart disease, they recommended maintaining a hemoglobin of 7–9 g/dL. For stable children undergoing biventricular repairs, TAXI recommended not transfusing if the hemoglobin is >7.0 g/dL. For infants and children undergoing staged palliative procedures with stable hemodynamics, the group recommended avoiding transfusions solely based upon hemoglobin if the hemoglobin is >9 g/dL. In all cases, the TAXI recommendations strongly supported the use of intra- and postoperative blood conservation measures.
- For children with sickle cell disease who are critically ill or at risk of critical illness,<sup>61</sup> the group recommended RBC transfusion to a goal of 10 g/dL prior to a surgical procedure requiring general anesthesia. For children with acute chest syndrome who are critically ill and deteriorating, TAXI recommended an exchange transfusion over a simple transfusion when possible; otherwise a

**Table 34.1** Pediatric RBC Transfusion Thresholds

Situation	RBC Transfusion Threshold (Hgb)	Comment
Stable child	<5 g/dL	Always consider transfusion
	5–7 g/dL	Clinical judgement
	>7 g/dL	No transfusion except for some situations (including bleeding, heart disease, and others)
Unstable child	<7 g/dL	Always consider transfusion
	>7 g/dL	Clinical judgment

simple transfusion was recommended. For children with stroke, the group recommended exchange transfusion if possible but did not recommend an optimal postexchange hemoglobin or percent S level.

- For children with cancer or those undergoing hematopoietic stem cell transplantation who are critically ill or at risk of critical illness,<sup>61</sup> the group recommended that a hemoglobin of 7–8 g/dL be considered a threshold for transfusion.
- For critically ill children on extracorporeal membrane oxygenation (ECMO),<sup>62</sup> TAXI recommended reporting hemoglobin concentration rather than hematocrit for transfusion algorithms, that blood sparing measures be adopted, and that physiologic metrics and biomarkers of oxygen delivery be taken into consideration. The group determined that there was insufficient evidence to recommend a specific transfusion threshold. Instead, RBC transfusions should be based on evidence of inadequate cardiorespiratory support or decreased oxygen delivery.

Outside of the critical illness setting, prophylactic RBC transfusions are administered to children with cancer, sickle cell disease, and thalassemia major. The Children's Oncology group updated their Supportive Care Guidelines in 2020,<sup>63</sup> though no specific recommendations were included for RBC transfusion thresholds. The American Society of Hematology published transfusion support guidelines for sickle cell disease in 2020;<sup>64</sup> those guidelines recommend automated RBC exchange transfusion over simple transfusion or manual exchange transfusion when feasible for chronic transfusion therapy (conditional recommendation, low certainty evidence). For children with thalassemia major, there is limited practice guidance and thus a broad range of RBC transfusion practices in the United States, with hemoglobin thresholds ranging from 8 to >10 g/dL in one survey.<sup>65</sup>

Existing studies of RBC transfusion (and other transfusion support) for pediatric trauma resuscitation have been summarized by Kamyszek *et al.*<sup>66</sup> and by Maw *et al.*<sup>67</sup> In brief, optimal transfusion thresholds and RBC:plasma:platelet ratios are not well established for pediatric trauma patients. Furthermore, unlike the situation in adults, pediatric massive transfusion protocols in and of themselves have not improved outcomes in critically ill children. Tranexamic acid is increasingly being utilized in pediatric trauma cases and has been shown to decrease mortality in children under 18 years old in the combat setting.<sup>68</sup> However, at least one study has reported an increased risk of seizures associated with its use.<sup>69</sup>

### Red blood cell product selection for infants and children

Beyond matching for ABO and Rh compatibility, storage solution and storage duration are variables to consider in RBC selection for infants and children. While Chapter 14 describes the history of storage and additive solution development, an entire chapter could be written on the history on optimization of storage solutions for transfusion of infants and children. The short summary is that most pediatric centers in the United States now utilize RBCs stored in additive solutions, and these products are presumed safe for “small-volume” (<10–15 mL/kg) RBC transfusions.<sup>70–72</sup> The amount of potassium infused during an RBC transfusion into small infants has been the subject of much debate over the years, with the consensus being that the actual amount of extracellular

(plasma) potassium infused during “small-volume” transfusions is relatively minimal. For example, an infant weighing 1 kg given a 15 mL/kg transfusion of RBCs at an Hct of 55–60% stored for 42 days in additive solution receives approximately 0.3 mEq total of potassium; these doses have been shown to be safe in infants.<sup>71</sup> Historically some centers used RBCs stored in CPDA-1, given the higher Hct of these units. However, CPDA-1 units contain more plasma and as such may at least theoretically be associated with more transfusion reactions. Further, due to a low demand nationwide, it has become more difficult for blood centers to maintain adequate production of CPDA-1 units.

From the perspective of component modification, irradiation is used to prevent transfusion associated graft versus host disease in at-risk pediatric patients. Given that infants may have undiagnosed immunodeficiencies,<sup>73</sup> some institutions irradiate all cellular products transfused to neonates and infants.<sup>74</sup> Other institutions selectively irradiate products transfused to neonates weighing less than 1200 g. However, the 2020 Guidelines on Irradiation from the British Society for Haematology Guidelines Transfusion Task Force state that routine irradiation is not required for cellular products transfused to preterm or term infants unless those infants received intrauterine transfusions.<sup>75</sup>

There are several specific subgroups of patients where irradiation is noncontroversial. Neonates or children with known or suspected cellular immunodeficiencies, undergoing bone marrow transplantation, and/or receiving directed donor products, require irradiated cellular blood products.<sup>74,76</sup> Children with certain malignancies (including Hodgkin disease) require irradiated cellular blood products, and those receiving intensive chemotherapy often receive irradiated cellular blood products as well. The impact of the post-irradiation storage period on outcomes in infants and children has not been studied in detail,<sup>77</sup> though metabolomic changes are evident in the RBC unit.<sup>78</sup> The British Society Guidelines recommend that RBCs be transfused within 24 hours of irradiation if the fetal/neonatal recipient is at risk for hyperkalemia (e.g., for large-volume transfusion, exchange transfusion, or intrauterine transfusion).<sup>75</sup>

A more common component modification, leukoreduction, is effective at decreasing HLA alloimmunization, febrile transfusion reactions, and transmission of viruses harbored in WBCs.<sup>79</sup> Thus, leukoreduced cellular products are typically recommended for transfusion to infants and children.<sup>63</sup> Leukoreduced products are also considered to be “CMV reduced risk.”<sup>80–85</sup> A birth cohort study of very low birth weight infants investigated the effectiveness of the so-called “belt and suspenders” approach of preventing transfusion transmitted CMV infection. In this study, leukoreduced components obtained from CMV-seronegative donors were found to be extremely effective at preventing CMV transmission through transfusion; almost all documented cases of postnatal CMV infection were attributable to virus in maternal breast milk.<sup>86</sup>

Historically, pediatric clinicians have been attentive to the storage time of units prior to transfusion. To address the importance of this variable, two randomized studies have evaluated the impact of storage duration on outcomes in infants and children, both with similar conclusions. The Age of Red Blood Cells in Premature Infants (ARIPI) study found no difference in the primary end point (a composite end point of major morbidities) in premature infants <1250 g transfused with fresher (<7-day-old) or standard issue (mean 14.6-day-old) RBCs. The ARIPI study sites used a relatively

liberal transfusion threshold, however (mean pretransfusion Hb 8.7–10.7 g/dL). Thus, it is unclear whether these results can be extrapolated to premature infants transfused at more conservative transfusion thresholds or to RBCs stored for up to 28 days post-irradiation. A biotin-labeled allogeneic RBC study in infants found that the slope of 24-hour post-transfusion recovery did not decrease with increasing storage duration, suggesting that the survival of transfused RBCs in circulation was not affected by storage duration.<sup>87</sup> The Age of Transfused Blood in Critically Ill children (ABC-PICU) study also found no difference in new or progressive multiorgan dysfunction (including mortality) in children in the ICU receiving fresh (median 5-day-old) versus standard issue (median 18-day-old) RBCs.<sup>88</sup>

Of note, nontransferrin bound iron has been shown to increase by three hours following RBC transfusion in preterm infants<sup>89</sup> and to remain increased for up to 24 hours.<sup>90</sup> Inflammatory cytokines (IL-1 $\beta$ , IL-8, TNF-alpha, and MCP-1) and measures of endothelial activation (increased macrophage inhibitory factor and soluble intracellular adhesion markers) have also been shown to increase in such infants post-transfusion.<sup>91–93</sup> A 2017 study demonstrated that very low birth weight infants are more likely to show increased levels of nontransferrin bound iron after RBC transfusion than are healthy adult volunteers. For example, when transfused with RBCs stored for 20 or more days, nontransferrin bound iron increased in preterm infants but not in adults. The adult transfusion volunteers did not experience an increase in nontransferrin bound iron until RBCs had been stored 35–42 days prior to transfusion.<sup>94</sup> Regardless of storage duration, these very low birth weight infants also had increased levels of serum iron, bilirubin, and MCP-1 post-RBC transfusion. Thus, very low birth weight infants seem to have a unique inability (compared to term infants, children, or adults) to respond to iron release from macrophages following consumption of transfused RBCs, presumably due to impaired hepcidin responses and low transferrin.<sup>95,96</sup> The long-term consequences of these findings remain under investigation.

## Platelet transfusion

### Pathophysiology of thrombocytopenia in infants

Platelet counts in developing fetuses are typically higher than 150,000/ $\mu$ L by the end of the first trimester,<sup>97,98</sup> and most healthy newborns have counts above this level at term.<sup>99</sup> However, 1–5% of all neonates are affected by thrombocytopenia, with platelet counts lower than 150,000/ $\mu$ L, although the prevalence varies by study.<sup>100–102</sup> Preterm infants and those born small for gestational age are more likely to be affected by thrombocytopenia than term infants,<sup>101</sup> with up to 25% of all neonates admitted to neonatal intensive care units (NICUs) having mild, moderate, or severe thrombocytopenia at some point during their hospital stay. Babies in the NICU with extremely low birth weights are quite likely to have low platelet counts, with 73% of infants weighing under 1000 g reported to have platelet counts lower than 150,000/ $\mu$ L.<sup>101</sup>

Neonatal thrombocytopenia can be caused by decreased platelet production, increased platelet destruction, or a combination of these. The pathophysiology of neonatal thrombocytopenia noted at or soon after birth may also be due in part to maternal hypertension, placental insufficiency, and/or perinatal hypoxemia;<sup>103</sup> such conditions are more likely to be present in preterm than term infants. Perinatal infections such as Group B Streptococcus or *Escherichia*

*coli* may also be associated with early neonatal thrombocytopenia as may congenital infections such as toxoplasmosis, rubella, CMV, or HSV.<sup>103</sup> Thrombocytopenia that develops after the first few days of life may be due to postnatally acquired sepsis, disseminated intravascular coagulopathy (DIC), or necrotizing enterocolitis (NEC).<sup>102</sup>

Unique to neonates, transplacental transfer of maternal alloantibodies may contribute to thrombocytopenia in otherwise “well-appearing” infants.<sup>104</sup> Maternal antibodies against human leukocyte antigens (HLA) as well as human platelet-specific glycoprotein antigens (HPA) are capable of crossing the placenta, binding to the platelets of fetuses, and producing neonatal alloimmune thrombocytopenic purpura (NAITP).<sup>105,106</sup> Antibodies against HPA-1a are the most well-known causes of NAITP in Caucasians, with women lacking the HPA-1a antigen and expressing the HLA DRB3\*0101 being at particularly high risk of developing these antibodies during pregnancy with HPA-1a expressing fetuses.<sup>106</sup> Antibodies against HPA-5b, HPA-15a, or other HPA proteins may also lead to NAITP.<sup>107</sup> Rarely, maternal autoantibodies such as those found in mothers with ITP or other autoimmune diseases like SLE may also lead to neonatal thrombocytopenia.<sup>108</sup> Thus, it is prudent to check the maternal history and the maternal platelet count in instances of unexpected neonatal thrombocytopenia.

Platelet dysfunction may also contribute to bleeding in infants and children, with etiologies including those that are congenital (such as in Glanzmann's thrombasthenia or Bernard Soulier) or medication-induced. For example, medications given to mothers prior to delivery, including Ketorolac,<sup>109</sup> can potentially impact neonatal platelet function. Medications used in NICUs, including indomethacin and nitric oxide, may also impact platelet function.

### Platelet transfusion in infants

Historically, neonatal platelet transfusion guidelines have been based largely on consensus.<sup>110,111</sup> A randomized trial of premature infants (<1500 g) in their first week of life, completed more than 25 years ago, demonstrated that a liberal pretransfusion threshold of 150,000/ $\mu$ L was not associated with fewer bleeding episodes than a more conservative transfusion threshold of 50,000/ $\mu$ L.<sup>112</sup> With those data in mind, some institutions have historically prophylactically transfused stable term and preterm infants at platelet counts of 25,000 and 30,000/ $\mu$ L, respectively,<sup>113</sup> whereas others have transfused stable infants at platelet counts of 50,000/ $\mu$ L. Table 34.2 lists potential guidelines for neonatal platelet transfusions.

In 2019, the landmark Platelets for Neonatal Transfusion-Study 2 (PlaNeT-2),<sup>114</sup> a randomized trial comparing preterm infants (median gestation age 26.6 weeks and median birth weight 740 g) receiving prophylactic platelet transfusions to maintain counts

**Table 34.2** Neonatal Platelet Transfusion Thresholds

Situation	Platelet Transfusion Threshold
Clinically stable term neonate	20,000–25,000/ $\mu$ L
Clinically stable preterm neonate	25,000–50,000/ $\mu$ L*
Clinically unstable neonate or bleeding neonate	50,000–100,000/ $\mu$ L
Need for invasive procedure	50,000/ $\mu$ L
NAITP	30,000–50,000/ $\mu$ L#
ECMO	50,000–100,000/ $\mu$ L#

\*Two studies found worse outcomes at higher thresholds

#Transfusion threshold dependent on past and present bleeding

above 25,000 or above 50,000/ $\mu\text{L}$ , reported a lower mortality and less intracranial bleeding in the restrictive arm. A subanalysis reported that these outcomes occurred in infants at both lower or higher baseline risk, with the authors suggesting that a threshold of 25,000/ $\mu\text{L}$  could be safely adopted in all preterm infants.<sup>115</sup>

A retrospective study of newborns <32 weeks of gestational age born in two different cities showed no difference in intracranial hemorrhage rates between neonates transfused with restrictive (transfusion for platelet count less than 50,000/ $\mu\text{L}$  in bleeding or sick neonates), compared to liberal (transfusion according to institutional guidelines) criteria.<sup>116</sup> Another study of preterm, low birth weight infants (<1500 g) demonstrated that more restrictive transfusion thresholds (transfusion for platelet count less than 50,000/ $\mu\text{L}$  in sick infants or less than 25,000/ $\mu\text{L}$  in stable infants) were associated with no greater bleeding than more liberal thresholds.<sup>117</sup> Furthermore, a 2009 study showed there is no clear relationship between platelet count and major intracranial hemorrhage; more than 91% of neonates with platelet counts under 20,000/ $\mu\text{L}$  demonstrating no bleeding.<sup>118</sup>

Two neonatal subpopulations worthy of additional discussion include those on ECMO and those with NAITP. Babies on ECMO are at risk of bleeding due in part to thrombocytopenia secondary to consumption, platelet dysfunction, and heparinization of the circuit. Thus, platelet transfusion thresholds between 50,000 and 100,000/ $\mu\text{L}$  are typically utilized in these babies, dependent on bleeding status.<sup>119,120</sup> Neonates with NAITP are also at high risk of bleeding. Babies with NAITP and a documented intracranial bleed should be maintained at platelet counts above 50,000/ $\mu\text{L}$  for at least the first week of life. Neonates with NAITP and no bleeding are typically maintained at platelet counts above 30,000/ $\mu\text{L}$  and often higher for the first week of life. Random donor ("off the shelf") platelets may be transfused for babies with NAITP and will typically increase the neonate's platelet count at least transiently.<sup>104,121,122</sup> Platelets lacking HPA-1a, HPA-5b, or other offending antigens may be requested from blood donor centers for neonates thought to have NAITP mediated by these respective antibodies, though emergent transfusions should never be withheld while antigen-negative platelets are being located. Maternal platelets are another therapeutic option, yet they may be difficult to obtain and they *must* be washed to remove offending antibodies and irradiated prior to transfusion.<sup>105</sup> IVIG may be given to affected neonates in an attempt to increase the platelet circulatory half-life; it is also administered during pregnancy to women carrying fetuses at risk for NAITP, with dose and schedule determined by risk stratification.<sup>122–124</sup>

### **Pathophysiology of thrombocytopenia in children**

Thrombocytopenia in children is typically caused by decreased production, increased destruction, or sequestration of platelets. Dilutional thrombocytopenia can also occur in situations of massive transfusion.

Hypoproliferative thrombocytopenia occurs in children treated with chemotherapy or radiation, with most platelet transfusions in childhood given in these patient populations. A subset of antiepileptic drugs, along with drugs in other categories, may decrease platelet production in children. Myelodysplasia, marrow infiltrative processes, and aplastic anemia also lead to decreased platelet production. Lastly, hereditary bone marrow failure syndromes and other congenital disorders such as Wiscott Aldrich Syndrome can lead to impaired platelet production.

The most common cause of destructive thrombocytopenia in children is idiopathic thrombocytopenic purpura (ITP). Most

children who present for medical care with acute ITP have isolated thrombocytopenia with platelet counts lower than 20,000/ $\mu\text{L}$  and may have the abrupt onset of bruising and bleeding. Platelet count alone has not been shown to predict bleeding severity in ITP.<sup>125</sup> Intracranial hemorrhage is a rare but life-threatening complication of acute ITP,<sup>126</sup> occurring in less than 1% of cases. A 2009 study of 40 children with ITP and intracranial hemorrhage showed that 90% had a platelet count lower than 20,000/ $\mu\text{L}$ , 33% had head trauma, and 22% had hematuria.<sup>127</sup>

Other destructive causes of thrombocytopenia in children include chronic ITP, autoimmune diseases, heparin induced thrombocytopenia, HIV, post-transplant thrombocytopenia, post-transfusion purpura, and neoplasm-associated immune thrombocytopenia. Nonimmune causes of destructive thrombocytopenias in children include hemophagocytic syndromes, Kasabach–Merritt syndrome, DIC, infectious etiologies, congenital heart disease, type 2B vWD, and platelet-type vWD. Thrombotic microangiopathies including HUS, TTP, and drug or bone marrow transplant-associated microangiopathy also occur in children.

Mild thrombocytopenia may occur in children with hypersplenism or splenic sequestration, although platelet counts rarely drop below 50,000/ $\mu\text{L}$  in these patients. Thrombocytopenia may also be associated with liver disease. Rarely, laboratory artifacts may lead to "spurious" thrombocytopenia (e.g., platelet counts reported to be low by automated counters, but not truly low); the main causes of such incorrect platelet counts include EDTA-dependent antibodies, cold agglutinins, and certain medications.

### **Platelet transfusion in children**

As with transfusions in infants or adults, platelet transfusions in children may be given: (1) in the setting of an acute hemorrhage or (2) as prophylaxis to decrease bleeding in at-risk patients. Thresholds for both situations continue to be debated, however. Young children with treatment-associated hypoproliferative thrombocytopenia may be at higher risk for bleeding than adults,<sup>128,129</sup> with factors in addition to low platelet counts likely contributing to this risk. These factors may include chemotherapy intensity as well as functional differences in interactions between the vascular endothelium and platelets.<sup>128</sup>

### **Platelet transfusion in children with active bleeding**

Platelet transfusion should be considered in any pediatric patient with thrombocytopenia and active bleeding, with transfusion thresholds being situation dependent (Table 34.3). Patients with

**Table 34.3** Pediatric Platelet Transfusion Threshold

Situation	Platelet Transfusion Threshold
Bleeding patient	Situation dependent
Bleeding on ECMO or during surgery	50,000–100,000/ $\mu\text{L}$
Prophylaxis for hypoproliferative thrombocytopenia	10,000/ $\mu\text{L}$
Prophylaxis for line placement	20,000/ $\mu\text{L}$
Prophylaxis for lumbar puncture	50,000/ $\mu\text{L}$ #
Prophylaxis for major surgery	50,000/ $\mu\text{L}$ %
Platelet dysfunction with bleeding or in need of invasive procedure	Not applicable

#May consider applying a higher threshold when circulating blasts are present

%May consider applying a higher threshold for CNS surgery

congenital platelet disorders and active bleeding may also benefit from platelet transfusions in addition to adjunctive therapies, regardless of platelet count.

Children with hypoproliferative thrombocytopenia from chemotherapy or radiation, those undergoing bone marrow transplantation, those on ECMO, or those undergoing surgery who are bleeding may also benefit from platelet transfusions. Children with ITP and acute, life-threatening bleeding should receive 1–2 doses of platelets (with a dose defined as 5–10 mL/kg) in addition to 0.8–1 g/kg of IVIG and 30 mg/kg of methylprednisolone.<sup>130–132</sup> Repeat platelet dosing may be needed due to rapid antibody-mediated removal post-transfusion. Rarely, emergent splenectomy is also considered in this setting. Though platelet transfusion thresholds are not applicable and alloimmunization may result, platelet transfusions may be effective at controlling bleeding in children with Glanzmann's thrombasthenia, Bernard Soulier, and other congenital causes of severe platelet dysfunction.<sup>133,134</sup> However, other treatments including topical therapies, antifibrinolytics, desmopressin, or recombinant Factor VIIa may also be used in some of these disorders.

### **Prophylactic platelet transfusion in children**

The 2015 AABB guidelines recommend that adult patients with hypoproliferative thrombocytopenia be transfused at a platelet count of 10,000/µL to prevent spontaneous bleeding<sup>135</sup> consistent with the 2018 American Society of Clinical Oncology guidelines for adults and children<sup>136</sup> and consistent with the 2020 Children's Oncology Group's Supportive Care Guidelines.<sup>63</sup> Despite these guidelines, some degree of equipoise exists regarding platelet transfusion thresholds for children with hypoproliferative thrombocytopenia.<sup>137,138</sup> A single institution study evaluating transfusion practice over 10 years showed the median platelet count for which pediatric oncology patients with hypoproliferative thrombocytopenia were transfused was 16,000/µL.<sup>139</sup> A 2013 survey of Children's Oncology Group Stem Cell Transplant Directors showed that 69% of institutions transfused nontransplant oncology patients for platelet counts below 10,000/µL, with 27% of institutions transfusing these patients for platelet counts below 15,000/µL.<sup>138</sup> This survey also found that transfusion thresholds for stem cell transplant patients were higher: 47% of institutions prophylactically transfused their transplant patients for platelet counts below 10,000/µL and 44% of institutions prophylactically transfused for platelet counts below 20,000/µL.<sup>138</sup>

Of the multiple large transfusion studies performed to date on patients with hypoproliferative thrombocytopenia, only one (Optimal Platelet Dose Strategy to Prevent Bleeding in Thrombocytopenia Patients [PLADO]) has included a separate pediatric analysis.<sup>140</sup> PLADO enrolled 200 pediatric patients from 0 to 18 years of age<sup>128</sup> and was designed to evaluate the relationship between platelet transfusion dose and bleeding. PLADO showed that platelet dose did not predict bleeding in pediatric patients. Of note, the children in the study had a higher risk of World Health Organization grade 2 or greater bleeding than the adults. Additionally, the children in this study had more days of grade 2 or higher bleeding than adults, with a median of three days (compared to one day in adults). Bleeding was most pronounced in the pediatric patients undergoing stem cell transplantation. Taken in combination, these results emphasize that factors beyond platelet counts impact bleeding risk.

Children with sickle cell disease undergoing stem cell transplantation are at risk of CNS bleeding<sup>141,142</sup> given vascular issues, prior CVAs, and transplant-associated hypertension. The prophylactic platelet transfusion threshold for this patient population has thus been set higher than that of other pediatric transplant patients;<sup>141,143</sup> many centers now use a transfusion threshold of 30,000/µL.<sup>144</sup>

Adult guidelines for platelet transfusion that are reasonable to translate to pediatric patients include transfusion for platelet counts less than 20,000/µL prior to central line placement and for platelet counts less than 50,000/µL prior to non-CNS surgeries.<sup>145</sup> The 2015 AABB guidelines recommend transfusing platelets for adult patient with platelet counts less than 50,000/µL prior to lumbar puncture; extrapolating these guidelines to lumbar punctures in children *without circulating blasts* is reasonable. It must be noted, however, that traumatic lumbar punctures in children with circulating blasts have been shown to decrease event-free survival from 7 to 17%.<sup>146–148</sup> Two studies have identified a platelet count under 100,000/µL as a risk factor for a traumatic lumbar puncture in pediatric leukemia patients.<sup>149,150</sup> As such, the optimal prelumbar puncture platelet count for pediatric patients with circulating blasts remains unclear.<sup>151</sup>

Prophylactic transfusions of functional platelets in the peri-surgery or peri-partum period may be indicated in patients with congenital or acquired platelet function defects; adjunctive therapies are often necessary as well. Careful care coordination and treatment planning between multiple specialties is necessary in these cases, to minimize the likelihood of operative or delayed postoperative bleeding. Conditions in which platelet transfusions should generally be avoided include those associated with consumptive coagulopathies such as TTP and HUS.

### **Platelet product selection for infants and children**

Platelets chosen for transfusion to infants and children in the United States may be from apheresis or whole blood donors, with many centers preferentially utilizing apheresis platelets. ABO compatible or identical platelets are ideally selected for transfusion into pediatric patients, in order to (1) minimize the passive transfer of incompatible plasma,<sup>152,153</sup> (2) minimize the destruction of platelets expressing incompatible antigens,<sup>154</sup> and (3) minimize transfusion reaction rates (including febrile and allergic reactions).<sup>155</sup> An additional consideration in neonates is the need for chosen platelets to be compatible with maternally derived isoantibodies that may be transiently present in the neonate's circulation. The same irradiation and leukoreduction indications reviewed in the RBC section also apply to platelets; HLA-matched or crossmatched platelets must always be irradiated. Pathogen reduction of platelets also inactivates T-cells, and thus pathogen-reduced platelets do not require irradiation. Of note, no increased rates of transfusion reactions have been reported in children transfused with pathogen-reduced platelets, though increased platelet utilization has been described.<sup>156</sup> However, a paucity of data currently exists regarding outcomes of preterm neonates transfused with pathogen-reduced platelets. Transfusion of Rh(D) positive platelet products into Rh(D) negative female children should be avoided when possible. Although the likelihood of an Rh(D) negative patient forming an anti-D after exposure to low amounts of residual RBCs in an Rh(D) positive apheresis platelet unit is extremely small,<sup>157–160</sup> prophylaxis with RhIg is appropriate in female children.<sup>136</sup> A 300 µg dose of RhIg can suppress immunization to 15 mL of packed RBCs.<sup>161</sup>

5–10 mL/kg of platelets are typically infused as a single “dose,” with a maximum dose regardless of weight typically being 1 apheresis unit or 1 “pool” of whole-blood-derived platelets (from 4 to 8 donors). Some centers provide fractions of apheresis platelet units in a weight-based fashion (e.g., 5–10 mL/kg to infants,  $\frac{1}{4}$  apheresis unit to children <15 kg,  $\frac{1}{2}$  apheresis unit to children between 15 and 30 kg, and a whole apheresis unit to children >30 kg), and others provide platelets derived from a single whole blood unit for every 10 kg of body weight. Small volumes of platelets are often transferred prior to transfusion from parent platelet units into syringes, with these aliquots retaining acceptable in vitro characteristics for up to six hours after transfer.<sup>162–164</sup> Volume reduction is not typically recommended, given the loss and/or activation of platelets that occurs during this modification.<sup>165</sup> Transfusion rates vary based on recipient condition, but each dose must be fully infused within four hours of being released from the blood bank. Post-transfusion platelet increases are dependent in part on the platelet count of the transfused unit, the recipient’s condition, the degree of donor/recipient ABO matching, and whether the unit was pathogen-reduced or not.

### Transfusion reactions in the pediatric population

In addition to infectious complications of transfusion, other noninfectious hazards of transfusion should also be considered.<sup>79</sup> Allergic transfusion reactions are relatively common in children compared with adults, and platelets are the most common component to cause such reactions. A 2015 study at a single institution found that 6.2/1000 pediatric transfusions were associated with a reported transfusions reaction compared to 2.4/1000 adult transfusions, with platelets being the most likely blood product to result in a transfusion reaction. 2.7/1000 pediatric transfusions (vs. 1.1/1000 adult transfusions) were associated with an allergic transfusion reaction, while 1.9/1000 pediatric transfusions (vs. 0.47/1000 adult transfusions) were associated with a febrile nonhemolytic transfusion reaction.<sup>166</sup> Another study evaluated composite data from multiple pediatric and adult US hospitals over a seven-year study period and reported an incidence of allergic transfusion reactions in children to be 3.23/1000, compared to 0.72/1000 in adults.<sup>167</sup> A final study reviewed the charts of pediatric patients at a single institution being transfused with platelets and found that 116/805 platelet transfusions were associated with a potential acute transfusion reaction; however, only 4 of these 116 potential reactions were reported to the hospital transfusion service.<sup>168</sup> In combination, these studies suggest that transfusion reaction rates in pediatric patients are likely higher than previously appreciated, with additional studies being warranted.

### Plasma transfusion

#### Development of the coagulation system

Quantitative and qualitative differences in coagulation factors, coagulation inhibitors, and fibrinolytic proteins exist in neonates compared to older children and adults. At one day of age, vitamin-K-dependent coagulation factors (II, VII, IX, and X) and the contact factors (XII, XI, high-molecular-weight-kininogen, and

prekallikrein) are 30% or more below the levels typically seen in adults.<sup>169,170</sup> By six months of age, levels of these coagulation factors in both premature and full-term infants are within normal adult ranges. On the other hand, levels of fibrinogen, FV, FVIII, and FXIII, and vWF are above 70% of adult values on the first day of life in both premature and full-term infants. Fibrinogen levels are lower in premature infants than in full-term infants. Inhibitors of coagulation (AT, protein C, and protein S) are also lower in infants than adults. In the fibrinolytic system, plasminogen levels are lower in infants than adults and significantly lower in preterm than term infants.<sup>171</sup>

Taken together, these alterations in factor concentrations affect the functioning of the coagulation system as well as standard laboratory coagulation tests. The activated partial thromboplastin time (aPTT) is most prolonged in premature and full-term infants on Day 1 of life as compared to adults, being 1.4–2.4 times and 1.2–1.5 times longer, respectively.<sup>171</sup> This prolongation has been attributed to the quantitative and qualitative deficiencies of contact factors. In premature and full-term infants, aPTT values normalize to adult values by six and three months of age, respectively. The prothrombin time (PT) is less prolonged in neonates, ranging from being not significantly different from adult values to being 1.15–1.3 times longer, depending on the study.<sup>171</sup> All diagnoses and treatment decisions must be made within the context of these age-dependent ranges.<sup>172</sup>

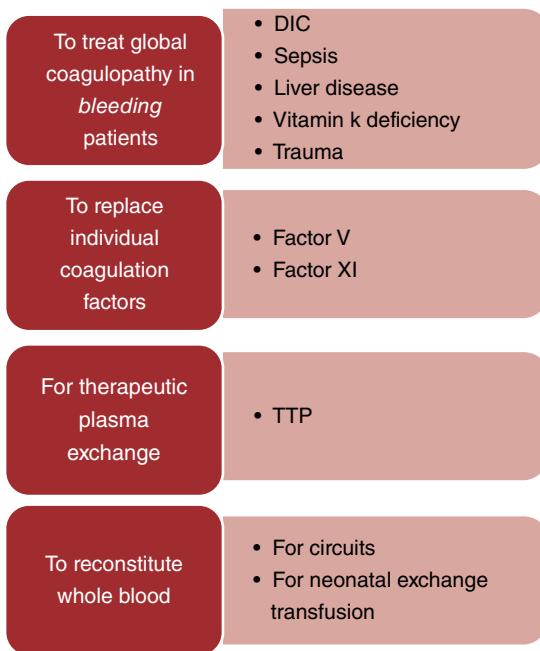
#### Plasma transfusion in infants and children

Plasma is one of the most inappropriately utilized blood products in pediatric transfusion. A 2014 study of multiple NICUs in Italy reported that 60% of 609 plasma transfusions given to neonates were noncompliant with published recommendations.<sup>174</sup> Other studies and surveys have also shown relatively high rates of plasma transfusion to nonbleeding infants and children despite limited evidence of efficacy.<sup>174–178</sup> The magnitude of this problem becomes apparent when one considers that 2.85% of children admitted to hospitals are transfused with plasma, based on a 2012 study of three million admissions of infants and children.<sup>173</sup>

Drawing on experience, past publications,<sup>76,179</sup> and adult guidelines,<sup>180</sup> Figure 34.1 lists acceptable plasma transfusion indications in infants and children. Generally, plasma may be used in infants and children to (1) treat global coagulopathy, (2) replace individual coagulation factors, (3) treat diseases such as TTP requiring therapeutic plasma exchange, and (4) to reconstitute whole blood for neonatal exchange transfusions. Other reasons for plasma transfusion are rarely supportable.

#### Plasma to treat global coagulopathy

Causes of coagulopathy in infants and children include DIC, liver disease, trauma, and dilutional effects. With the understanding that treatment of the underlying cause of DIC is essential, plasma may be transfused to mitigate DIC-associated bleeding. Plasma is also indicated for liver failure with active bleeding and prior to invasive procedures, though the hemostasis observed after FFP infusion is typically quite transient. Although vitamin K is the first-line therapy for neonates with congenital or acquired vitamin K deficiency, plasma or prothrombin complex concentrates are also indicated in cases of life-threatening bleeding in such patients.<sup>171,181,182</sup> Trauma-associated coagulopathy has been demonstrated in children



**Figure 34.1** Considerations for Plasma Transfusion in Infants and Children. Source: Based on Roback *et al.*<sup>180</sup>

independent of dilutional effects and is associated with adverse outcomes.<sup>183</sup> Pediatric massive transfusion protocols typically treat trauma and dilutional coagulopathy by providing plasma, cryoprecipitate, and platelets for resuscitation, in addition to RBCs, as described earlier in this chapter.<sup>67</sup> Infants with hypoxic ischemic encephalopathy treated with therapeutic hypothermia can also benefit from plasma transfusion to treat their well-described coagulopathy.<sup>184</sup> In contrast to the above clinical scenarios, empiric infusion of plasma is not recommended to correct an elevated INR in the absence of bleeding.<sup>185,186</sup>

#### Plasma to replace individual coagulation factors

Plasma is indicated for replacement of coagulation factors in situations where specific factor concentrates are not available.<sup>185</sup> If a child has a suspected congenital bleeding deficiency but the etiology is not apparent, initial therapy with 10–15 mL/kg of plasma is reasonable while awaiting definitive factor testing.<sup>171</sup> Plasma remains the treatment of choice in the United States for the replacement of Factors V and XI,<sup>182,187,188</sup> though two Factor XI concentrates are available outside the United States.<sup>189,190</sup> Reviewed in more detail in Chapters 37, the administration of recombinant or virally inactivated plasma-derived Factors VII, VIII, IX, XIII, vWF, and fibrinogen have replaced the use for plasma or cryoprecipitate in patients congenitally deficient in these factors.

#### Plasma as a replacement fluid for therapeutic plasma exchange

Plasma is typically used as a replacement fluid in therapeutic plasma exchange procedures for children with acquired TTP.<sup>191,192</sup>

The act of removing the patient's plasma reduces levels of IgG autoantibodies against ADAMTS13, while the plasma components used for replacement replenish the ADAMTS13 cleaving enzyme.

#### Plasma to reconstitute whole blood (for circuits or neonatal exchange transfusions)

Transfusion support of pediatric cardiothoracic surgery patients is complex, with dual risks of thrombosis and bleeding. RBCs, fresh whole blood, plasma in combination with RBCs, or nonblood products are used to prime cardiothoracic surgery circuits.<sup>193</sup> Studies show conflicting data with regard to which components in a prime result in less bleeding, fewer transfusion requirements, and better outcomes.<sup>194–197</sup>

Similarly, the priming and maintenance of ECMO circuits, which are used to treat a number of pediatric conditions nonresponsive to traditional ventilatory support,<sup>119</sup> are also complicated with some institutions combining albumin, RBCs, and/or other additives for this purpose.<sup>74</sup> Plasma can be transfused to children on ECMO for bleeding and/or elevated INR.<sup>120,198</sup> A protocol that includes Factor Xa monitoring, thromboelastography, and antithrombin monitoring, in addition to traditional coagulation testing, has been shown to decrease bleeding and transfusion requirements, while increasing circuit life in pediatric ECMO patients.<sup>199</sup>

Neonatal exchange transfusions are typically performed using group O RBCs (lacking offending cognate minor antigens), reconstituted with AB plasma to an Hct of 50–55%. The primary indication for exchange transfusion in neonates, reviewed in detail in Chapter 33, is hemolytic disease of the fetus and newborn with antibody-mediated hemolysis and hyperbilirubinemia.<sup>200</sup>

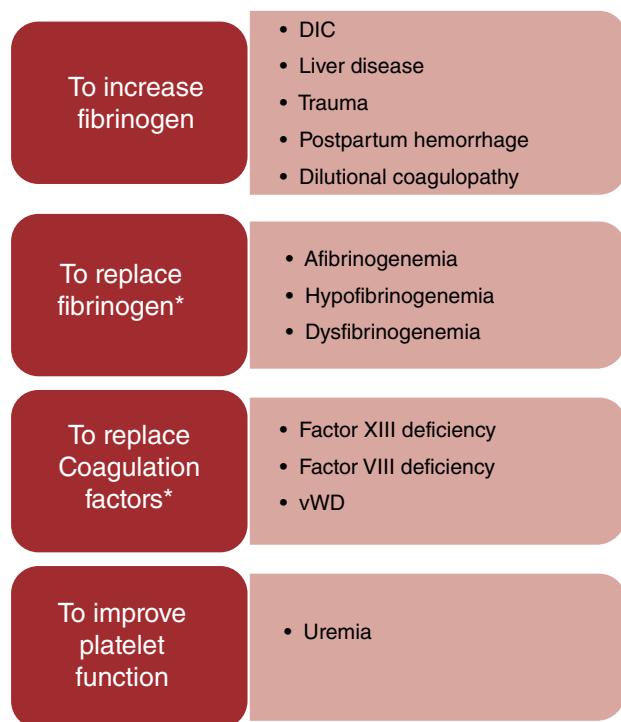
#### Plasma product selection for infants and children

FFP is the proper name for plasma frozen to  $-18^{\circ}\text{C}$  within 6–8 hours of phlebotomy, while PF24 is used to denote plasma frozen within 24 hours of phlebotomy. These products can be used interchangeably, unless single Factor V or VIII replacement is needed.<sup>201,202</sup> Thawed plasma is a term used to describe FFP or PF24 stored at  $1\text{--}6^{\circ}\text{C}$  for up to four days since these products show degradation of coagulation factors over time. This product may be useful in situations such as traumas, in which plasma is rapidly needed;<sup>203</sup> however, few pediatric studies have been completed involving thawed plasma. Plasma cryoprecipitate reduced (also known as cryo-poor plasma) is utilized in some centers as a second-line product and in others as a product equivalent to FFP or PF24 for TTP plasmapheresis.

Plasma chosen for transfusion should ideally be ABO compatible, though Rh(D) matching is not required. Plasma is considered an acellular product and thus irradiation is not necessary. 10–15 mL/kg of plasma typically raises coagulation factors by approximately 30%.

#### Cryoprecipitate transfusion

Cryoprecipitate is made from the cold insoluble high-molecular-weight proteins removed from thawed plasma, including fibrinogen, Factor VIII, Factor XIII, vWF, and fibronectin. Each individual bag of cryoprecipitate must include 80 IU of Factor VIII and 150 mg of fibrinogen, typically in 5–20 mL of plasma.<sup>201</sup> Many blood manufacturers prepool 2–10 individual bags of cryoprecipitate for transfusion convenience.



**Figure 34.2** Considerations for Cryoprecipitate Transfusion in Infants and Children

\* If recombinant or human-derived concentrates are not available

Platelets for infants	• 5–10 mL/kg
Platelets for children	<ul style="list-style-type: none"> <li>• 5–10 mL/kg*</li> <li>• Fractions of apheresis units may be ordered:           <ul style="list-style-type: none"> <li>• <math>\frac{1}{4}</math> apheresis unit if 10–15 kg</li> <li>• <math>\frac{1}{2}</math> apheresis unit if 15–30 kg</li> <li>• 1 apheresis unit if &gt;30 kg</li> </ul> </li> </ul>
Plasma for infants and children	• 10–15 mL/kg
Cryoprecipitate for infants and children	• 2–3 mL/kg

\* Higher platelet doses increase the length of time between transfusion episodes in patients with hypoproliferative thrombocytopenia, but have not been shown to decrease bleeding rates

**Figure 34.3** Platelet, Plasma, and Cryoprecipitate Dosing for Infants and Children. Source: Based on Poterjoy & Josephson<sup>177</sup> and Fasano & Luban.<sup>204</sup>

Cryoprecipitate may be used to increase fibrinogen levels in infants and children with hypofibrinogenemia and hemorrhage due to liver disease or DIC<sup>204</sup> (Figure 34.2). Cryoprecipitate may also be used to increase fibrinogen levels in older adolescents/young women with postpartum hemorrhage<sup>205</sup> and to replenish fibrinogen levels in pediatric trauma patients, a subset of whom have been reported to have trauma-associated hypofibrinogenemia independent of dilutional coagulopathy.<sup>185</sup> In addition, cryoprecipitate can be

used in combination with FFP to replace fibrinogen plus coagulation factors in instances of dilutional coagulopathy. Cryoprecipitate has also been used as a second-line therapy to treat uremia associated bleeding.<sup>206</sup>

While cryoprecipitate has historically been used to treat bleeding or for prophylaxis in patients with hemophilia, vWD, Factor XIII deficiency, or congenital afibrinogenemia, hypofibrinogenemia, or dysfibrinogenemia, these diseases are now largely treated with recombinant

or human-derived concentrates.<sup>207</sup> Nonetheless, cryoprecipitate remains a second-line therapy should such concentrates be unavailable.

Transfusion thresholds for fibrinogen have not been extensively studied in children, though extrapolation of adult guidelines would suggest that fibrinogen levels should be maintained above 80–100 mg/dL.<sup>181,208</sup> Recent adult studies suggest that fibrinogen levels above 150 mg/dL may be more ideal,<sup>205,209</sup> depending on the transfusion indication.

In children, 1 unit of cryoprecipitate per 5–10 kg of body weight is estimated to raise the fibrinogen level by 60–100 mg/dL (Figure 34.3);<sup>177,204</sup> this equates to approximately 2–3 mL of cryoprecipitate per kg.<sup>181,210</sup> The half-life of fibrinogen (3–5 days), along with the indication for transfusion, will determine the recommended frequency of cryoprecipitate transfusion. Cryoprecipitate is considered an acellular product and thus does not require irradiation for recipients at risk for transfusion associated graft-versus-host disease. Potential adverse effects of cryoprecipitate transfusion include the risk of thrombosis.

## Disclaimer

The authors have disclosed no conflicts of interest.

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## CHAPTER 35

# Thrombocytopenia and platelet transfusion

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Thrombocytopenia is caused by disorders impairing platelet production, causing platelet destruction, or leading to platelet sequestration. In addition, there are both congenital and acquired disorders of platelet function. These latter conditions may lead to the functional equivalent of thrombocytopenia despite the presence of a normal platelet count. Platelet transfusion represents an important therapeutic option for most of these disorders. However, under certain circumstances, platelet transfusion is contraindicated. Therefore, correct diagnosis of the underlying etiology of thrombocytopenia is important.

Methods for the preparation of platelet products for transfusion include platelet pools derived from multiple whole blood donations and apheresis units from individual donors. These components may be further manipulated depending on the requirements of the recipient. Platelet refractoriness represents a significant problem that develops in some patients who require repeated platelet transfusions. While refractoriness is most frequently due to nonimmune platelet consumption, immune responses to platelet antigens also occur. HLA alloimmunization is the most important immune cause, and it is managed by the provision of HLA-matched or crossmatched platelets. The development of platelet substitutes and the administration of thrombopoiesis-stimulating agents represent alternatives to platelet transfusion that are in various stages of clinical development and may ultimately reduce the need for donor-derived platelets.

### Thrombocytopenia

*Thrombocytopenia* refers to any reduction in platelet number below the lower limit of the normal range, which is about  $150 \times 10^9/L$  in most laboratories. In the absence of other factors, however, the risk of bleeding is considered to be relatively modest until the platelet count is less than  $50 \times 10^9/L$ , and it is not considered to be severe until the platelet count is less than  $25 \times 10^9/L$ .<sup>1</sup> However, congenital or acquired abnormalities in platelet function may be associated with bleeding even when the platelet count is in the normal range.<sup>2,3</sup> A systematic diagnostic approach facilitates the correct diagnosis and appropriate management of thrombocytopenia and platelet dysfunction. Careful assessment of the medical history is imperative, including a review of concomitant medications. The peripheral

blood smear should be reviewed for morphologic abnormalities in any of the three lineages (myeloid, erythroid, and megakaryocytic), which may provide important insights into the underlying diagnosis, be it congenital or acquired. The causes of thrombocytopenia are summarized in Table 35.1.

### Impaired platelet production

Disorders of platelet production may be congenital or acquired. Congenital disorders are relatively rare, whereas acquired disorders are much more commonly encountered in clinical practice. In the absence of an apparent etiology, distinguishing decreased platelet production from increased platelet destruction or from splenic sequestration can be challenging. Decreased platelet production is generally characterized by normal platelet size on the peripheral blood smear and a normal mean platelet volume (MPV). In addition, the reticulated platelet count as measured by flow cytometry is low.<sup>4</sup> A bone marrow aspirate and biopsy may be required in order to determine the underlying disorder associated with thrombocytopenia.

Hematologic and nonhematologic malignancies, and myelofibrosis, are associated with replacement of the marrow space and a reduction in blood cell production and pancytopenia. However, sometimes reduction in the platelet count is the first indication of one of these abnormalities. Platelet transfusions may be used appropriately for supportive care as needed while the cause of the underlying disorder is identified.

### Platelet sequestration

About one-third of the platelet mass is normally sequestered in the spleen.<sup>5</sup> Splenomegaly from any cause tends to further increase splenic platelet pooling. Hematologic malignancies including the myeloproliferative disorders may result in marked splenomegaly. In particular, myelofibrosis may be associated with thrombocytopenia.<sup>6</sup> Portal hypertension from liver disease may also be associated with marked splenomegaly leading to thrombocytopenia or even pancytopenia. The thrombocytopenia associated with splenic sequestration alone is generally moderate and is not associated with bleeding. However, concomitant conditions, such as the coagulopathy of liver disease or dysfibrinogenemia, can exacerbate the tendency to bleed in this setting.<sup>7</sup> Individuals with alcoholic cirrhosis

**Table 35.1** Causes of Thrombocytopenia

<b>Impaired Production</b>
Selective megakaryocyte depression:
Rare congenital defects
Drugs, chemicals, and viruses
As part of general bone marrow failure:
Cytotoxic drugs and chemicals
Radiation
Megaloblastic anemia
Leukemia
Infection
Myelodysplastic syndromes
Myeloma
Myelofibrosis
Solid tumors
Aplastic anemia
<b>Excessive Destruction or Increased Consumption</b>
Immune
Autoimmune: ITP
Drug induced (e.g., GP IIb/IIIa inhibitors, penicillins, and thiazides)
Secondary immune (SLE, CLL, viruses, drugs, e.g., heparin and bivalirudin)
Neonatal alloimmune thrombocytopenia
Posttransfusion purpura
Disseminated intravascular coagulation
Thrombotic thrombocytopenic purpura
<b>Sequestration</b>
Splenomegaly
Hypersplenism
<b>Dilutional</b>
Massive transfusion

and portal hypertension who continue to consume alcohol may also have exacerbated thrombocytopenia resulting from suppression of platelet production.<sup>8</sup>

### Congenital platelet disorders

Hereditary disorders may affect platelet number, platelet function, or both. Some congenital disorders leading to severe thrombocytopenia or markedly abnormal platelet function are identified early in life, but others are identified only after excessive bleeding is encountered in adulthood at the time of trauma or surgery.<sup>9</sup> Although platelet transfusion is effective in the treatment of bleeding in congenital platelet disorders, it can lead to the production of platelet antibodies and thus should be reserved for serious bleeding episodes.  $\epsilon$ -Aminocaproic acid (EACA) or desmopressin (DDAVP) may be useful for minor bleeding or for perioperative management of minor procedures, depending on the severity of the underlying disorder. In the absence of any bleeding, patients should be counseled to avoid aspirin and nonsteroidal anti-inflammatory drugs (NSAIDs) and to take appropriate prophylactic measures before procedures (e.g., use of EACA before minor dental procedures).<sup>9,10</sup>

### Acquired platelet disorders

Thrombocytopenia may be the primary manifestation of several different hematologic disorders. These include immune thrombocytopenic purpura (ITP) and the microangiopathic hemolytic anemias.

#### Immune thrombocytopenic purpura

ITP, covered in more detail in Chapter 36, is an acquired immune-mediated disorder characterized by isolated thrombocytopenia in the absence of any obvious underlying cause.<sup>11,12</sup> In particular, red cell morphology is normal, and large platelets are often seen. In

children, this condition is typically acute, is associated with an antecedent viral illness, and resolves spontaneously. However, in adults it often follows a chronic course and there are usually no precipitating factors. Signs and symptoms vary widely; some patients have little or no bleeding, whereas others can experience life-threatening hemorrhage.<sup>11</sup>

The standard initial treatment in adults is with corticosteroids (1 mg/kg/day). Individuals with bleeding, at high risk for bleeding, who require a surgical procedure, or who are unresponsive to steroids may benefit from additional treatment with intravenous immunoglobulin at a dose of either 1 g/kg/day for one or two days or 0.4 g/kg/day for up to five days.<sup>11</sup> In cases of severe or life-threatening bleeding, platelet transfusion is indicated. Although the survival of transfused platelets is greatly reduced, they are likely to have some beneficial effect on active bleeding.<sup>11</sup>

### Microangiopathic hemolytic anemia

Several different conditions can cause a similar appearance on the blood smear: thrombocytopenia and the presence of red cell fragmentation (schistocytes).<sup>13</sup> However, clinical distinction between TTP/HUS and disseminated intravascular coagulation (DIC) is critical. In addition, malignant hypertension and valvular hemolysis may result in a similarly appearing smear.

TTP represents a medical emergency. It is a rare condition that has been characterized by a pentad of clinical findings:

- microangiopathic hemolytic anemia (MAHA),
- thrombocytopenia,
- neurological deficits,
- fever, and
- renal abnormalities.

However, its onset is insidious and the classic pentad is rarely present at diagnosis.<sup>14</sup> The pathophysiology of this disorder has become elucidated as resulting from reduction or inhibition in function of the von Willebrand factor (vWF)-cleaving protease ADAMTS13.<sup>14</sup> Decreased ADAMTS13 activity leads to an accumulation of ultralarge von Willebrand factor multimers, which bind to platelets and induce aggregation. Microthrombi cause tissue ischemia and organ dysfunction (commonly involving the brain, heart, and kidneys), which may result in long-term as well as early complications.<sup>15</sup> Early diagnosis and treatment are essential, and TTP is now often diagnosed and treated when MAHA and thrombocytopenia are present, without the other findings in the pentad, in the absence of any other known cause.<sup>14</sup> Coagulation tests are almost always normal.

Platelets are activated in TTP, leading to a hypercoagulable state. Therefore, transfusion with platelets should be reserved for individuals with thrombocytopenia and life-threatening hemorrhage. The therapy of choice is plasma exchange.<sup>14</sup> Steroids are useful to suppress the autoantibodies inhibiting ADAMTS13 activity in severe ADAMTS13 deficiency.<sup>14</sup> Relapsing and refractory TTP, a less common presentation of this disease, has been treated successfully with the CD20 monoclonal antibody rituximab.<sup>14</sup> Caplacizumab, a humanized single-variable-domain antibody against vWF, prevents interaction with the platelet glycoprotein Ib-IX-V receptor and has been shown to speed the resolution of acute TTP.<sup>16</sup> A recent study found that the treatment of acquired TTP with caplacizumab, but without plasma exchange, increased platelet counts and improved surrogate parameters of organ damage in select patients.<sup>17</sup>

HUS is characterized by the triad of thrombocytopenia, MAHA, and acute renal failure.<sup>18,19</sup> The most common form of HUS is

associated with diarrheal illness with Shiga-toxin-producing bacteria such as *Escherichia coli* H:0157.<sup>19</sup> Supportive care is usually all that is required because spontaneous recovery is the rule. Atypical HUS (aHUS), a term applied to any HUS not caused by Shiga toxin, has been associated with a variety of precipitating events including infections, drugs, autoimmune conditions, and pregnancy. Until the beginning of the 2010s, plasma exchange was considered the gold standard for aHUS. Eculizumab has now been shown to be highly effective in aHUS, with up to 95% of patients becoming disease free.<sup>20</sup>

DIC is most commonly associated with infection or malignancy, although it can also be observed in the setting of vascular lesions such as hemangiomas and intravascular dissections. In contrast to TTP and HUS, the consumptive coagulopathy that occurs is not exacerbated by the administration of platelets and other blood components.<sup>21</sup> Although primary therapy should address the underlying condition, transfusions of platelets and other blood components may be safely administered to treat bleeding.

### Malignancy

Thrombocytopenia is also a side effect of the cytotoxic chemotherapy administered for many different hematologic and nonhematologic malignancies. However, in addition, several hematologic malignancies are associated with inherent impairment in platelet function. Both the myelodysplastic and myeloproliferative syndromes are potentially associated with impaired platelet function out of proportion to any reduction in platelet number that is present.<sup>22</sup> Platelet transfusions are sometimes indicated in the bleeding patient despite apparently acceptable platelet numbers but are not indicated to treat stable thrombocytopenia in nonbleeding patients.<sup>23</sup> It should also be noted, however, that myeloproliferative syndromes are also associated in some situations with platelet activation and thrombosis. Because of the latter, agents such as EACA and tranexamic acid should be used cautiously in these disorders and reserved for situations with symptomatic bleeding due to reduced platelet function or severe thrombocytopenia.

### Uremia

Several nonmalignant acquired conditions are associated with platelet dysfunction. Of these, uremia is most commonly encountered in clinical practice. Treatment of uremia with dialysis restores platelet function toward normal. For the patient with acute bleeding in the setting of uremia, several therapies have potential benefit.<sup>24</sup> DDAVP releases additional vWF from endothelial stores and is reasonably safe and effective in this setting. Administration of cryoprecipitate facilitates the same end. For less acute bleeding, high doses of conjugated estrogens and correction of anemia may be effective.

### Dialysis

Although dialysis improves platelet function in uremia, it is also associated with the development of thrombocytopenia. In particular, continuous venovenous dialysis or hemofiltration has been associated with a reduction in platelet number in some patients. It is important to distinguish thrombocytopenia caused by the dialysis procedure from heparin-induced thrombocytopenia (see below) because patients are often treated with this anticoagulant in the intensive care setting.

### Cardiac interventions and abnormal vascular surfaces

Cardiac procedures, use of ventricular assist devices, and cardiopulmonary bypass may all be associated with thrombocytopenia and/or defects in platelet function.<sup>25,26</sup> A predisposition to bleeding is

exacerbated by the concomitant use of antiplatelet agents. There is increasing recognition of the role of point-of-care viscoelastic and functional platelet testing, and these along with the use of antifibrinolytics may reduce the need for transfusion.<sup>27</sup> As in the dialysis setting, the distinction of heparin-induced thrombocytopenia from other causes is critical. Platelet transfusion is indicated if simple platelet consumption is suspected, whereas it is relatively contraindicated when heparin-associated thrombocytopenia is present.

### Heparin-induced thrombocytopenia

Heparin-induced thrombocytopenia is a special type of immune-mediated thrombocytopenia. It is a prothrombotic drug reaction caused by antibodies to platelet factor 4 (PF4)/heparin complexes that activate platelets.<sup>28</sup>

Although perhaps more commonly associated with unfractionated heparin, this syndrome is also associated with the use of low-molecular-weight heparins. The diagnosis is suggested when the platelet count decreases by 50%, or to less than  $100 \times 10^9/L$ , in a patient receiving one of these drugs. Diagnosis requires a high clinical likelihood of the condition (e.g., 4Ts scoring system) plus the presence of platelet-activating antibodies.<sup>29</sup>

When heparin-induced thrombocytopenia is suspected, treatment consists of immediate discontinuation of any heparin-containing product and institution of a nonheparin anticoagulant such as argatroban, bivalirudin, danaparoid, fondaparinux, or a direct oral anticoagulant.<sup>30</sup> Use of warfarin alone is contraindicated because it decreases protein C levels and can lead to catastrophic thrombosis. Platelet transfusion is contraindicated even in patients with low counts on anticoagulation except in the circumstance of life-threatening bleeding.

### Other medications and supplements

A number of medications, herbal remedies, and nutritional substances result in disorders of platelet function or number.<sup>31,32</sup> Aspirin and NSAIDs are among the most commonly used over-the-counter medications. In the case of aspirin, irreversible acetylation of cyclooxygenase in the platelet results in loss of function for the remainder of the 5–7-day lifespan of the platelet. Conversely, NSAIDs are reversible inhibitors. This distinction is important in the bleeding patient. If a patient with life-threatening bleeding has consumed aspirin during the past several days, platelet transfusion may be indicated. However, in the case of NSAIDs, once at least 4–5 half-lives have elapsed (the half-life of ibuprofen is 1.8–2 hours and that of acetaminophen is 1–4 hours), there is generally little utility in platelet transfusion unless a concomitant condition is present.

Immune-mediated thrombocytopenia (see Chapter 36) has been associated with the use of many different drugs. Most notably, quinine, quinidine, sulfonamides, sulfonamide derivatives such as furosemide, and vancomycin have been associated with autoantibody formation.<sup>33,34</sup> Although platelet transfusion may be necessary in patients with active bleeding, treatment consists primarily of discontinuation of the agent and observation. If necessary, treatment with intravenous immunoglobulin or corticosteroids may be initiated to hasten the recovery of the platelet count.

Many other commonly encountered classes of drugs are associated with qualitative or quantitative changes in platelets. These include traditional anticonvulsants such as valproic acid, which has also been associated with immune-mediated thrombocytopenia, and more recently approved agents such as gabapentin.<sup>35,36</sup> Simple discontinuation of these agents is generally all that is required in order to facilitate the resolution of the defects.

### Posttransfusion purpura

PTP is a rare but serious complication of blood transfusion, characterized by the sudden onset of severe thrombocytopenia (platelet count often  $<10 \times 10^9/L$ ) usually within 5–10 days of a blood transfusion.<sup>37</sup> It usually affects HPA-1a-negative women who have been previously alloimmunized by pregnancy. The transfusion precipitating PTP causes a secondary immune response, boosting the HPA-1a antibodies, although the mechanism of destruction of the patient's own HPA-1a-negative platelets remains uncertain. On average, 10 cases per year of PTP were reported to the UK hemovigilance scheme (SHOT) prior to the introduction of universal leukoreduction of blood components in 1999.<sup>38</sup> Since then, PTP has been rarely reported.<sup>39</sup>

Although the thrombocytopenia usually resolves spontaneously within a few weeks, there is a relatively high rate of bleeding associated with PTP so that treatment should be considered. Platelet transfusions are usually ineffective in raising the platelet count.<sup>40,41</sup> Administration of IVIg (0.4 g/kg for five days or the equivalent) has been shown to have some efficacy in this setting.<sup>42</sup>

### Neonatal alloimmune thrombocytopenia

About 2% of women have platelets of the HPA-1b/1b phenotype. When they become pregnant with an HPA-1a-positive fetus, about 10% develop HPA-1a antibodies. These antibodies cross the placenta and can lead to fetal/neonatal thrombocytopenia even during the first pregnancy. This is unlike the situation with RhD-negative mothers in which sensitization is caused by the first RhD-positive fetus, but the RhD antibodies do not cause adverse effects until the following RhD-positive pregnancies. Neonatal alloimmune thrombocytopenia (NAIT) is most commonly manifest as skin and other minor bleeding after birth. However, it may cause intracranial hemorrhage either at the time of delivery or in utero usually after 30 weeks of gestation. The precise incidence of intracranial hemorrhage is unknown, but conservative estimates suggest that it is around 1 in 20,000 live births.<sup>43</sup> For the management of an affected neonate, if the platelet count is  $<30 \times 10^9/L$  or if there are signs of bleeding with a low count, it is recommended that the neonate be transfused with donor platelets that are ideally both HPA-1a and -5b-negative as these will be compatible with the maternal HPA alloantibody in ≥95% of cases.<sup>44</sup> If HPA-1a-negative and HPA-5b-negative platelets are not immediately available and there is an urgent clinical need for transfusion, then random, ABO- and RhD-compatible donor platelets should be used.<sup>44</sup> Washed maternal platelets are a less readily available alternative. The results of laboratory investigations should not delay immediate platelet transfusion as full investigation may be time-consuming and the risk of intracranial hemorrhage is highest in the first 48 hours postdelivery. In a typical case, the platelet count should recover to normal within a week, although a more protracted recovery can occur. Intravenous immunoglobulin is not recommended as first-line treatment as it is only effective in about 75% of cases and there is a delay of 24–48 hours before a satisfactory count is achieved.

For the antenatal management of women with a history of NAIT in a previous pregnancy, referral to specialists in fetal medicine and hematology is required. Treatment during the subsequent pregnancy involves the administration of immunoglobulin and steroids; the timing of the initiation of treatment and the doses depend on the history of hemorrhage and fetal/neonatal thrombocytopenia in previous pregnancies.<sup>44,45</sup>

### Platelet transfusion

#### Platelet components

In the United States, whole-blood-derived platelet units (also called random-donor units) are prepared as platelet-rich plasma (see Chapter 17).<sup>46,47</sup> Outside of the United States, the buffy coat method is often used for platelet preparation.<sup>48</sup> In this case, after centrifugation analogous to that used to separate plasma from the red cell pellet, the buffy coat layer atop the red cells is harvested. Both platelet preparation methods involve the pooling of 4–6 units and result in a similar quality product. Each unit contains at least  $5.5 \times 10^{10}$  platelets.<sup>49</sup> Apheresis platelets (often called single donor platelets) are harvested from an individual through use of an apheresis device.<sup>50</sup> A six-unit random-donor pool or an apheresis unit contains around  $3 \times 10^{11}$  platelets, and this platelet dose generally leads to a platelet increment of  $30–60 \times 10^9/L$  in the platelet-naive patient when measured one hour after administration. Although the different platelet products may be considered interchangeable in terms of efficacy and safety,<sup>51</sup> the use of whole-blood-derived platelets in some settings may be preferred to the use of apheresis platelets in the absence of special circumstances such as the need for HLA matching.<sup>52</sup>

Platelet storage and processing are described in detail in Chapter 17.

#### Platelet administration

Platelets contain ABO antigens on their surface. Transfusion of ABO-mismatched platelets, however, is not associated with a major reaction. Nonetheless, there are data to indicate that naturally occurring anti-A or anti-B in the recipient may lead to the destruction of group A or B mismatched platelets and to poorer platelet increments.<sup>53,54</sup> When the supply allows, ABO-matched platelets should be transfused.

In contrast to ABO, Rh blood group antigens are not expressed on the surface of the platelet. However, red cells with these antigens are invariably contained in platelet products, albeit to a much lower extent in modern platelet products. Therefore, the transfusion of platelets from RhD-positive donors to RhD-negative recipients can lead to RhD alloimmunization. Because of immune suppression, this is uncommonly encountered in patients with hematologic malignancies receiving RhD-mismatched platelets. However, it is recommended to administer RhD-negative platelets to RhD-negative recipients whenever available and to administer intravenous Rh immunoglobulin to female children and females of childbearing age who have hypoproliferative thrombocytopenia. A dose of 1500 IU will cover multiple platelet exposures.

Specific platelet transfusion products, such as for patients with acute leukemia, may be needed e.g., leukocyte-reduced, irradiated platelet products (see other Chapters including 28, 42, 48, and 50). Because of the potential reduction in the number of platelets, washing of platelets should be reserved for individuals with severe allergic reactions that cannot otherwise be overcome by appropriate medications.

Although acetaminophen 650–1000 mg and diphenhydramine 25–50 mg are frequently administered before platelet transfusion as primary prophylaxis against febrile and allergic reactions, few data exist to support this as routine practice.<sup>55,56</sup> However, once an allergic reaction has been observed (hives, wheezing, or dyspnea), diphenhydramine is the drug of choice to prevent additional reactions in the future. If a moderate allergic reaction recurs in an adult despite the administration of these premedications, hydrocortisone 100 mg IV is often effective. Recurrence of severe allergic reactions

despite premedication is an indication for the use of washed platelets. Platelet concentrates must be administered to adults through a blood filter. This is generally a standard blood set with an infusion time of about 30 minutes.

Platelet transfusion is not without risk. Data from the UK hemovigilance system, Serious Hazards of Transfusion (SHOT), indicates that platelets are the most commonly implicated component in reported reactions and adverse events.<sup>57</sup> Aside from the development of platelet refractoriness and allergic reactions that can be observed with any blood component, platelets are the component most commonly associated with the transmission of bacterial infection (Chapter 46).<sup>58</sup> In the past, it has been estimated that about 1 in 5000 platelet units were contaminated with bacteria. Newer methods for bacterial testing have reduced the incidence of bacteremia associated with platelet transfusion. Nonetheless, there should be a heightened sense of awareness for the possibility of bacterial transmission in those receiving platelet transfusions, particularly if individuals are neutropenic and become febrile during or shortly after a platelet transfusion.

### **Prophylactic platelet transfusions**

*Prophylactic transfusion* refers to the maintenance of the platelet count above a certain threshold in patients who are neither bleeding nor actively consuming platelets because of immune destruction or infection. In patients with acute myeloid leukemia who have received 25 platelet transfusions, the mean one-hour platelet increment is reduced by about one-third, and the 18–24-hour platelet increment is reduced by more than half in comparison to that observed after the first platelet transfusion.<sup>59</sup> Therefore, reduction in the number of unnecessary platelet transfusions is of significant benefit, including cost and supply implications. As an example, around 300,000 units for platelet transfusion are issued by UK Blood Services each year, for an estimated annual cost of over \$90 million (\$300/unit, excluding storage, administration, and patient monitoring costs in hospital).

Several of the questions raised regarding prophylactic platelet transfusions in patients with reversible bone marrow failure are addressed below.

### **Should prophylactic platelet transfusions be given?**

A number of trials have addressed this first key question, as to whether a policy of prophylaxis has benefit for patients. These trials have compared prophylactic versus therapeutic platelet transfusion strategies.<sup>60</sup> The early studies used outdated methods of platelet component production and patient supportive care. More recently two larger randomized controlled trials have addressed the question of whether prophylactic platelet transfusions should be used.<sup>61,62</sup> Both showed that prophylactic platelet transfusions reduced the risk of bleeding. However, this effect was less marked in the prespecified subgroup of patients receiving autologous hematopoietic stem cell transplants (HSCTs).<sup>63</sup> When considering these results as a number needed to treat (NTT), five patients receiving intensive chemotherapy or an allogeneic stem cell transplant would need to receive prophylactic platelet transfusions over a 30-day period to prevent one patient from having clinically significant bleeding (WHO grade 2 or above) (95% CI, 3–18), whereas for autologous HSCT patients 43 patients would need to receive prophylactic platelet transfusions to prevent one patient from bleeding.<sup>63</sup> One further observation from both studies was that bleeding continued to be frequently observed in patients despite a policy of prophylaxis.

### **What platelet transfusion threshold should be used?**

The largest groups of trials of platelet transfusions have evaluated different platelet transfusion thresholds in patients with hematological malignancies. Two trials compared a threshold of  $20 \times 10^9/\text{L}$  versus  $10 \times 10^9/\text{L}$ ,<sup>64,65</sup> and other trials different thresholds. A meta-analysis of all four studies (658 patients) showed no increase in the proportion of patients who bled and also showed a significant reduction in the number of platelet transfusions given when using lower thresholds.<sup>66</sup> However, this meta-analysis may not be sufficiently powered to detect an increased bleeding risk in the  $10 \times 10^9/\text{L}$  threshold arm of less than 50%. There has been a suggestion that platelet transfusion thresholds should be lowered, but automated hematology analyzers are not yet accurate enough in counting very low platelet numbers.<sup>67</sup> No randomized studies in adult patients have assessed the use of other transfusion metrics, such as platelet mass, absolute immature platelet number, or immature platelet fraction.

For patients with chronic bone marrow failure, there is little evidence on which to base practice. A retrospective study considered platelet transfusion in outpatients with stable chronic severe aplastic anemia.<sup>68</sup> Prophylactic platelets were given if the count was  $5 \times 10^9/\text{L}$  or less, or if the patient was unwell with fever (temperature  $>38^\circ\text{C}$ ) or recent hemorrhage when the count was  $10 \times 10^9/\text{L}$  or less. Patients with significant bleeding or prior to minor surgery received platelets at counts  $>10 \times 10^9/\text{L}$ . In total, 55,239 patient days were reviewed with 18,706 days when the platelet count was  $10 \times 10^9/\text{L}$  or less. All deaths from hemorrhage were associated with alloimmunization or withdrawal from treatment. Three nonlethal major bleeding episodes occurred. The authors concluded that this restrictive policy, with a median transfusion interval of seven days, was feasible, safe, and economical.

Both national and international guidelines vary in their advice for platelet transfusion in stable nonbleeding patients with bone marrow failure resulting in chronic thrombocytopenia. Recommendations include a threshold of  $<10 \times 10^9/\text{L}$ , as used in patients with reversible bone marrow failure;<sup>69</sup> a threshold of  $5 \times 10^9/\text{L}$  or less;<sup>70,71</sup> or no prophylaxis.<sup>24,72,73</sup> National recommendations for adult intensive care patients are not based on randomized trials.<sup>74</sup>

A number of trials have tested different thresholds in groups of patients outside hematological malignancies. Two RCTs have assessed whether prophylactic platelet transfusions are beneficial in dengue hemorrhagic fever.<sup>75,76</sup> Platelet transfusion did not prevent progression to severe bleeding, nor did it appear to shorten time to cessation of bleeding. It was also associated with an increased risk of severe side effects. Other studies have shown no correlation between the platelet count and risk of bleeding;<sup>77,78</sup> therefore, the use of platelet transfusions in dengue fever should not be based solely on a platelet count.

Platelet transfusion thresholds in neonates are varied.<sup>79</sup> The Platelets for Neonatal Thrombocytopenia (PlaNeT-2) study compared platelet count thresholds that were relevant to neonatal practice, and which are typically much higher than found in adult patients with hematological malignancies. This trial recruited 660 babies and reported an overall benefit of a prophylactic platelet transfusion threshold of  $25 \times 10^9/\text{L}$  compared with  $50 \times 10^9/\text{L}$  for major hemorrhage and/or death in preterm neonates (7% absolute-risk reduction).<sup>80</sup> This unexpected result should reinforce the recommendations for more restrictive practices in neonatal intensive care units. Additional analyses found that these harmful effects occurred in neonates with both high and low baseline risks of death or hemorrhage, supporting the generalizability of the main trial

findings to all groups of preterm babies.<sup>81</sup> However, a limitation of the study was the lack of mechanistic studies to understand why platelets might cause harm. Hypotheses include the use of adult platelet donations into a neonatal hemostatic system or the effects of platelets through immunomodulatory pathways.<sup>82</sup>

### What platelet transfusion dose should be used?

Multiple randomized trials have compared different platelet transfusion doses in patients with hematological malignancies.<sup>83</sup> Four studies have assessed clinically significant bleeding as an outcome measure (usually defined as WHO grade 2 or above), including the largest trial to date (PLADO).<sup>84</sup> There was no evidence of a difference in the risk of bleeding between low-dose ( $1.1 \times 10^{11}/m^2$ ) and standard-dose transfusions ( $2.2 \times 10^{11}/m^2$ ) and between standard-dose and high-dose platelet transfusions ( $4.4 \times 10^{11}/m^2$ ). Low-dose transfusions decreased the total amount of platelets that patients received but at the expense of a higher number of transfusion episodes. The UK dose for platelet transfusions is that at least 75% of units contain  $>2.4 \times 10^{11}$  platelets per adult dose and should continue to be used as standard. Increasing the platelet dose from a standard-dose ( $2.2 \times 10^{11}/m^2$ ) to a high-dose ( $4.4 \times 10^{11}/m^2$ ) transfusion regimen does not increase the transfusion interval (a median of five days for both regimens). Again, as mentioned earlier, it should be noted that despite different policies of platelet transfusion by dose, patients continued to experience significant rates of bleeding. The lack of a dose-effect response perhaps continues to argue for a limited role of platelet transfusions to impact clinical bleeding in thrombocytopenic patients with hematological malignancies. With this in mind, new clinical studies are ongoing to assess the role of alternatives to platelet transfusion, for example tranexamic acid.<sup>85</sup>

### Additional risk factors for bleeding

Numerous clinical factors have been associated with an increased risk of bleeding (Table 35.1). However, the majority of these postulated risk factors are based on low-level evidence, such as expert opinion or retrospective analysis of patient databases. Currently, the platelet transfusion threshold is commonly raised to  $20 \times 10^9/L$  when patients have an infection or fever. While inflammation has been shown to be associated with an increased risk of bleeding in mice,<sup>86</sup> studies have differed on whether fever increases the risk of bleeding in humans.<sup>87</sup> Further studies are required to identify clearly which factors should prompt an increase in the transfusion threshold, and what this threshold should be.

### Preprocedure platelet transfusions

#### Bone marrow aspirates and trephines

According to the UK confidential registry of complications after bone marrow aspirates and trephines, the risk of significant bleeding is very low (less than 1:1000), and the majority of patients had bleeding when they did not have a significant thrombocytopenia.<sup>88–92</sup>

#### Central venous catheters

Twenty observational studies have reported bleeding outcomes in thrombocytopenic patients after insertion of central venous catheters (CVCs).<sup>93–112</sup> At least 1450 procedures were performed when the platelet count was  $\leq 50 \times 10^9/L$ . Only one case of severe bleeding (Hb drop  $>15$  g/L) was reported throughout all of these studies.<sup>110</sup> Three of these studies looked at risk factors associated with bleeding. The two studies that did not perform the CVC procedure with ultrasound guidance found that the number of attempts, site of insertion (jugular

vs. subclavian), and failed guidewire insertion significantly increased the risk of bleeding on multivariable analysis,<sup>93,99</sup> whereas the third study that used ultrasound guidance did not.<sup>111</sup> Systematic reviews of complications of CVC placement have found that ultrasound guidance significantly reduced failure and complication rates.<sup>113,114</sup> A small study in thrombocytopenic patients that compared ultrasound-guided versus landmark insertion techniques reiterated this finding.<sup>109</sup> The Zeidler study<sup>111</sup> looked at risk of bleeding according to platelet count thresholds with multivariable analysis. The risk of bleeding only increased when the platelet count was less than  $20 \times 10^9/L$  (OR, 2.88; 95% CI, 1.23–6.75;  $p = 0.015$ ); this analysis controlled for sex, type of leukemia, insertion site, and use of prophylactic platelet transfusions. All CVCs in this study were un tunneled and inserted by experienced individuals.<sup>111</sup> In the large Haas study, no patient with thrombocytopenia experienced bleeding that could not be treated with simple pressure at the site of the tunneled CVC; their platelet count threshold for insertion was  $25 \times 10^9/L$ .<sup>101</sup>

A small RCT (57 participants) comparing three transfusion policies in patients with liver disease showed that a restrictive transfusion policy reduced costs with no increase in the risk of bleeding or other harms.<sup>115</sup>

One additional prospective study assessed peripherally inserted central catheters (PICCs) without prophylactic platelet transfusions. Of the 50 patients who had a line inserted with a platelet count less than  $20 \times 10^9/L$ , only one bleeding episode occurred (minor oozing).<sup>116</sup>

One prospective nonrandomized study has assessed the risk of bleeding after traction removal of tunneled cuffed CVCs in patients with abnormal platelet counts or international normalized ratio (INR).<sup>117</sup> Fourteen of the 179 patients enrolled in the study had a time to hemostasis of over five minutes, and only one of these patients had a platelet count  $<100 \times 10^9/L$ .

### Lumbar punctures and epidural anesthesia

Reviews of the literature have been performed of the risk of spinal hematoma following lumbar puncture or neuraxial anesthesia.<sup>118,119</sup> New small studies have contributed to the more recent review. Resulting recommendations suggest that lumbar punctures can be performed safely when the platelet count is  $>40 \times 10^9/L$ , and that epidural anesthesia can be performed safely when the platelet count is  $>80 \times 10^9/L$ .<sup>119</sup> Platelet transfusions should be administered to raise the platelet count above these thresholds if it is below these levels.

### Liver biopsy

Percutaneous liver biopsies are considered safe when the platelet count is at least  $50–60 \times 10^9/L$ .<sup>120</sup> 2740 percutaneous liver biopsies were conducted in the HALT-C trial;<sup>121</sup> only 16 patients (0.6%) had a serious adverse event due to bleeding after the biopsy. A single center retrospective study has shown no increase in harm using a lower threshold of  $25 \times 10^9/L$ , none of the 21 participants who had a platelet count  $<50 \times 10^9/L$  had bleeding that required an intervention, and only 2/21 had any bleeding.<sup>122</sup> Transjugular liver biopsy has been shown to be safe in patients with low platelet counts, and with more modern techniques it can produce comparable histological samples to percutaneous liver biopsies.<sup>123–125</sup>

### Renal biopsy

Uncontrolled hypertension, high serum creatinine, anemia, older age, and female sex have all been shown to be risk factors for bleeding post renal biopsy as well as a prolonged bleeding time.<sup>126–129</sup> There have been no systematic studies that have assessed the effect

of acutely lowering blood pressure prior to renal biopsy.<sup>127</sup> Dialysis may improve the bleeding tendency in patients with uremia. DDAVP can be used successfully to prevent bleeding before invasive procedures.<sup>24,130–132</sup> Conjugated estrogens are a long-acting alternative to DDAVP because they shorten the bleeding time with a more sustained effect.<sup>24,133</sup> Correcting anemia will also decrease the bleeding time.<sup>24</sup> Transjugular renal biopsy has been used in patients in whom percutaneous renal biopsy has failed or been contraindicated, and has produced a similar diagnostic yield and safety profile.<sup>134</sup> Given the possibility that patients with kidney disease will go on to transplant, it is recommended that platelet transfusions be avoided if possible because of the risk of alloimmunization.<sup>135</sup>

### Dental extraction

One recent small RCT (36 patients) has shown a low rate of bleeding complications with no blood products transfused, in patients with liver disease.<sup>136</sup> Patients were randomized to desmopressin, platelet or plasma transfusions, or both. There was no evidence of a difference in minor or major bleeding. Two observational studies also found that local hemostatic measures (e.g., gauze soaked in tranexamic acid) were sufficient to stop bleeding post dental extraction without the need for platelet transfusions.<sup>137,138</sup>

### Surgery

There remains a lack of evidence to guide the prophylactic use of platelet transfusions before major surgery.<sup>139</sup> Guidelines from around the world suggest a threshold of  $50 \times 10^9/L$  before major surgery,<sup>24,140,141</sup> and a threshold of  $100 \times 10^9/L$  prior to neurosurgery or ophthalmic surgery involving the posterior segment of the eye, because of the critical sites involved.<sup>24,140,141</sup> A platelet count increment after any platelet transfusion is desirable, but it may be limited by the circumstances of the conduct of the procedure.

### Therapeutic platelet transfusions

The predominant usage of platelet transfusions is to prevent bleeding (up to 67%),<sup>142</sup> but therapeutic platelet transfusions are also needed to treat active bleeding from thrombocytopenia and/or

platelet dysfunction in a wide range of clinical scenarios. Although most clinical research of the use of platelet transfusions has been in the area of prophylactic transfusions in patients with hematologic malignancies, the management of the actively bleeding patient with severe thrombocytopenia and/or platelet dysfunction has received less attention and is arguably more challenging. The clinical scenario is often complex, and management depends on the nature and site of the bleeding, the severity of the thrombocytopenia, the presence or absence of other disorders of hemostasis, and the presence or absence of anemia, as well as the clinical condition of the patient.

Consensus guidelines have generally recommended a threshold for therapeutic platelet transfusions of at least  $50 \times 10^9/L$  if a patient is actively bleeding (Table 35.2). However, there are few definitive studies to substantiate these platelet transfusion triggers, and the level of evidence to support the recommendations is poor.

### Platelet refractoriness

*Platelet refractoriness* is defined as the repeated failure to achieve satisfactory responses to platelet transfusions; this topic has been comprehensively reviewed.<sup>143</sup> The effectiveness of platelet transfusions can be assessed clinically in a bleeding patient by monitoring cessation of bleeding or not. However, where platelet transfusions have been given prophylactically, the response is assessed by measuring the posttransfusion platelet count increment. Various formulas have been devised to refine this assessment of the response to platelet transfusion.<sup>144</sup>

- 1 The percentage platelet recovery ( $R$ ) is calculated from the platelet increment  $\times 10^9/L$  (PI), the blood volume (BV) in liters, and the platelet dose transfused  $\times 10^9$  (PD):

$$R (\%) = PI \times BV \times PD^{-1} \times 100$$

The corrected count increment  $\times 10^9/L$  (CCI) is calculated from the platelet increment (PI), the body surface area of the patient in square meters (BSA), and the dose of platelets transfused  $\times 10^{11}$  (PD):

**Table 35.2** Platelet Thresholds for Therapeutic Transfusion during Active Bleeding

Clinical Indication	Treatment Trigger ( $\times 10^9/L$ )	Level of Evidence*
Major bleeding and thrombocytopenia or massive hemorrhage	50–75	Grade C, level IV
Massive transfusion and multiple trauma or TBI	75–100	Grade C, level IV
Surgery	50–100	Grade B, level III, to Grade C, level IV
DIC	50	Grade C, level IV
DIC in neonates	100	Grade C, level IV
Intracerebral bleeding	100	Grade C, level IV
Platelet function defects	No threshold	Grade C, level IV

\* Levels of evidence originate from the US Agency for Healthcare Research and Quality ([www.ahrq.gov](http://www.ahrq.gov)):

#### Statements of evidence

Ia: Evidence obtained from meta-analysis of randomized controlled trials.

Ib: Evidence obtained from at least one randomized controlled trial.

IIa: Evidence obtained from at least one well-designed controlled study without randomization.

IIb: Evidence obtained from at least one other type of well-designed quasi-experimental study.

III: Evidence obtained from well-designed nonexperimental descriptive studies, such as comparative studies, correlation studies, and case studies.

IV: Evidence obtained from expert committee reports or opinions and/or clinical experiences of respected authorities.

#### Grades of recommendations

A: Requires at least one randomized controlled trial as part of a body of literature of overall good quality and consistency addressing the specific recommendation. (Evidence levels Ia and Ib.)

B: Requires the availability of well-conducted clinical studies but no randomized clinical trials on the topic of recommendation. (Evidence levels IIa, IIb, and III.)

C: Requires evidence obtained from expert committee reports or opinions and/or clinical experiences of respected authorities. It indicates an absence of directly applicable clinical studies of good quality. (Evidence level IV.)

TBI: traumatic brain injury; DIC: disseminated intravascular coagulation.

$$\text{CCI} = \text{PI} \times \text{BSA} \times \text{PD}^{-1}$$

A platelet recovery of about 67% in a stable patient is considered an appropriate response, but the minimum platelet recovery to define a successful transfusion is considered to be >30% at 1-hour post transfusion and >20% at 20–24 hours. When using CCI, thresholds for successful transfusions are  $>7.5 \times 10^9/\text{L}$  at 1 hour and  $>4.5 \times 10^9/\text{L}$  at 20–24 hours. In practice, a 24-hour platelet increment of  $<5 \times 10^9/\text{L}$  following a platelet transfusion on two or more occasions is a good indicator of refractoriness to random-donor platelets.

It is sometimes stated that the absence of a response one hour after transfusion indicates refractoriness from immunologic causes, and lack of a response at a later time following transfusion is caused by underlying clinical problems, but this rule is an unreliable way of determining the cause of platelet refractoriness.

There are many causes of platelet refractoriness, and they can be subdivided into immune and nonimmune. The main immune cause is HLA alloimmunization, which occurs predominantly in females with a history of pregnancy or in patients who have received multiple transfusions. Other immune causes include HPA alloimmunization, ABO incompatibility with high-titer ABO antibodies in the recipient, platelet autoantibodies, and drug-related platelet antibodies. On the other hand, a lack of response to platelet transfusion may be the result of an underlying acute illness, such as infection, or it may result from a more chronic condition, such as splenomegaly. Fever, sepsis, DIC, hemorrhage, and conditions associated with marrow transplantation such as veno-occlusive disease and graft-versus-host disease all contribute to platelet refractoriness.

The incidence of HLA alloimmunization varies with the type of blood components transfused, the patient's underlying condition, and the previous history of pregnancy and transfusion. For example, HLA alloimmunization is more frequent in patients with aplastic anemia than in patients with acute leukemia. The Trial to Reduce Alloimmunisation to Platelets found that in patients with acute myeloblastic leukemia receiving nonleukocyte-reduced blood components the incidence of HLA alloimmunization was 33% in those who had never been pregnant and 62% in those who had been pregnant; in patients receiving leukocyte-reduced blood components, it was 9% and 32%, respectively.<sup>145</sup> The incidence of alloimmune platelet refractoriness caused by HLA antibodies has declined due to the implementation of leukocyte reduction of blood components and more aggressive treatment for patients with hematological malignancies and other cancers.

The role of platelet-specific (or HPA) antibodies in platelet refractoriness is less clear. HPA antibodies have been detected at a frequency of 8%<sup>145</sup> to 20–25%<sup>146</sup> in various studies of multitransfused hematology patients and are usually found in combination with HLA antibodies. Most commonly, HPA alloimmunization is directed toward antigens with phenotypic frequencies below 30%.<sup>147</sup> Some studies have suggested that there is no clear correlation between HPA antibodies and poor responses to platelet transfusions,<sup>148,149</sup> but others have found that matching for platelet-specific antigens in patients refractory to HLA-matched platelets may be beneficial.<sup>150</sup>

While immune causes are often prioritized during the investigation of platelet refractoriness, the observed reduction in platelet survival is most commonly due to nonimmune clinical factors, including DIC, splenomegaly, and intravenous antibiotics (especially antifungal drugs such as amphotericin B).<sup>59,151,152</sup> Fever has

also been implicated in causing poor responses to platelet transfusions, although whether this is a reflection of sepsis, associated DIC, or antibiotic therapy rather than the temperature itself is unclear.<sup>59,153</sup>

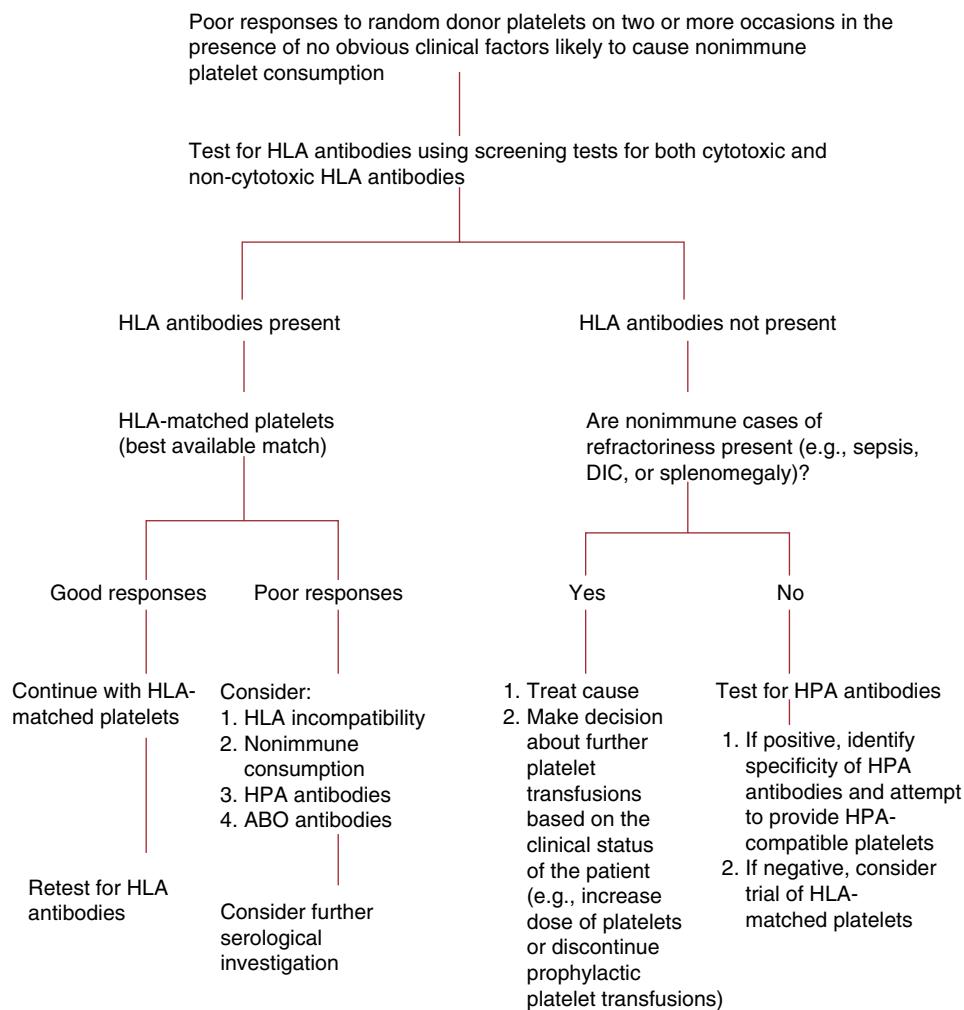
The appropriate investigation and management of platelet refractoriness require consideration of information from a clinical assessment of the patient as well as laboratory investigations (Figure 35.1). The first step is a clinical evaluation for possible nonimmune clinical causes. Any significant clinical factors such as infection should be treated if possible, and prophylactic platelet transfusions from random donors continued as standard care. If poor responses to platelet transfusions persist, the patient should be tested for HLA antibodies, and, if present, platelet transfusions selected for the HLA-A and HLA-B antigens of the patient should be used.<sup>154</sup>

HLA matching of platelets uses databases of HLA-typed platelet donors who can then be asked to donate by apheresis. This requires logistic coordination and may result in a time lag before the product is available. There are a number of ways to select HLA-matched platelet transfusions. Traditionally, recipient and donor are matched for HLA-A and HLA-B antigens as the most important antibodies in causing platelet refractoriness are directed against these antigens.<sup>154</sup> Refinements to HLA matching have been made, including grading of the quality of HLA match and its revision to include "permissive" mismatches,<sup>155,156</sup> the identification of the specificity of HLA antibodies and the issue of HLA-matched platelets based on their specificity,<sup>155</sup> and more recently the use of software tools such as *HLA matchmaker* to predict HLA compatibility by identifying immunogenic epitopes in antibody-accessible regions of HLA molecules.<sup>157,158</sup> A systematic review found that HLA-matched platelet transfusions improved one-hour posttransfusion platelet increments but did not consistently improve the 24-hour increments, and failed to demonstrate any reduction in mortality or bleeding as the studies were inadequately powered for these outcomes.<sup>159</sup>

HPA antibodies are rare in the absence of HLA antibodies and do not always cause platelet refractoriness. It is not necessary to test for HPA antibodies during the initial serological investigation of platelet refractoriness.

The use of HLA-matched platelet transfusions is also justified if there has not been time to carry out serological testing, particularly when platelet refractoriness is associated with bleeding. However, HLA-matched platelet transfusions are not indicated when full serological testing has failed to detect HLA antibodies. In this case, further consideration should be given to the identification of nonimmune clinical factors, and if they still appear to be absent, testing for HPA antibodies should be undertaken.

Responses to HLA-matched platelet transfusions should be carefully monitored, ideally with posttransfusion platelet counts both 1 and 20–24 hours post transfusion. If there are improved responses, HLA-matched platelet transfusions should continue to be used for further transfusions. If there are poor responses to HLA-matched platelet transfusions, the reasons should be sought, including residual HLA incompatibility, which is most likely to occur in patients with unusual HLA types with few well-matched donors, nonimmune platelet consumption, and HPA and ABO incompatibility. Further serological investigations including testing for HPA antibodies may be useful to differentiate between these possibilities. Depending on the results of these investigations, the appropriate management could be the use of ABO-identical or HPA-matched platelet concentrates if the specificity of the HPA antibodies can be identified.



**Figure 35.1** Algorithm for the investigation and management of patients refractory to platelet transfusions. Source: Modified from Phekoo *et al.* (1997).<sup>154</sup>

Platelet crossmatching of the patient's plasma against the lymphocytes and platelets of donors of HLA-matched platelet transfusions that have failed to produce satisfactory responses may be very helpful in identifying the cause of the poor responses.<sup>160</sup> Platelet crossmatching can also be used as an alternative approach to the management of refractory patients with HLA-matched platelet transfusions.<sup>161–163</sup> Typically, the patient's plasma is tested against platelet samples of ABO-compatible apheresis platelet donors. Donor platelets lacking reactivity are considered to be *crossmatch compatible*, and the associated platelet concentrates selected for transfusion in preference to those from random donors. An advantage of platelet crossmatching is its timeliness when the HLA types of platelet refractory patients are not yet known. A disadvantage is the need to carry out testing each time a platelet transfusion is required.

The management of patients with HLA and/or HPA alloimmunization with no compatible donors may be very difficult. There is no evidence that alloimmunized patients benefit from prophylactic transfusions of incompatible platelets that do not produce an increase in the platelet count, and prophylactic platelet support should be discontinued. If bleeding occurs, platelet transfusions from random donors or the best-matched donors, despite being incompatible, may reduce the severity of hemorrhage, although large doses of platelets may be required. Other management

approaches for severe alloimmune refractoriness, such as the use of high-dose intravenous immunoglobulin, splenectomy, and plasma exchange, have not been shown to be effective.<sup>73</sup>

The management of patients with nonimmune platelet consumption is similarly problematic. Treatment of the underlying illness is indicated. Common practice is to continue with daily platelet transfusions as prophylactic platelet support, but it is not known whether this approach is effective, or whether platelet transfusions should be discontinued or the dose of platelets increased. Intravenous immunoglobulin is ineffective in this scenario. However, EACA and tranexamic acid may be useful in reducing bleeding in patients with severe thrombocytopenia (although tranexamic acid is not available in the United States). Even at relatively low doses of 1 g every six hours, EACA appears to be effective in this setting.

### Platelet substitutes and storage technologies

The possibility of providing various forms of platelet substitutes has been proposed for many years, but currently there are no alternatives in clinical use.<sup>164,165</sup>

More research has been performed on different storage methods to increase shelf-life or hemostatic activity.<sup>166</sup> Pilot studies have shown that cryopreserved platelets are safe, and further work is being performed to assess their effectiveness.<sup>167</sup>

### Thrombopoietin receptor agonists

Thrombopoietin is the primary regulator of megakaryocyte progenitor expansion and differentiation.<sup>168</sup> Its cloning in the mid-1990s led to the development of thrombopoietic drugs, and there was initial success in reducing the duration of thrombocytopenia with its use in cancer patients. However, trials were discontinued because healthy volunteers developed severe thrombocytopenia as a result of an immune response to the drug where antibodies crossreacted with endogenous thrombopoietin. Focus shifted to the development of small-molecule peptide (romiplostim; Nplate marketed by Amgen) and nonpeptide thrombopoietin mimetics (eltrombopag; Promacta marketed by GlaxoSmithKline), and these have proved successful in the treatment of autoimmune thrombocytopenia. Several trials have assessed the use of thrombopoietin mimetics in patients with liver disease prior to procedures. Eltrombopag has been shown to reduce the requirement for platelet transfusions; however, the study was stopped early due to an increased risk of thrombosis.<sup>169</sup> More recent studies using lusotrombopag and avatrombopag have also shown decreased platelet use but without any evidence of an increased risk of thrombosis.<sup>170</sup> Thrombopoietin mimetics probably slightly decrease the risk of bleeding in patients with myelodysplastic syndrome,<sup>171</sup> but the evidence is much more limited for patients with other cancers.<sup>172,173</sup>

### Summary

A variety of diverse congenital and acquired conditions are associated with thrombocytopenia or its functional equivalent. Congenital causes are rare, but acquired causes are relatively common. In particular, thrombocytopenia or platelet dysfunction associated with the administration of chemotherapy or with surgical procedures is a leading indication for platelet transfusion. The availability of a variety of platelet products for transfusion facilitates the supportive care of individuals receiving chemotherapy and the treatment of bleeding individuals with thrombocytopenia or its functional equivalent. Leukocyte-reduced whole-blood-derived platelets and apheresis platelets are associated with similar rates of infection and alloimmunization, and they can be used interchangeably in most settings. A prophylactic platelet transfusion threshold of  $10 \times 10^9/L$  is generally acceptable for afebrile patients receiving cancer chemotherapy. Maintenance of a higher platelet count is indicated in a number of situations, including the settings of sepsis, DIC, hemorrhage, trauma, and surgery. Febrile and allergic reactions to platelet

transfusion can often be managed conservatively with the administration of acetaminophen and diphenhydramine. Platelet refractoriness based on nonimmune and immune mechanisms remains a major issue when repeated transfusions are required. After platelet refractoriness following two transfusions have been documented, ABO matching, platelet crossmatching, and HLA matching may be used as initial strategies to address the situation. Because of the limitations of current storage techniques, research and development continue on platelet storage and substitutes. Thrombopoiesis-stimulating agents provide additional therapeutic alternatives for the management of thrombocytopenia in specific settings. However, additional research and development will be necessary in order to establish their safety and efficacy in patients receiving cancer chemotherapy.

### Disclaimer

The authors have disclosed no conflicts of interest.

### Key references

- A full reference list for this chapter is available at: [www.wiley.com/go/simon/Rossi6](http://www.wiley.com/go/simon/Rossi6)
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## CHAPTER 36

# Management of immune-mediated thrombocytopenia

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## Introduction

Thrombocytopenia, commonly defined as a platelet count below the normal range, is one of the most common reasons for hematological consultation. Low circulating platelet numbers can be caused by platelet underproduction, sequestration, hemodilution, consumption, or destruction. This chapter explores the problem of immune-mediated thrombocytopenia (platelet “destruction”), including autoimmune conditions (e.g., primary immune thrombocytopenic purpura [ITP]), secondary immune conditions (e.g., drug-induced immune thrombocytopenia [DITP] and heparin-induced thrombocytopenia [HIT]), and alloimmune conditions (e.g., neonatal alloimmune thrombocytopenia [NAIT]). Immune thrombocytopenia is characterized by a shortened platelet lifespan caused by platelet–antibody interactions and, in primary ITP, reduced platelet production likely because of immune-mediated alterations to bone marrow megakaryocytes. We begin with a review of routine and specialized laboratory testing used for the investigation of thrombocytopenic conditions, followed by a description of the most common immune-mediated thrombocytopenic syndromes and a review of current management.

## Laboratory tests for the investigation of thrombocytopenia

### Complete blood cell count and blood film

Platelets are usually quantitated during a complete blood cell count with a particle counter. A normal platelet count is usually 150,000–400,000/ $\mu$ L, although the reference range may be lower in Mediterranean populations (125,000–300,000/ $\mu$ L) that have larger sized platelets.<sup>1</sup> The platelet count usually remains fairly stable throughout a normal human lifespan.<sup>2</sup> An exception occurs during pregnancy, when the platelet count decreases somewhat, perhaps the result of increased plasma volume (hemodilution).<sup>3</sup> An elevated platelet count is also normal 10–14 days after a major surgical procedure (postoperative thrombocytosis, 250,000–1,000,000/ $\mu$ L) before return to preoperative baseline by three weeks after the operation.<sup>4,5</sup> It is important to understand these expected excursions from a normal platelet count. For example, while a platelet count of 170,000/ $\mu$ L is within the normal range, if the sample was collected 10 days after an

operation in a patient with dyspnea who had received postoperative heparin prophylaxis, the diagnosis of pulmonary embolism complicating HIT should be considered. A useful general rule is that isolated thrombocytopenia is usually caused by increased platelet consumption or destruction, whereas bycytopenia or pancytopenia is usually attributable to marrow dysfunction, hypersplenism, or hemodilution. Furthermore, isolated, severe thrombocytopenia (platelet count less than 20,000/ $\mu$ L) often indicates platelet destruction by autoantibodies, alloantibodies, or drug-dependent immunoglobulin G (IgG) antibodies. While severe thrombocytopenia can occasionally occur in patients with HIT<sup>6</sup> or septicemia, platelet count nadirs are typically more than 20,000/ $\mu$ L in these two disorders characterized by in vivo platelet activation caused by heparin-dependent platelet-activating IgG antibodies or thrombin, respectively.

Examining the blood film is important to exclude pseudothrombocytopenia (spurious thrombocytopenia resulting from antibodies that cause ex vivo platelet agglutination) and to suggest various nonimmune causes of thrombocytopenia, such as toxic leukocytes indicating infection or fragmented red cells suggesting microangiopathic hemolysis. In contrast, primary immune thrombocytopenia is usually characterized by a reduction in platelet number with otherwise unremarkable morphologic features of all cell lines.

### Platelet size and platelet RNA

A particle counter is also used to determine average platelet size, or mean platelet volume (MPV), which usually ranges from 7.0 to 10.5 fL. Disorders of increased platelet destruction usually are characterized by large platelets, and MPV ranges from 10 to 15 fL. Normal-sized or small platelets are common in disorders of underproduction or sequestration of platelets.

Young platelets contain residual amounts of RNA, which can be detected by means of flow cytometric analysis of platelets labeled with either thiazole orange, auramine-O, or SYTO-13. However, such quantitation of reticulated platelets (immature platelet count [IPC]) has not gained the acceptance that red cell reticulocyte assays have. Elevated IPC is associated with greater risk of major adverse cardiovascular events<sup>7</sup> (although a causal vs. confounded relationship remains uncertain).<sup>8</sup> Elevated IPC can be seen in consumptive/destructive thrombocytopenic disorders,<sup>9</sup> including

ITP,<sup>10</sup> and reduced IPC may identify subgroups of patients with ITP who have a marked defect in platelet production.<sup>11</sup>

### Bone marrow examination

Disorders of increased platelet destruction are characterized by normal or increased numbers of megakaryocytes in the marrow.<sup>12</sup> Sometimes examination of the marrow yields enough information to determine the cause of the thrombocytopenia, such as myelodysplasia or megaloblastic anemia.

### Measurement of platelet lifespan

A platelet survival study is the definitive test for classifying the cause of thrombocytopenia and determining the organ site(s) of platelet destruction (or sequestration). Indium-111 is the radiolabel of choice because of its higher labeling efficiency and efficient range of  $\gamma$  emissions. Three patterns of platelet survival can be observed: (1) normal platelet recovery (60–75%) and a normal survival time (7–10 days) characterize thrombocytopenia caused by underproduction; (2) markedly reduced platelet lifespan (hours) is found in patients with thrombocytopenia caused by increased platelet destruction; and (3) reduced platelet recovery (10–30%) with a normal or near-normal platelet survival time is consistent with platelet sequestration (hypersplenism). Platelet survival studies are rarely performed because these tests are complex and experienced physicians can usually infer the mechanism of the thrombocytopenia from the clinical situation. Nonetheless, platelet survival studies can be informative. For example, one study<sup>13</sup> found that spleen-predominant platelet sequestration in ITP predicted a durable remission following splenectomy.

### Platelet-antibody assays

There are two broad categories of platelet antibody assays: (1) platelet-associated IgG assays and (2) assays that identify the protein target of the antibody (protein-specific assays).

#### Platelet-associated IgG assays

Measurement of platelet-associated IgG (PAIgG) has been widely available for many years. It can be used to detect surface-associated immunoglobulin or complement directly on the patient platelets using a labeled anti-immunoglobulin (and/or anticomplement) probe or total PAIgG measured after platelet lysis. Although these assays are simple, a disadvantage is that platelet membranes nonspecifically adsorb IgG and other proteins from the plasma and can transfer them to internal compartments. Furthermore, even monoclonal anti-immunoglobulin probes may not differentiate specific from nonspecific bound IgG. These assays have limited diagnostic usefulness because a positive test result does not differentiate immune from nonimmune thrombocytopenia.<sup>14</sup> Nevertheless, this type of assay can be diagnostically useful in certain situations, such as the laboratory evaluation of drug-induced ITP, by detecting a drug-dependent increase in platelet surface-bound IgG in the presence of patient serum and implicated drug, using an anti-immunoglobulin probe labeled with a fluorescent marker.<sup>15</sup>

#### Protein-specific platelet-antibody assays

The diagnostic usefulness of platelet-antibody assays has increased dramatically with the introduction of various protein-specific assays that help identify the platelet protein target of antibodies with either monoclonal antibodies or electrophoretic techniques. Current protein-specific antibody assays use detergents to extract the glycoprotein (GP) target from the membrane. This process increases the specificity of the test, but may affect antigenicity

because the use of detergents and certain inhibitors (e.g., EDTA) may alter the structure of certain GP targets, such as the GPIIbIIIa complex, reducing the reactivity of some antibodies to HPA-1a.<sup>16</sup>

These assays are only available in specialized reference laboratories (e.g., Versiti, Milwaukee, WI, USA; McMaster Platelet Immunology Laboratory, Hamilton, Canada). Further, the assays are usually developed “in-house” using reagents that can vary among laboratories. Although commercial kits are available for some tests for use in larger, nonreference laboratories, they generally have a reduced repertoire of antigens for testing and may be limited in their availability (e.g., the PakAuto<sup>®</sup> [Immucor] assay was discontinued in the USA). Although sensitivity of some assays is limited (e.g., protein-specific platelet-antibody assays for the diagnosis of ITP), other tests can be crucial for diagnosis and management, mandating referral of patient material to reference laboratories with the appropriate expertise (e.g., diagnosis and management of neonatal alloimmune thrombocytopenia).

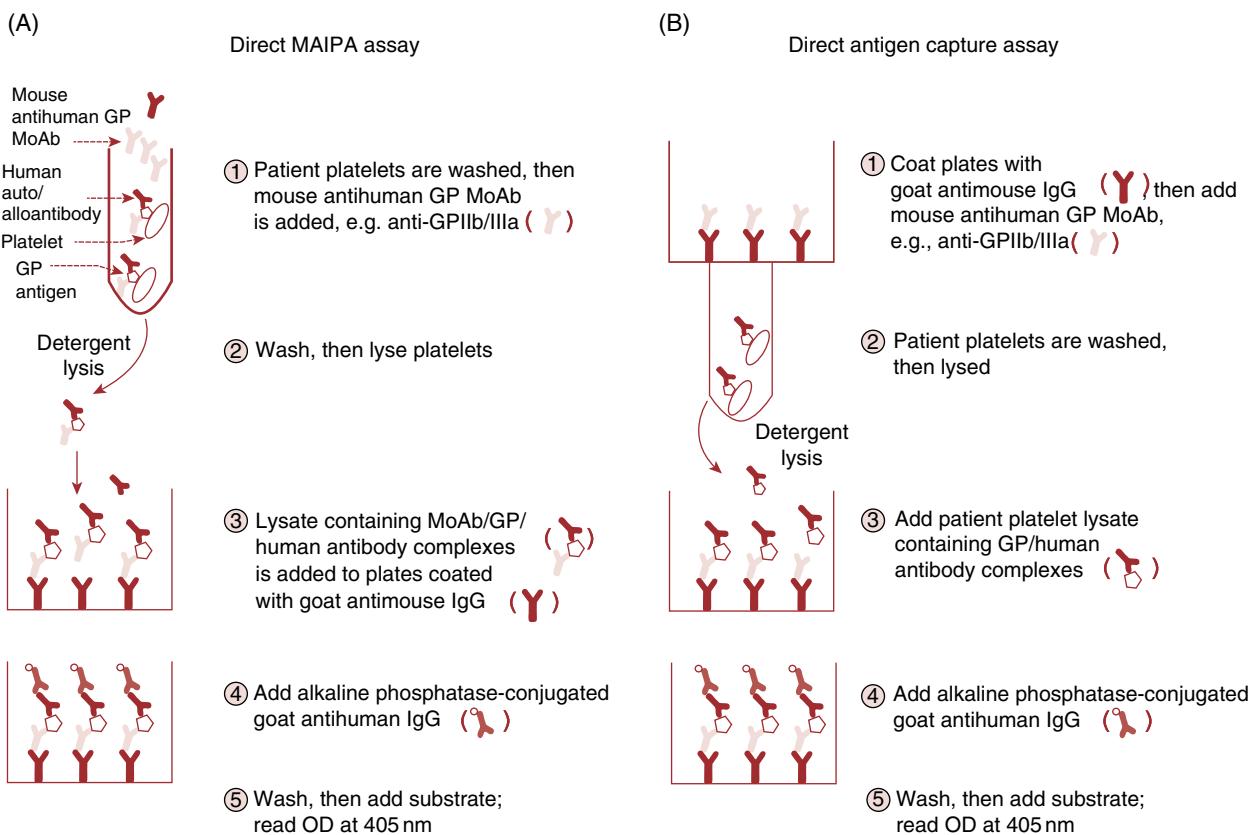
#### Monoclonal antibody-based assays

Various monoclonal antibody-based assays can be used to detect platelet antibodies. Perhaps most widely used is the monoclonal antibody immobilization of platelet antigen (MAIPA) assay. An improved modified MAIPA assay<sup>17</sup> is shown in Figure 36.1A. A technically simpler assay is the antigen capture enzyme immunoassay, in which platelet GP monoclonal antibodies interact with detergent-solubilized platelet samples (Figure 36.1B) rather than intact platelets.<sup>15,18</sup> Simplicity and improved specificity are advantages of these assays, especially the antigen capture assay. In addition to investigation of autoimmune thrombocytopenia, these assays can be adapted to study alloimmune disorders with a panel of platelet glycoproteins of known alloantigen phenotype, or drug-induced thrombocytopenia through demonstration of drug-dependent binding of antibody to specific platelet glycoproteins.<sup>15</sup> A disadvantage is that the identity of the target protein (and thus the monoclonal antibody to be used in the test) must be known in advance, and a number of different monoclonal antibodies are often needed. These assays also can give false-negative results if the patient antibody competes for the same epitope recognized by the monoclonal antibody. Some human sera contain antibodies that recognize murine IgG, which is why the modified MAIPA and antigen capture assays are preferred to the original MAIPA (to avoid a false-positive result).<sup>17</sup>

#### Immunoprecipitation

Immunoprecipitation has the advantage of being able to identify novel protein targets of platelet antibodies and does not require the use of a known monoclonal antibody in advance. It is performed by incubating patient serum or plasma with platelets labeled with iodine-125 or tagged with nonradioactive biotin.<sup>19</sup> The proteins are then solubilized by the addition of detergent, and the antibody-protein complex is precipitated by addition of an anti-immunoglobulin bound to a solid phase (e.g., immobilized staphylococcal protein A or G). The labeled protein-antibody complexes are washed, eluted from the beads, and the platelet proteins are separated by denaturing gel electrophoresis and detected by autoradiography or use of enzyme-conjugated streptavidin. The target antigen is identified according to its electrophoretic mobility. Either the patient's platelets are used (direct immunoprecipitation), or patient serum or plasma is mixed with target platelets (indirect immunoprecipitation).

Immunoprecipitation offers advantages over immunoblotting because the antibody reacts with native, rather than denatured, platelet proteins. In particular, this technique has allowed detection of clinically significant antibodies against previously unrecognized



**Figure 36.1** Modified monoclonal antibody immobilization of platelet antigen (MAIPA) assay and antigen capture assay. “Direct” versions of both assays are shown, that is, the patient’s platelets are tested for the presence of platelet glycoprotein (GP)-bound autoantibodies. (In indirect assays, the patient’s serum or plasma is added to target platelets to detect the presence of serum or plasma platelet glycoprotein antibodies.) (A) In the MAIPA assay, the antihuman GP monoclonal antibodies (MoAbs) are added to washed intact patient platelets before detergent lysis.<sup>17</sup> (B) In the antigen capture assay, the patient’s platelets are washed, then lysed, and added to capture GP MoAb precoated on the EIA plate.<sup>18</sup> However, both assays resemble each other in the final stages in which enzyme-conjugated antihuman IgG is added followed by substrate (steps 4 and 5). A relative advantage of the antigen capture assay is that the platelet lysate can be stored and the assay performed later using any MoAb of choice, making this a technically simpler and more versatile assay. OD: optical density.

platelet proteins, such as anti-HPA-15 (anti-Gov) on CD109, a 175-kD glycosylphosphatidylinositol (GPI)-anchored protein.<sup>20</sup> The main disadvantage of immunoprecipitation is technical difficulty. In addition, not all platelet proteins are optimally labeled (e.g., GPVI).

#### Immunoblotting (Western blotting)

In immunoblotting, patient serum is allowed to interact with platelet proteins that have been electrophoretically separated and then immobilized onto a solid phase (nitrocellulose). The test serum is added, and binding of patient antibody to specific protein bands is detected with labeled anti-immunoglobulin. Although immunoblotting offers the advantage of simplicity and the opportunity to store the immobilized proteins for long periods, a major disadvantage is that protein antigens are denatured in this method. This can destroy some platelet antigens (e.g., HPA-5 and HPA-15) and may modify or expose epitopes to which normal sera can react (e.g., vinculin and talin), making interpretation difficult.<sup>20,21</sup>

#### Surface plasmon resonance (SPR)

A problem with many immunoassays is the requirement for wash steps. Even relatively gentle washes and dilutions can disrupt the binding of lower affinity antibodies to their glycoprotein targets. SPR was developed to investigate interactions between biomolecules

in real time. The change in refractive index at the interface between a gold surface and media can be measured as molecules in the fluid phase are passed over their targets bound to the surface. SPR has been used to investigate serum platelet antibodies, which may be low avidity and thus may escape detection by conventional protein binding assays. For example, SPR was used to confirm the diagnosis of NAIT in women who were suspected of having the disease but tested negative in other conventional immunoassays.<sup>22,23</sup> An alternative method for measuring low affinity binding uses a dip-and-read platform, and has the potential for higher throughput than SPR. Biolayer interferometry (BLI) measures interference patterns associated with binding of protein in a well to its partner immobilized on the biosensor.<sup>24</sup> A disadvantage of both SPR and BLI is the requirement for purified target protein to bind to the gold surface or sensor, limiting the method to abundant platelet glycoproteins that can be isolated efficiently. SPR and BLI have not yet been adopted into routine practice for platelet antibody investigations.

#### Tests for heparin-induced thrombocytopenia

Special tests are required to detect the antibodies that cause HIT. These assays are classified as platelet activation or platelet factor 4 (PF4)-dependent antigen assays (or PF4-dependent immunoassays).<sup>25</sup>

### Platelet activation assays

Heparin-induced thrombocytopenia antibodies have potent heparin-dependent, platelet-activating properties. Aggregation of platelets (prepared as citrate-anticoagulated platelet-rich plasma) by patient plasma detected with conventional platelet aggregometry once was widely used for HIT; however, the sensitivity of this assay is relatively low (~70%) for detecting HIT antibodies.<sup>26</sup> In contrast, assays performed with washed platelets that have been resuspended in calcium-containing buffer, such as the platelet serotonin release assay (SRA) or heparin-induced platelet activation assay (HIPA), have both high sensitivity (~95%) and specificity (~95%) for detecting clinically significant HIT antibodies.<sup>25</sup> These assays are not widely available because they are technically demanding, require careful platelet donor selection, and require a panel of strong and weak positive serum controls. HIT antibodies have a characteristic activation profile: platelet activation at therapeutic, but not high, heparin concentrations and inhibition of activation by Fc receptor-blocking monoclonal antibodies. It should be noted that strong serum-induced platelet activation that occurs in the absence of heparin is a feature of severe HIT, including “delayed-onset” and “spontaneous” HIT (discussed in this chapter). In recent years, PF4-dependent platelet activation assays have been developed;<sup>27–29</sup> here, PF4 (with or without heparin) is added, so as to improve diagnostic sensitivity, given that occasional HIT sera test is negative in the SRA (so-called “SRA-negative HIT”).<sup>30,31</sup>

### PF4-dependent enzyme immunoassays

In 1992, Amiral *et al.*<sup>32</sup> identified PF4-heparin complexes as the antigen of HIT. Both commercial and in-house antigen assays based on enzyme immunoassay (EIA) methods have since become available. An intriguing feature of the HIT antigen is that certain polyanions other than heparin render PF4 antigenic. This is the basis of the PF4-polyvinylsulfonate EIA for PF4-heparin antibodies.<sup>33</sup> PF4-heparin EIAs are highly sensitive for HIT (>99%) but have relatively low diagnostic specificity.<sup>25</sup> An important phenomenon is that greater reactivity in a PF4-heparin EIA (expressed in optical density units) predicts for a greater likelihood of platelet-activating antibodies being detectable, and hence a diagnosis of HIT.<sup>34,35</sup>

### Rapid (on-demand) PF4-dependent immunoassays

Although EIAs can be performed in a few hours, in most situations, samples are tested in batches, and so results are often obtained one or more days after ordering the test. In contrast, several rapid “on-demand” PF4-dependent immunoassays have become available in recent years. Two are available in the United States: the latex-enhanced immunoturbidimetric assay (LIA)<sup>35</sup> and the chemiluminescence immunoassay (CLIA).<sup>36</sup> Both assays have high (~90–98%) sensitivity, and specificity that is higher than EIAs. For both the LIA and CLIA, results are reported semiquantitatively (in “units per mL”), and—as with EIAs—greater reactivity predicts for a higher likelihood of SRA-positive status and hence a diagnosis of HIT. Two other rapid PF4-dependent immunoassays that are widely used outside of the United States are the particle gel immunoassay (PaGIA)<sup>37</sup> and the lateral flow immunoassay (LFI).<sup>38</sup> Recently, approaches have been described in which clinical probability has been integrated with testing using two different immunoassays (e.g., LIA and CLIA;<sup>39</sup> PaGIA and CLIA<sup>40</sup>) so as to optimize diagnostic sensitivity and provide real-time Bayesian (integrated clinical/laboratory probability-based) diagnosis.

### Platelet genotyping

Serologic assays are generally reliable for detecting platelet alloantibodies. They also have been used to identify platelet antigens (platelet phenotyping). However, a disadvantage of antibody-based analysis is that there may not be sufficient platelets available from a patient with severe thrombocytopenia (e.g., post-transfusion purpura [PTP]) to allow determination of the reciprocal platelet alloantigen phenotype.<sup>41</sup> Second, specific phenotyping sera are available for only a few of the platelet antigens. Molecular techniques provide a reliable alternative to serologic phenotyping. Genomic DNA is used to determine the corresponding platelet alloantigen genotype and is readily available from a number of sources. Molecular techniques are particularly useful in the evaluation of suspected NAIT. Analysis of fetal cells (obtained by amniocentesis, chorionic villus sampling, or sampling of fetal blood) can determine whether a fetus is at risk for this complication. Small amounts of tissue can be studied (e.g., 5–10 mL of amniotic fluid) because the technique of polymerase chain reaction (PCR) greatly amplifies the DNA. The possibility of significant maternal DNA contamination of a fetal sample can be assessed with the forensic technique of variable-number tandem repeat analysis of the sample.<sup>42</sup>

### Polymerase chain reaction and restriction fragment length polymorphism

For all platelet alloantigen polymorphisms but one (HPA-4), the single-base substitution responsible for the change in the expressed amino acid is associated with a restriction endonuclease recognition site.<sup>41</sup> Accordingly, restriction fragment length polymorphism (RFLP) analysis was the first genotyping assay developed to identify platelet alloantigens. In the PCR-RFLP method, a section of DNA that encompasses the polymorphism is amplified using a pair of sense–antisense primers. The amplified product is subjected to restriction enzyme digestion, which cuts the amplified DNA into fragments depending on the nucleotide sequence. These are separated using agarose gel electrophoresis to identify the size and number of fragments, which indicate the platelet antigen genotype. One limitation is the possibility that another polymorphism within the amplified fragment could confound the genotyping by interfering with the restriction enzyme.<sup>43</sup> Phenotyping methods do not appear to be affected, suggesting that multiple methods for antigen typing may be warranted.<sup>44</sup>

### Allele-specific polymerase chain reaction

Because PCR-RFLP is a comparatively labor-intensive technique, allele- or sequence-specific PCR (SSP-PCR) is commonly used. For this technique, one of the primers is specific for the particular allele to be amplified.<sup>45</sup> When the specific nucleotide corresponding to the allelic polymorphism is positioned at the 3' end of the oligonucleotide primer, efficient amplification occurs only when the primer is 100% complementary to the genomic sequence. The advantage of this method is that the PCR product is visualized directly in agarose gels to determine the genotype. Appropriate controls must be included in the assay, including additional primers to amplify a ubiquitous gene (e.g., human growth hormone) to ensure that all assay constituents are working properly.

### Real-time polymerase chain reaction

A modification of SSP-PCR is real-time PCR. Real-time PCR has improved both the speed and accuracy of genotyping.<sup>46</sup> The use of specifically designed hybridization probes tagged with fluorescent

dyes for the PCR allows direct determination of the platelet genotype without gel electrophoresis or restriction enzymes in a single reaction. One hybridization probe, the donor, is tagged with a dye (fluorescein) and emits light at a specific wavelength when excited by a light source. The other probe, the acceptor, is tagged with a different dye. It straddles the single nucleotide polymorphism of interest and is 100% homologous to one of the platelet alleles. The acceptor probe usually is one nucleotide away from the donor probe in a head-to-tail arrangement, that is, the dyes are juxtaposed. The energy emitted by the dye of the donor probe is transferred to the adjacent dye of the acceptor probe, which emits light at a different wavelength. The intensity of the second light emitted is proportional to the amount of double-stranded DNA present. Unbound donor probe in the mixture can be excited but cannot transfer energy to the acceptor probe. On completion of the PCR, the platelet genotype is determined by means of melting curve analysis. Because the acceptor probe straddles the polymorphism and is 100% homologous to one of the alleles, the melting curve has a different temperature midpoint ( $T_m$ ) depending on whether a mismatch is present. The acceptor probe can have as much as 5–8 °C difference in  $T_m$  between the two platelet alleles. In all, three melting curves are seen, one for each polymorphism and a composite melting curve when the heterozygous situation is present.<sup>46</sup> The advantage of fluorescence-based real-time PCR with melting curve analysis is that the PCR product is measured directly, and no further manipulation is required. Any additional polymorphism in the region of the acceptor probe is detected in the melting curve analysis. Rapid thermal cycling and DNA extraction permit the determination of a platelet genotype within two hours of specimen collection. Noninvasive prenatal diagnosis requires higher sensitivity to detect fetal DNA in maternal plasma. PCR methods using next-generation sequencing (NGS), bead chip techniques, or droplet digital PCR have shown promise for these investigations.<sup>47,48</sup>

### Immune-mediated thrombocytopenic syndromes

The principal mechanism for immune-mediated thrombocytopenia is antibody-mediated destruction of platelets. The IgG-sensitized platelets are phagocytosed by monocytes and macrophages of the reticuloendothelial system. Reticuloendothelial cells are located throughout the body but are concentrated in the spleen, liver, lungs, and marrow. Pathogenic antibodies bind to platelets via their Fab termini, usually against specific autoantigen or alloantigen epitopes. Sometimes the target antigen is induced by a drug or drug metabolite. The result is a ternary complex that involves IgG, a drug, and a specific region on a platelet glycoprotein. Heparin-induced thrombocytopenia is an exception to these generalizations: Although HIT antibodies bind to PF4-heparin complexes via the Fab terminus, the Fc portion of IgG interacts with platelet Fc $\gamma$ IIa receptors, resulting in platelet activation.<sup>6</sup>

The rate of platelet destruction is determined by the quantity and subclass distribution of IgG on the platelet, the amount of complement, and the efficiency of reticuloendothelial clearance. The severity of thrombocytopenia reflects the balance between the rate of platelet destruction and the compensatory marrow thrombopoiesis. Immune mechanisms can also target megakaryocytes in the bone marrow leading to reduced platelet production.<sup>49</sup>

### Immune thrombocytopenia (ITP)

Immune thrombocytopenia (ITP), previously known as primary idiopathic thrombocytopenic purpura, is a common disorder characterized by increased platelet destruction and impaired platelet production. It is a diagnosis of exclusion, defined by isolated thrombocytopenia with no other clinically apparent cause. Secondary ITP can occur in the setting of human immunodeficiency virus (HIV), hepatitis C, and *Helicobacter pylori* infections; systemic lupus erythematosus (SLE); certain drugs; and lymphoproliferative disorders. Causes of nonimmune thrombocytopenia that are often confused with ITP include myelodysplastic syndrome, splenomegaly, and familial thrombocytopenia.<sup>50–52</sup> Platelet-antibody assays detect platelet GP-reactive autoantibodies in 40–80% of patients;<sup>53</sup> this variability results from heterogeneity of the patient population studied and the particular tests performed. Overall, low assay sensitivity indicates that a negative test does not exclude the diagnosis of ITP.<sup>18,54</sup>

### Pathogenesis

Over 50 years ago, Harrington *et al.*<sup>55</sup> showed that ITP plasma infused into healthy volunteers caused acute severe thrombocytopenia. The platelet-destroying plasma factor was later shown to be IgG, although in some patients, IgM and IgA antibodies may be pathogenic. The immune target of the autoantibodies usually is one of the two major platelet glycoprotein complexes, with GPIIb/IIIa implicated more often than GPIb/IX.<sup>56</sup> Other less common autoantigen targets include GPIa/IIa,<sup>56</sup> GPV,<sup>57</sup> and, possibly, nonprotein targets such as glycosphingolipids. The autoantibodies bind to platelets by way of their Fab terminus and cause premature platelet destruction via Fc receptor (Fc $\gamma$ R)-mediated phagocytosis by macrophages in the spleen and other reticuloendothelial tissues. Platelet autoantibodies can also activate complement in vitro. Another proposed mechanism of platelet destruction is direct platelet lysis by cytotoxic T cells.<sup>58,59</sup>

Platelet production is also impaired in ITP. Platelet turnover is lower than expected, as shown by radiolabeled autologous platelet studies,<sup>60</sup> and some patients who respond to thrombopoietin (TPO)-based treatments have demonstrated an increase in the number of reticulated platelets, suggesting that this compensation was not maximal.<sup>11</sup> An increased megakaryocyte mass that has been observed in some patients may account for the relative TPO deficiency because these cells bind free TPO. Conversely, platelet underproduction may result from megakaryocyte injury caused by autoantibodies.<sup>61–63</sup> Another mechanism that may cause impaired platelet production is antibody-mediated desialylation of platelet glycoproteins, which leads to platelet clearance by the Ashwell-Morell receptors in the liver<sup>64</sup> as well as to impaired platelet production.<sup>65</sup>

The cause of autoantibody formation in ITP is not known. Light chain and immunoglobulin subclass restriction of platelet-reactive antibodies suggests an oligoclonal origin of autoantibodies in chronic ITP.<sup>66</sup> Furthermore, the autoepitopes involved may be fairly restricted in scope. Autoantibodies are produced by B lymphocytes, and the loss of T-cell tolerance to platelet proteins is a key feature.

### Clinical and laboratory features

Stages of ITP have been defined by the international ITP Working Group: newly diagnosed ITP (within three months), persistent ITP (3–12 months), and chronic ITP (more than 12 months).<sup>67</sup> The prevalence of ITP has been estimated at 12.1 (95% confidence interval [CI], 11.1–13.0) per 100,000 adults.<sup>68</sup> If the clinical evaluation

reveals other signs or symptoms, e.g., weight loss, fever, lymphadenopathy, hepatomegaly, or splenomegaly, other diagnoses should be considered. Laboratory testing for patients with ITP should include evaluation of the peripheral blood film and testing for HIV, hepatitis C, and (when rituximab treatment is anticipated) hepatitis B, as well as quantitative immunoglobulin levels, thyroid function screening, and antiphospholipid antibody testing.<sup>51,52</sup> *H. pylori* testing should be considered, especially for patients from Japan.

Mucocutaneous bleeding is the hallmark of ITP and manifests as purpura (petechiae and ecchymosis), epistaxis, menorrhagia, oral mucosal bleeding, and gastrointestinal bleeding. Severe bleeding occurs in 9.6% of adults and 20.2% of children, and the incidence of intracerebral hemorrhage is 1.4% for adults and 0.4% for children.<sup>69</sup> The frequency of severe bleeding is highest among patients with chronic ITP, patients with previous bleeding, and patients over 60 years of age.<sup>70</sup> Male sex and heavily pretreated ITP are also risk factors for severe bleeding.<sup>71</sup> On the other hand, many patients have minimal or no bleeding despite very low platelet count levels, possibly because of the presence of young, reticulated platelets with enhanced  $\alpha$ -granule release to platelet agonists.<sup>72</sup> When choosing whether and how to treat ITP, clinicians should remember that morbidity in ITP patients is often attributable to adverse effects of the treatments administered rather than the disease itself.<sup>73</sup>

### Treatment overview

Treatment strategies for ITP are based on the following principles:

- 1** Many patients do not bleed, even with low platelet counts ( $<30 \times 10^9/L$ ), and many patients may not need treatment.
- 2** The goal of treatment is to achieve a safe platelet count, not necessarily a normal count.
- 3** The urgency of treatment will dictate the type of therapy: platelet transfusions may be useful when immediate (but transient) hemostasis is needed, intravenous immunoglobulin (IVIG) will start to increase the platelet count in most patients by 12–24 hours, and corticosteroids typically result in a platelet count response within 3–7 days.
- 4** Most adults have chronic ITP and only approximately 20% will have a spontaneous remission, whereas most children have acute ITP that often improve spontaneously or with minimal treatment.
- 5** Many adults will require treatment beyond first-line therapies, but the least amount of treatment should be used to maintain a hemostatic platelet count in patients with chronic ITP.
- 6** Drugs that interfere with platelet function—particularly aspirin, nonsteroidal anti-inflammatory agents, and alcohol—should be avoided.

### First-line therapies

#### Corticosteroids

Corticosteroids, either prednisone (0.5–2 mg/kg/day, maximum dose 80 mg, followed by a taper) or high-dose dexamethasone (40 mg/day for four consecutive days, repeated monthly if necessary), are considered first-line treatments. To avoid toxicity from long-term exposure, the prednisone dose should generally be tapered and discontinued after eight weeks (and perhaps more rapidly in patients who do not achieve a platelet count response). Two prospective studies<sup>74,75</sup> compared conventional and lower dose prednisone therapy (1 mg/kg/day vs. 0.25 mg/kg/day in one trial; 1.5 mg/kg/day vs. 0.5 mg/kg/day in the other), and in neither study was there a significant difference in remission at six-month follow-up evaluation. In the larger study,<sup>75</sup> however, the higher dose

regimen showed a trend to a higher rate of complete remission (46% vs. 35%) as well as higher platelet counts at 14-day follow-up evaluation (77% vs. 51% having a platelet count greater than 50,000/ $\mu L$ ).

High-dose dexamethasone has been used with success in patients with newly diagnosed ITP. In one study ( $n = 125$ ), 84% of patients with acute ITP treated with a single course of high-dose dexamethasone (40 mg/day for four days) achieved a platelet count response ( $>50,000/\mu L$ ), and of those, 50% had a response that lasted from 2 to 5 years.<sup>76</sup> In another study ( $n = 37$ ), six courses of monthly high-dose dexamethasone resulted in an overall response rate of 83.8%, and a sustained response of 64.9% after two years.<sup>77</sup> However, repeated cycles of high-dose dexamethasone were often poorly tolerated, and these high rates of durable response have not been consistent in practice. In patients with chronic ITP, responses with high-dose dexamethasone are variable. In one study, all 10 treated patients achieved a response that lasted six months following six cycles of high-dose dexamethasone (40 mg/day for four days).<sup>78</sup> However, other subsequent studies were less encouraging. Using the same regimen, Stasi *et al.*<sup>79</sup> reported that 13 of 32 patients (40.6%) had transient responses only, and in the study by Warner *et al.*,<sup>80</sup> none of nine patients responded, and five could not tolerate the treatment.

In a systematic review<sup>81</sup> of randomized trials comparing high-dose dexamethasone and prednisone in adults with previously untreated ITP ( $n = 533$ ), treatment with dexamethasone resulted in improved overall (79% vs. 59%;  $p = .048$ ) and complete platelet count response (64% vs. 36%;  $p = .040$ ) without excess toxicity. Platelet count responses occurred more rapidly with high-dose dexamethasone (within 14 days); however, sustained responses at six months were not different between groups.

Adverse effects of corticosteroids include facial swelling, weight gain, and behavioral changes in up to 20% of patients.<sup>50,82</sup> Less common (1–5%) complications include infection, myopathy, hyperglycemia, psychosis, hypertension, hypokalemia, and osteoporosis. Osteonecrosis, most commonly involving the femoral head, occurs as a late side effect in approximately 5% of patients who undergo prolonged therapy, but it may occur even after intensive short-term exposure.<sup>83</sup>

#### Intravenous immunoglobulin

High-dose IVIG has been used to treat patients with ITP since 1981.<sup>84,85</sup> Reticuloendothelial blockade is the principal mechanism of action of IVIG, but many other mechanisms have been proposed, including the reduction of antibody synthesis, IgG molecules against the Fab regions of pathogenic autoantibodies (“anti-idiotypic antibodies”),<sup>86</sup> cytokine-induced pro- or anti-inflammatory effects,<sup>87</sup> up- or downregulation of various Fc $\gamma$ Rs, or the formation of soluble immune complexes. In a mouse model of ITP, the inhibitory IgG receptor, Fc $\gamma$ RIIB, was required for IVIG to cause an elevation in platelet count,<sup>88</sup> and the transfer of IVIG-primed dendritic cells has been shown to recapitulate the effect of IVIG.<sup>89</sup>

The use of IVIG is indicated for patients with ITP with bleeding or for whom an invasive procedure is planned to raise the platelet count quickly. A French study of 122 adults with ITP showed that IVIG (0.7 g/kg/day for three days) raised the platelet count to over 50,000/ $\mu L$  within five days more frequently than did corticosteroids (methylprednisolone, 15 mg/kg/day for three days).<sup>90</sup>

The usual dose of IVIG is 1–2 g/kg, given either as 0.4 g/kg for five consecutive days or as 1 g/kg over two days. One trial showed no difference between one and two doses of 1 g/kg.<sup>91</sup> Thus, our approach

**Table 36.1** Comparison of High-Dose IVIG and Intravenous RhIG for Management of Immune Thrombocytopenic Purpura

Characteristic	High-Dose IVIG	Intravenous RhIG
Side effects	Common: headache, hypertension, fever, and chills	Common: mild hemolysis
Response rate	Rare: hemolysis, renal failure, myocardial infarction, and stroke	Rare: severe hemolysis necessitating transfusion or hemodialysis, and DIC
Response duration	Usually transient	Usually transient
Pattern of platelet increase	Rapid increase (12–24 h), high platelet peak, and shorter duration of response	Slower increase, lower peak, and longer duration of response
Influence of ABO, Rh type	No influence	Only RhD-positive patients respond
Influence of splenectomy	Unknown	Minimal or no response in patients without a spleen
Suitability for emergency management of ITP	Recommended	Not recommended

IVIG: intravenous immune globulin; RhIG: Rh immune globulin; DIC: disseminated intravascular coagulation; ITP: immune thrombocytopenic purpura.

is to give 1 g/kg as a single dose and to repeat the dose one or two days later if no significant platelet count increase has occurred.<sup>92</sup>

IVIG increases the platelet count to greater than 50,000/ $\mu$ L in approximately 80% of adult patients. Responses are usually transient, lasting 2–6 weeks. Tachyphylaxis after repeated courses may be observed in patients with severe ITP.

Common but mild side effects include headache in 10% of patients, backache, nausea, flushing, and fever.<sup>93</sup> Aseptic meningitis may occur rarely. Chest pain, hypertension, hypotension, bronchospasm, and laryngeal edema have been reported. A boxed warning concerning the risk of thrombosis with IVIG was issued by the US Food and Drug Administration (FDA) in 2013 (<http://www.fda.gov>) because arterial and venous thrombosis have rarely been reported, particularly in elderly patients<sup>94</sup> (Table 36.1). Because IVIG is prepared from pooled plasma from thousands of donors, infection transmission is theoretically possible, but this risk has been significantly mitigated by use of viral inactivation methods. Transfusion-related acute lung injury following IVIG has also been described.<sup>95</sup>

#### RH immune globulin

Like IVIG, the mechanism of action of Rh immune globulin (RhIg) is believed to be reticuloendothelial blockade, which occurs through occupancy of the reticuloendothelial cell Fc $\gamma$ Rs by IgG-sensitized red cells.

Doses of 50–75  $\mu$ g/kg of RhIg have been shown to produce a rapid increase in platelet count.<sup>96</sup> Hemolysis is an expected side effect, and rarely severe hemolysis with disseminated intravascular coagulation and renal failure has been reported with fatal outcomes<sup>97,98</sup> (Table 36.1). Because of this, RhIg was voluntarily withdrawn from some European markets in 2009. Its use is usually restricted to nonsplenectomized patients who are Rh-positive and have a negative direct antiglobulin test.

#### Second-line therapies

The principal second-line therapies for ITP are splenectomy, rituximab, and TPO receptor agonists. Immune suppressant medications can also be used as second-line therapy. A new treatment option is fostamatinib, an oral SYK inhibitor (see below). Randomized trials comparing different second-line treatments for ITP are lacking; thus, the choice of therapy depends on individual factors such as patient characteristics and preferences, access, and cost, as described in evidence-based guidelines.

#### Splenectomy

Complete remission of ITP occurs in 70% of patients within 4–6 weeks of splenectomy,<sup>99</sup> presumably because in many patients the spleen is the major site of both autoantibody production and

platelet destruction. In complete responders, normal platelet counts are reached within seven days in 90% and within six weeks in 98% of patients; remission is encountered rarely thereafter. In a further 5–10% of patients, partial remission is achieved. In a systematic review of the efficacy of splenectomy for patients with ITP, younger age was an independent predictor of response to splenectomy;<sup>99</sup> however, other investigators have shown that response to IVIG<sup>100–102</sup> and splenic clearance of platelets (determined by radionuclide platelet imaging techniques)<sup>103</sup> are also useful predictors. The risk of relapse after a complete remission following splenectomy is low (approximately 10–15% at 10 years). Splenectomy is commonly performed using a laparoscopic approach.

Perioperative management of splenectomy involves (1) optimization of the platelet count before surgery, (2) vaccination against encapsulated bacteria to minimize the risk of overwhelming post-splenectomy infection, and (3) prevention of thromboembolism. Adequate perioperative hemostasis can usually be achieved with preoperative high-dose IVIG or corticosteroids, and TPO receptor agonists may be a suitable alternative.<sup>104</sup> However, most patients do not have excessive bleeding; therefore, platelet transfusions should not be administered prophylactically but rather be reserved for the treatment of perioperative bleeding.

The presence of residual accessory spleen should be considered when splenectomy fails, or when patients relapse months or years after surgery.<sup>105</sup> Postsplenectomy blood film changes (e.g., Howell-Jolly bodies) do not necessarily eliminate the possibility of a residual accessory spleen. Unfortunately, a durable platelet count response following accessory splenectomy performed because of postsplenectomy relapse of ITP is uncommon and can be expected in less than 50% of patients.

Perioperative morbidity after splenectomy is less than 10%. The most frequent complications are pleuropulmonary (pneumonia, subphrenic abscess, and pleural effusion) in 4% of patients, major bleeding in 1.5%, and thromboembolism in 1%. Morbidity is lower with a laparoscopic approach by an experienced surgeon. Overall mortality is approximately 1% following laparotomy and 0.2% following laparoscopic splenectomy.<sup>99</sup>

The risk of bacterial infection is higher among asplenic patients. The risk of overwhelming postsplenectomy infection is approximately 1–2%.<sup>106</sup> In a population-based study of 3812 patients who underwent splenectomy, the overall incidence rate of infection was 7.7 per 100 person-years (odds ratio compared to nonsplenectomized patients with an indication for splenectomy, 1.7 [95% CI, 1.5–2.1] in the first 90 days).<sup>107</sup> In a review of the literature, the frequency of infection was 2.1% and mortality was 1.2% among 484 patients who had splenectomy for ITP.<sup>108</sup> Life-threatening infections may occur many years after splenectomy, suggesting that the

risk of severe infection may persist.<sup>109</sup> To reduce the risk of bacterial infection, all patients who undergo splenectomy should receive vaccinations against the encapsulated bacteria *Streptococcus pneumoniae*, *Hemophilus influenzae* type B (HIB), and *Neisseria meningitidis*.

Postoperative thromboembolism is the most common cause of postoperative mortality; thus, for patients with delayed postoperative mobilization, thromboprophylaxis should be considered.

After the diagnosis of ITP, it is recommended to wait at least 12 months before considering splenectomy, as about 20% of patients can achieve a remission within the first year of the disease. Now that additional treatments are available including the TPO receptor agonists, the use of splenectomy has declined; however, it remains a therapeutic option with a high response rate.<sup>110</sup>

### **Rituximab**

Rituximab is a chimeric monoclonal anti-CD20 indicated for the treatment of lymphoma, chronic lymphocytic leukemia, rheumatoid arthritis, and Wegener's granulomatosis. The Fab portion binds to CD20 on B lymphocytes, which results in Fc<sub>Y</sub>R-mediated B-cell lysis by complement-dependent<sup>111</sup> and antibody-dependent pathways.<sup>112–114</sup> In ITP, rituximab depletes CD20-positive B cells, which are responsible for platelet autoantibody production. It can also correct T-cell dysfunction which has been associated with platelet count improvements after rituximab.<sup>115</sup> Rituximab has been associated with durable remissions in up to 20–30% of patients.<sup>116,117</sup>

A systematic review<sup>118</sup> of observational studies described outcomes with rituximab in adults who had ITP for 1–360 months (50% had splenectomy). A complete response to rituximab (platelet count greater than 150,000/ $\mu$ L) was seen in 43.6% of patients (95% CI, 29.5–57.7%) and an overall response (platelet count >50,000/ $\mu$ L) in 62.5% (95% CI, 52.6–72.5%). The median time to response was 5.5 weeks (some responses may occur up to 8–12 weeks), and responses lasted a median of 10.5 months. Data from randomized trials were less optimistic. In a meta-analysis, rituximab plus standard of care was more often associated with a complete platelet count response (platelets >100  $\times$  10<sup>9</sup>/L without rescue treatment) compared with standard of care alone (46.8% vs. 32.5%;  $p = 0.002$ ); however, responses were generally not sustained past 12 months.<sup>119</sup> In some studies, responses were more frequent in females, younger patients (<40 years), patients who had ITP for less than 12 months, and patients with other autoimmune conditions.<sup>120–123</sup>

Results with rituximab in children are less encouraging. In a prospective single-arm study of 36 children with chronic ITP treated with rituximab, 11 (30.6%) achieved a platelet count of 50,000/ $\mu$ L or greater for four consecutive weeks.<sup>124</sup>

Toxicities of rituximab include infusional reactions, hepatitis B reactivation, and a possible association with progressive multifocal leukoencephalopathy.<sup>125</sup> Rituximab has been shown to impair vaccine responses for up to six months.<sup>126</sup>

### **Thrombopoietin receptor agonists**

TPO receptor agonists (TPO-RAs) bind to and activate the c-Mpl receptor on hemopoietic stem cells and megakaryocytes, ultimately leading to increased platelet production. They do not resemble endogenous TPO, and thus do not induce the formation of anti-TPO antibodies (which had previously occurred with pegylated recombinant human megakaryocyte growth and development factor [PEG-rHuMGDF]).<sup>127</sup>

In 2008, two TPO-RAs—romiplostim and eltrombopag—were approved for the treatment of patients with chronic ITP. In 2019,

another TPO-RA, avatrombopag, was also approved by the FDA. Romiplostim is administered by weekly subcutaneous injection, while eltrombopag and avatrombopag are oral daily medications. The clinical success of these medications has been one of the most important advances in ITP treatments since the discovery of IVIG. In phase III trials, each has been shown to be effective in increasing platelet counts compared with placebo or standard of care<sup>128–130</sup> in 60–80% of patients. Responses are sustained as long as the medication is continued even after prolonged use.<sup>131,132</sup> Once the medication is stopped, the platelet counts may drop to pretreatment levels, at which point patients may be at increased risk of bleeding.<sup>133</sup> However, up to 20–30% of patients maintain durable platelet count responses even after TPO-RA cessation.<sup>134</sup>

Side effects of TPO-RAs include thrombosis and bone marrow reticulin formation,<sup>135</sup> however, the risk of thromboembolism appears to be only slightly higher than the baseline risk in patients with ITP. The risk of clinically meaningful bone marrow reticulin formation is low and reticulin formation generally improves when the medication is stopped. Eltrombopag has been associated with serum liver function test abnormalities in approximately 10% of patients. Due to the high response rates, the use of these medications in ITP patients has steadily increased. Their use early in the course of ITP has been associated with improved short-term outcomes.<sup>136</sup> Despite being a safe and effective treatment option, the high cost may limit the prolonged use of TPO-RAs.

### **Fostamatinib**

In April 2018, the spleen tyrosine kinase (Syk) inhibitor, fostamatinib, was approved by the FDA as a novel therapy for the treatment of chronic ITP with an insufficient response to at least one prior therapy. The mechanism of action is to block platelet destruction secondary to antibody-mediated phagocytosis. Two parallel randomized trials<sup>137</sup> for ITP patients with a median disease duration of 8.5 years (many of whom had failed splenectomy, TPO agents, and/or rituximab) showed an overall response rate (platelet count  $\geq$ 50,000/ $\mu$ L) of 43% (vs. 14% on placebo) with the initial dose of fostamatinib 100 mg twice daily. The time to response was a median of 15 days. Stable response (platelets  $\geq$ 50,000/ $\mu$ L for at least four of six weeks) was achieved in only 18% of patients. Diarrhea and hypertension were the most frequent side effects.<sup>138</sup>

### **Immunosuppressive agents**

Mycophenolate, azathioprine, and cyclosporin are immunosuppressive agents that have been used to treat patients with refractory ITP. Cyclophosphamide, an alkylating agent given in doses of 1–2 mg/kg/day, has also been used. Azathioprine and mycophenolate are purine antimetabolites that inhibit lymphocyte proliferation. Cyclosporin is a calcineurin inhibitor that selectively blocks T-cell-dependent biosynthesis of lymphokines, particularly interleukin-2, at the level of messenger RNA transcription.

These drugs, alone or in combination, have shown moderate success, with up to two-thirds of patients exhibiting a platelet count response.<sup>139–141</sup> The combination of azathioprine, mycophenolate, and cyclosporin may achieve an overall response rate of 73.7%.<sup>142</sup> However, only 20–30% have responses that persist after stopping the drug(s). Responses may occur as early as two weeks and as late as three months after initiation of treatment. The early use of mycophenolate added to corticosteroids is currently being evaluated in a randomized trial.<sup>143</sup> Azathioprine can have myelosuppressive effects on hematopoietic cells and may cause severe bone marrow toxicity in patients who lack activity of the enzyme

thiopurine methyltransferase (TPMT) (1 in 300 individuals).<sup>144</sup> Mycophenolate may also be associated with leukopenia. Cyclosporin can be associated with hypertension and renal failure. Concerns over leukemic transformation and other toxicities (hepatic, hemorrhagic cystitis) have limited the use of cyclophosphamide.<sup>145</sup>

### Danazol

Danazol is an attenuated androgen with mild virilizing effects that can be used to treat men and nonpregnant women with ITP. Its mechanism of action is to decrease Fc<sub>γ</sub>R numbers and the rate of Fc<sub>γ</sub>R-mediated clearance of IgG-sensitized cells. Danazol decreases the number of monocyte IgG Fc receptors.<sup>146</sup> Usually, 400–800 mg is administered daily in divided doses. Time to response is approximately one to two months.

Danazol can produce an increase in platelet count in approximately 30–40% of patients. The response rate may be higher among patients with associated rheumatologic disorders.<sup>147</sup> Danazol must be continued to maintain the platelet count response, although attempts at dose reduction should be made. Danazol is generally well tolerated but may be associated with virilizing side effects in women, liver dysfunction, and rash.

### Vinca alkaloids

Vinca alkaloids (vincristine and vinblastine) can produce generally short-lived increases in platelet count in approximately 65% of patients.<sup>148</sup> These drugs bind to platelet microtubules and may work by being delivered to, and thereby inhibiting, reticuloendothelial macrophages. Repeated doses of vinca alkaloids are rarely used to treat patients with chronic ITP, mostly because of dose-dependent neuropathy.

### Special treatment situations

#### *Emergency treatment of a bleeding patient*

For life-threatening bleeding, platelet transfusions may transiently increase platelet counts and improve hemostasis. Other treatments should be used in combination to provide a rapid increase in platelet count, including high-dose IVIG to block the reticuloendothelial system and corticosteroids for longer-term disease control. TPO receptor agonists can also be considered, in addition to other hemostatic medications such as tranexamic acid and recombinant factor VIIa if life-threatening bleeding cannot be controlled.

#### *Preparation for invasive procedures*

High-dose IVIG usually is the treatment of choice for severely thrombocytopenic patients with ITP who need urgent surgery or an invasive procedure. When at least two or three days are available before the planned procedure, less expensive corticosteroids may be an option, but these are associated with toxicities that are particularly concerning in the perioperative period, including delayed wound healing and hyperglycemia. In a randomized trial, eltrombopag was shown to be noninferior to IVIG at achieving and maintaining surgical platelet count thresholds (50,000/ $\mu$ L for minor surgery or 100,000/ $\mu$ L for major surgery) during the perioperative period.<sup>149</sup>

#### *Immune thrombocytopenia in pregnancy*

Thrombocytopenia during pregnancy is usually not caused by ITP. Rather, a benign condition known as *gestational thrombocytopenia*, or *incidental thrombocytopenia of pregnancy*, is more common, occurring in approximately 5% of pregnancies at term. In this condition, maternal platelet counts fall moderately, but usually not

below 70,000/ $\mu$ L.<sup>150</sup> Newborns of mothers with incidental thrombocytopenia are not at increased risk of neonatal thrombocytopenia.<sup>151</sup> The second most likely cause of thrombocytopenia is pregnancy-related thrombocytopenic disorders (e.g., preeclampsia and syndrome of hemolysis, elevated liver enzymes, and low platelet count [HELLP syndrome]). Preeclampsia occurs in approximately 10% of pregnancies and causes thrombocytopenia in one-fourth of affected mothers. Preeclampsia is associated with increased maternal and fetal morbidity and mortality. Secondary causes of immune thrombocytopenia that can occur in young women, such as SLE or HIV infection, should also be considered in the appropriate clinical context.

Nonetheless, when ITP occurs in pregnancy it is an important disorder to recognize because of the treatment implications for the mother and the possibility of fetal or neonatal thrombocytopenia caused by transplacental passage of IgG platelet autoantibodies. In the past, infants of mothers with ITP were delivered by cesarean section; however, today, this procedure is not routinely recommended for two reasons. First, the frequency of severe fetal thrombocytopenia (platelet count less than 20,000/ $\mu$ L) is low (approximately 4%).<sup>152</sup> Second, there is no evidence that cesarean section leads to less intracranial bleeding compared with vaginal delivery.

Many pregnant women with ITP do not need specific treatment, unless the platelet count decreases below 20,000/ $\mu$ L or there is evidence of impaired hemostasis. The two preferred treatment options are intermittent high-dose IVIG and prednisone; however, IVIG is generally the treatment of choice (1 g/kg every 2–4 weeks) because of its favorable side effect profile compared with prednisone (which is associated with maternal hypertension and hyperglycemia and, possibly, fetal complications). Splenectomy is rarely indicated in pregnancy and should only be considered in the second trimester.<sup>50</sup> Azathioprine is safe in pregnancy but other immunosuppressant medications should generally be avoided. Rituximab is not recommended, although it appears to be relatively safe. Similarly, TPO receptor agonists are not recommended in pregnancy, but cohort studies have demonstrated success without significant toxicities.<sup>153</sup>

The mode of delivery should depend on obstetrical indications since vaginal deliveries are safe for mothers with ITP. Reasonable platelet count thresholds are 20,000–30,000/ $\mu$ L for vaginal delivery, 50,000/ $\mu$ L for caesarean section, and 80,000–100,000/ $\mu$ L for epidural anesthesia. In a relatively large cohort of infants born to mothers with ITP (119 pregnancies in 97 women), 10% of infants had a platelet count below 50,000/ $\mu$ L at birth, 15% required hemostatic treatments, and 1% died.<sup>154</sup> Unfortunately, there are no good predictors of fetal thrombocytopenia, not even the severity of maternal thrombocytopenia (indeed, women cured of ITP by splenectomy can bear infants with passive autoimmune thrombocytopenia).

### Immune thrombocytopenia in children

Acute ITP of childhood is a relatively common and generally self-limited autoimmune disorder.<sup>155</sup> In children, ITP has a peak incidence between 2 and 6 years of age. Boys and girls are equally affected, except for infants, in which males predominate.<sup>156</sup> Most children (80–90%) with acute ITP recover completely within six months; the others have persistent thrombocytopenia.

The typical clinical manifestations are bleeding and bruising following a viral infection. The mortality is approximately 0.4%, and most deaths are caused by intracranial hemorrhage. Laboratory abnormalities include isolated thrombocytopenia and normal or increased MPV. A marrow examination is usually not performed

on a child with typical clinical features of ITP but is indicated for unexpected clinical or laboratory findings. Normal or increased numbers of megakaryocytes are observed. Sometimes, morphologically distinct lymphoid cells, called *hematogones*, constitute up to one-half of the marrow cells and may cause confusion with acute leukemia.<sup>157</sup> These nonneoplastic cells have the surface immunophenotypic profile of immature lymphocytes.

Acute ITP in children is usually a benign disease. Although rare, intracranial hemorrhage is the most feared complication. Clinical trials have focused on the time to increase the platelet count to safe levels as a surrogate end point for avoiding intracranial hemorrhage.

In a randomized trial of IVIG versus observation for children aged 3 months to 16 years with severe acute ITP (platelet count 20,000/ $\mu$ L or less) and mild to moderate bleeding ( $n = 206$ ), the frequency of chronic ITP—defined as a platelet count <150,000/ $\mu$ L at 6 months—was 18.6% in the IVIg group and 28.9% in the observation group (relative risk 0.64; 95% CI, 0.38–1.08).<sup>158</sup> Grade 4 to 5 bleeding occurred in 9% of the patients in the observation group versus only 1% in the IVIg group.

In a meta-analysis of six randomized trials ( $n = 410$  children), IVIG was associated with a more rapid platelet count increase compared with corticosteroids.<sup>159</sup> On the basis of these results, high-dose IVIG is generally recommended for initial treatment of severe acute ITP in children. Oral prednisone or an additional dose of IVIG is added if the platelet count remains below 20,000/ $\mu$ L by 48 hours. It appears that lower doses of IVIG (e.g., 250–500 mg/kg/day for two days rather than the standard 1 g/kg/day) may also be effective.<sup>160</sup>

High-dose corticosteroid therapy (e.g., intravenous methylprednisolone at 30 mg/kg/day for three days; or oral methylprednisolone at 30–50 mg/kg/day for seven days) also rapidly increases the platelet count in children with acute severe ITP.<sup>161</sup> Combined treatment with IVIG and pulse methylprednisolone may be indicated for those children felt to be at very high risk of intracranial hemorrhage.<sup>162</sup>

Approximately 10–20% of children will develop chronic ITP, defined as a platelet count less than 100,000/ $\mu$ L for more than six months. As many as one-third of children who meet this definition can still enter late spontaneous remission, sometimes 5–10 years after diagnosis. Chronic ITP in children resembles chronic ITP in adults.

Some children have no symptoms despite marked thrombocytopenia. These children generally do not need treatment. For children with symptoms, options include rituximab, TPO receptor agonists, intermittent IVIG or RhIG, splenectomy, and immunosuppressive agents.<sup>163</sup>

Intravenous immune globulin (2 g/kg over 2–5 days) increases the platelet count of most children with chronic ITP<sup>84,163</sup> and repeated courses of IVIG have been used to defer or avoid splenectomy.<sup>164</sup> Low-dose, alternate-day corticosteroid therapy may improve the results with maintenance IVIG.

Intravenous RhIG is effective in increasing the platelet count for many Rh-positive children with chronic ITP<sup>165,166</sup> and, at a dose of 75  $\mu$ g/kg, it appears to be as effective as IVIG.<sup>167</sup> The benefit is transient, however, with a median duration of approximately three weeks. RhIG can also be considered splenectomy sparing in some patients.<sup>166</sup> RhIG is generally ineffective after splenectomy.<sup>166</sup>

Splenectomy is sometimes performed in children with chronic ITP who cannot be maintained on other treatments. However, in general, splenectomy is avoided in children, especially young

children, because of the long-term risk of postsplenectomy sepsis, the possibility of late spontaneous remissions, and the efficacy of other medical treatments. As with adults, children must receive vaccines for *S. pneumoniae*, *N. meningitidis*, and *H. influenzae* type b at least two weeks before splenectomy.<sup>168</sup>

Results of use of immunosuppressive agents (azathioprine or cyclophosphamide) and vinca alkaloids to treat children with chronic ITP are variable and based on uncontrolled studies.<sup>163</sup> About one-third of pediatric patients with chronic ITP may respond to rituximab.<sup>124</sup>

### Drug-induced immune thrombocytopenia

Drugs can produce several immune-mediated thrombocytopenic disorders<sup>6,169–174</sup> (Table 36.2). The most common is HIT (discussed separately below), which paradoxically is associated with increased risk of thrombosis but not bleeding. In contrast, many other drugs in rare instances cause severe thrombocytopenia and bleeding, a syndrome called *drug-induced immune thrombocytopenic purpura* (DITP). Some drugs have atypical clinical features (e.g., abciximab-induced thrombocytopenia) or trigger an illness that resembles TTP or HUS.

### Typical drug-induced immune thrombocytopenia

The most common drugs that have been confirmed by laboratory testing to cause DITP are quinine, quinidine, trimethoprim/sulfamethoxazole, vancomycin, penicillin, rifampin, carbamazepine, and ceftriaxone.<sup>171,173</sup> The risk of DITP is approximately 1 case in 1000 for quinine and 1 in 25,000 for sulfamethoxazole-trimethoprim.<sup>175</sup> Quinine is present in certain beverages (e.g., tonic water), and consequently patients may not be aware of exposure.

Clinical criteria supporting a diagnosis of DITP are as follows: (1) thrombocytopenia occurs during drug treatment and is corrected completely after discontinuation of the drug; (2) the implicated drug was the only one used when thrombocytopenia occurred, or platelet count recovery occurred or persisted despite continuation or reintroduction of the other drugs used; (3) other causes of thrombocytopenia are excluded; and (4) re-exposure to the implicated agent resulted in recurrent thrombocytopenia.<sup>170</sup> For reasons of patient safety, the fourth criteria, drug re-exposure, is rarely evaluated deliberately, but sometimes the outcome of unintentional re-exposure can provide important diagnostic information (e.g., recurrent thrombocytopenia following ingestion of tonic water suggests quinine-induced thrombocytopenia). Meeting all four criteria provides definite evidence of causation, whereas meeting the first three criteria suggests a probable cause.<sup>170</sup>

Although many dozens of drugs have been suspected as causing DITP, laboratory evidence confirming the presence of drug-dependent platelet antibodies exists for relatively few drugs.<sup>171,173</sup> Supporting evidence for the drugs claimed to cause DITP is available (<http://www.ouhsc.edu/platelets/>).

Drug-induced immune-mediated thrombocytopenia typically begins abruptly and is severe; most patients have a platelet count less than 20,000/ $\mu$ L.<sup>6,172,176</sup> Although the interval between starting the drug and development of thrombocytopenia usually is one or two weeks, occasionally it can be abrupt (if the patient was previously exposed to the drug and drug-dependent antibodies remain present),<sup>177</sup> or begin several months or longer after administration of the drug is started. Sometimes, thrombocytopenia persists for several weeks even after the drug is stopped, possibly because some of the IgG antibodies formed have drug-independent platelet reactivity.

**Table 36.2** Drug-Induced Immune Thrombocytopenic Syndromes

Syndrome and Drug(s)	Comment
<b>Heparin-induced thrombocytopenia</b>	Prothrombotic reaction caused by heparin-dependent platelet-activating IgG antibodies that recognize platelet factor 4/heparin complexes; caused less often by low-molecular-weight heparin and fondaparinux than by unfractionated heparin
<b>Drug-induced immune thrombocytopenic purpura (DITP)</b>	Prohemorrhagic reaction caused by IgG antibodies that recognize drug (or drug metabolite) bound to platelet glycoprotein (GP); patients have severe thrombocytopenia and mucocutaneous bleeding.
• Quinine • Quinidine • Rifampin • Sulfa antibiotics • Vancomycin • Iodinated contrast • Acetaminophen • Many others	Quinine-dependent anti-GPIIb/IIIa and GPIb/IX IgG implicated; drug is widely available (e.g., tonic water) Antibodies usually distinct from quinine-dependent antibodies Rifampin-dependent anti-GPIIb/IIIa and GPIb/IX IgG implicated Occurs in <1 of 25,000 patients receiving trimethoprim-sulfamethoxazole Vancomycin-dependent anti-GPIIb/IIIa IgG; most common cause of DITP in hospitalized patients Severe thrombocytopenia begins after radiologic procedure IgG recognizes metabolite of acetaminophen See published lists
<b>Atypical DITP</b>	Abrupt onset of severe thrombocytopenia (platelet count nadir, 15,000–35,000/ $\mu$ L) by naturally occurring antibodies that recognize murine sequences within chimeric (human-murine) abciximab “Fibin” drug (ligand) reacts with GPIIb/IIIa, inducing neoepitope(s) (ligand-induced binding sites) recognized by antibodies Thrombocytopenia can persist for months after gold therapy is stopped (mimics chronic ITP) Transient autoimmune thrombocytopenia (anti-GPIIb/IIIa) that occurs a few weeks after vaccination (resembles childhood acute ITP)
<b>DITP: hapten mechanism</b>	Indicates that IgG recognizes drug that remains bound to platelet surface even following platelet washing Not well established
<b>Drug-induced TTP/HUS</b>	Estimated frequency, 1/2000 to 1/5000 Estimated frequency, 1/20,000 Quinine-dependent IgG against platelets and other cells found May be pathogenic factor in transplantation-associated TTP/HUS See text

MMR: measles–mumps–rubella; ITP: immune thrombocytopenic purpura; TTP: thrombotic thrombocytopenic purpura; HUS: hemolytic uremic syndrome.

The pathogenesis of DITP involves the formation of a ternary complex involving a platelet glycoprotein (usually, the GPIIb/IIIa complex, less often GPIb/IX), drug (or drug metabolite), and the Fab terminus of IgG.<sup>15,178,179</sup> Such a mechanism has been invoked for quinine, quinidine, sulfonamide, rifampin, vancomycin,<sup>178</sup> and pentamidine, among others. Unlike the mechanism of HIT, platelet Fc $\gamma$ Rs are not involved. Furthermore, the drug does not function as a hapten, that is, drug-dependent IgG does not bind to platelets that have been washed after pretreatment with the implicated drug.<sup>172</sup> Limited evidence of a hapten mechanism of DITP has been suggested only for penicillin (and possibly for piperacillin and certain cephalosporins),<sup>171</sup> that is, penicillin-dependent IgG binds to platelets that have been washed after pretreatment with penicillin. A proposed model suggests that drug-dependent antibodies are derived from a pool of naturally occurring antibodies with weak affinity for self-antigens residing on platelet membrane glycoproteins; certain drugs are able to affect both antibody and antigen in such a way that their interaction is greatly enhanced, provided that B cells expressing such antibodies are induced to produce these.<sup>172,180</sup>

In a case of suspected DITP, as many drugs as possible should be discontinued. If further drug treatment is necessary, an immunologically non-crossreactive substitute should be prescribed. Platelet transfusions should be given to patients with life-threatening bleeding or who are judged to be at high risk of bleeding (e.g., severe thrombocytopenia plus “wet purpura”). High-dose IVIG, 1 g/kg given over 6–8 hours for two consecutive days, may be of value, but can be ineffective if the relevant drug is not discontinued.<sup>178</sup> Corticosteroids are relatively ineffective in the management of this condition.<sup>176</sup>

### Atypical drug-induced immune thrombocytopenia

#### Glycoprotein IIb/IIIa antagonist-induced thrombocytopenia

Thrombocytopenia is a relatively common side effect of the three approved GPIIb/IIIa antagonists—abciximab, tirofiban, and

eptifibatide.<sup>181</sup> Often, the thrombocytopenia begins within hours of a first exposure; this is caused by naturally occurring antibodies.<sup>171,182</sup> In other patients, the thrombocytopenia begins about one week after initial—or abruptly upon repeat—drug administration; this clinical presentation reflects drug-induced antibody formation.<sup>183</sup>

Abciximab (ReoPro, Eli Lilly) is a humanized chimeric Fab fragment of a murine monoclonal antibody specific for an epitope on GPIIIa. It is used to prevent restenosis after coronary angioplasty. Approximately 0.5% of patients have moderate or severe thrombocytopenia within several hours of treatment with this drug.<sup>181</sup> Although approximately 20% of the normal population have antibodies that react against the papain cleavage site in abciximab, the remainder (1.6% overall) react against murine sequences incorporated into abciximab that confer specificity for GPIIb/IIIa. It is this latter group of antibodies that evinces pathogenicity. Perhaps surprisingly, given the degree of thrombocytopenia and use of a major platelet glycoprotein inhibitor, most patients do not have petechiae or bleeding<sup>181</sup> (although fatal bleeding episodes have been reported). Treatment with platelet transfusions and, perhaps, IVIG may benefit bleeding patients.

For approximately one-third of patients with apparent abciximab-associated thrombocytopenia, the examination of the blood film shows platelet clumping. This finding suggests that pseudothrombocytopenia (ex vivo platelet clumping) is caused by abciximab.<sup>184</sup> Such patients are not at risk for bleeding and do not need treatment.

Tirofiban and eptifibatide are synthetic compounds that mimic or contain the RGD (arg-gly-asp) peptide and bind tightly to the RGD recognition site in GPIIb/IIIa.<sup>181</sup> As with abciximab, the frequency of naturally occurring antibodies (approximately 1–2%) correlates roughly with their risk of inducing abrupt-onset thrombocytopenia upon first exposure. Delayed-onset thrombocytopenia beginning about one week after exposure has also been reported.

For tirofiban, some drug-dependent antibodies produce platelet activation and increased risk of ischemic events.<sup>185</sup>

#### **Drug-induced autoimmune thrombocytopenia**

Approximately 1–3% of patients treated with gold-containing medications have thrombocytopenia that sometimes persists for weeks or months despite stopping the drug; thus, the disorder resembles chronic ITP.<sup>186</sup> It remains uncertain whether this is true drug-induced autoimmune thrombocytopenia or is caused by gold-dependent IgG antibodies that are slowly released from tissues. Procainamide,  $\alpha$ -methyldopa, sulfonamide antibiotics, and interferons alfa and beta may also cause autoimmune thrombocytopenia.<sup>172,187</sup> In rare instances (~1/20,000 exposures),<sup>188</sup> measles-mumps-rubella vaccination causes an acute ITP-like illness in which anti-GPIIb/IIIa IgG is formed.<sup>189</sup>

#### **Drug-induced thrombotic microangiopathy**

Drugs may cause a thrombotic microangiopathy (TMA) that closely resembles TTP or HUS.<sup>190</sup> Two general mechanisms for drug-induced TMA include immune (idiosyncratic, non-dose-dependent) and toxic (dose-dependent) etiologies. Immune-mediated drug-induced TMA is caused by quinine,<sup>191</sup> with endothelium and other target cells affected by quinine-dependent antibodies. Paradoxically, immune-mediated TMA may be caused by the antiplatelet agents ticlopidine<sup>192</sup> and clopidogrel.<sup>193</sup> Ticlopidine-induced TTP is estimated to occur in 1 in 2000–5000 patients who receive this drug after coronary stenting. The characteristic onset is between one and eight weeks after administration of the drug is started. Clopidogrel-induced TTP occurs in approximately 1 in 20,000 recipients, generally within the first two weeks of treatment. Autoantibodies to von Willebrand factor-cleaving metalloproteinase have been identified in these patients and may contribute to the pathogenesis.<sup>194</sup>

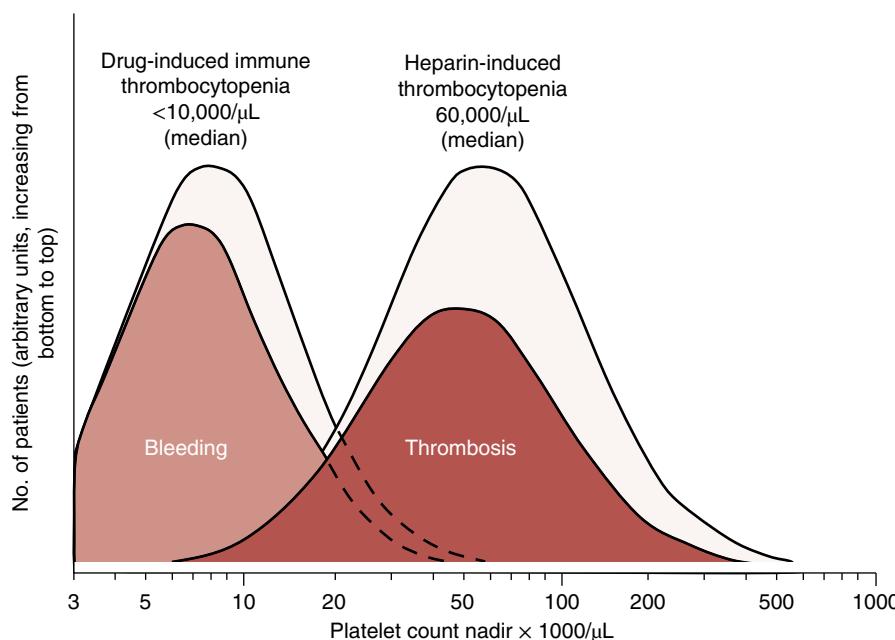
Other drugs that cause an illness that resembles HUS due to toxic effects include antineoplastic agents (e.g., gemcitabine, mitomycin C, cisplatin, bleomycin, and proteasome inhibitors such as carfilzomib,<sup>195</sup> ixazomib,<sup>196</sup> and bortezomib<sup>196</sup> used to treat multiple

myeloma) and immunosuppressive agents (e.g., cyclosporine and tacrolimus). Because some of the medical conditions leading to use of these drugs can be associated with microangiopathic blood film changes (neoplasia, organ rejection, graft-vs.-host disease, and vasculitis), causal relationships to the various drugs listed can be problematic.

The mortality of drug-induced TMA is high. Early recognition and discontinuation of the drug are essential. Response to plasma exchange is usually poor, particularly if ADAMTS13 levels are not severely reduced. Many physicians would also give corticosteroids, although the efficacy remains unproven. Specific therapy for drug-induced HUS (corticosteroids, eculizumab) is individualized depending on the clinical situation. The prognosis is poor for drug-induced TTP in the setting of hematopoietic stem cell transplantation (HSCT).

#### **Heparin-induced thrombocytopenia**

Heparin-induced thrombocytopenia (HIT) is a relatively common, IgG-mediated, adverse reaction to heparin that has a strong association with venous and arterial thrombosis.<sup>4–6,197,198</sup> HIT is a clinicopathologic syndrome, that is, the diagnosis is made most reliably on both clinical and serologic grounds.<sup>198–200</sup> Thus, HIT antibody formation without thrombocytopenia or other abnormalities is not HIT, whereas HIT antibody formation accompanied by an otherwise unexplained 50% or greater postoperative decrease in platelet count (even if the platelet count remains greater than 150,000/ $\mu$ L)<sup>5</sup> or complicated by necrotizing skin lesions at heparin injection sites<sup>197</sup> are examples of HIT syndrome. Indeed, the thrombocytopenia usually is much less severe in HIT<sup>6</sup> than in DITP<sup>176</sup> or GPIIb/IIIa antagonist-induced thrombocytopenia<sup>181</sup> (Figure 36.2). Another contrast from DITP is that even when severe thrombocytopenia occurs in HIT, petechiae and other types of bleeding typically are not observed.<sup>197,201</sup> Indeed, even the one characteristic hemorrhagic complication of HIT—bilateral adrenal hemorrhage—is caused by thrombosis (adrenal vein thrombosis leading to hemorrhagic necrosis).<sup>197</sup> HIT is an antibody-mediated disorder, and a minimum of five days is required for an immunizing exposure



**Figure 36.2** Platelet count nadirs ( $\log_{10}$  scale) and clinical profile of classic drug-induced immune thrombocytopenic purpura (DITP) and heparin-induced thrombocytopenia (HIT). Classic DITP (e.g., caused by quinine, vancomycin, or glycoprotein IIb/IIIa antagonists, among many others) typically produces severe thrombocytopenia (platelet count nadir, ~10,000/ $\mu$ L) and associated mucocutaneous bleeding. In contrast, HIT typically results in mild to moderate thrombocytopenia (median platelet count nadir, ~60,000/ $\mu$ L) and associated venous or arterial thrombosis. Note that the relative heights of the two peaks are not drawn to scale; HIT is much more common than all other causes of DITP combined. Source: Warkentin (2007).<sup>6</sup> Reproduced with permission of Massachusetts Medical Society.

to heparin to generate sufficient levels of antibodies to cause thrombocytopenia.<sup>202</sup>

Sometimes, onset of thrombocytopenia and thrombosis begins—or worsens—after all heparin has been stopped (“delayed-onset” or “autoimmune-like” HIT),<sup>203–205</sup> in some of these patients, thrombocytopenia and thrombosis risk persist for several weeks (“persistent” or “refractory” HIT).<sup>206,207</sup> Occasionally, small amounts of heparin (“flushes”) are implicated.<sup>208</sup> Some patients who have not previously been exposed to heparin will develop abrupt onset of thrombocytopenia, thrombosis and have detectable platelet-activating anti-PF4/heparin antibodies; such patients with so-called “spontaneous HIT syndrome”<sup>209–211</sup> have often had recent infection<sup>212,213</sup> or surgery, particularly, knee replacement surgery.<sup>214–216</sup> It is now recognized that such atypical clinical pictures of HIT—now named “autoimmune HIT” [aHIT]<sup>210</sup>—are explained by anti-PF4/polyanion antibodies that are able to strongly activate platelets in vitro and in vivo even in the absence of heparin (“heparin-independent platelet activation”). As discussed later, there are special treatment considerations for such patients, most notably, adjunctive therapy with high-dose intravenous immunoglobulin (IVIG).

The target antigen recognized by HIT antibodies consists of a multimolecular complex between PF4 (a platelet  $\alpha$ -granule protein of the CXC family of chemokines) and heparin.<sup>32</sup> The HIT antibodies bind to one or more PF4 regions that have undergone conformational modification through binding to heparin. The formation of the antigen is somewhat nonspecific because PF4 can be rendered antigenic by binding to certain other polyanions, such as pentosan polysulfate or polyvinylsulfonate.<sup>33</sup> At least 12–14 saccharide units are needed for heparin to form the antigen complex with PF4. This may explain why LMW heparin preparations are less immunogenic than unfractionated heparin and are less likely to cause HIT.<sup>4,5</sup> Although LMW heparin sometimes causes HIT, it is likely that very small heparin preparations (e.g., fondaparinux, a synthetic antifactor Xa-binding pentasaccharide) or specially engineered heparins (e.g., highly sulfated heparin moieties bridged with nonsulfated spacer regions) only rarely cause HIT.<sup>217,218</sup>

Figure 36.3 illustrates several possible mechanisms to explain the intense thrombin generation that occurs in HIT.<sup>219</sup> These include an important role for the classic complement pathway both in triggering the HIT immune response (by facilitating binding of PF4/heparin/natural IgM complexes to B lymphocytes)<sup>220,221</sup> as well as in facilitating activation by HIT antibodies of cells other than platelets.<sup>222</sup> Further, platelet-activating anti-PF4/heparin antibodies induce formation of procoagulant, platelet-derived microparticles<sup>223</sup> that result from cell signaling triggered by clustering of platelet Fc $\gamma$ RIIa. Recent data suggest that in situ formation of IgG/PF4/heparin complexes on the platelet surface leads to progressive platelet activation.<sup>224</sup> In vivo platelet activation is indicated by expression of P-selectin by circulating platelets in HIT.<sup>225</sup> Tissue factor expression by endothelium<sup>226,227</sup> or monocytes<sup>228,229</sup> activated by HIT antibodies that recognize PF4 bound to surface glycosaminoglycans, as well as HIT antibody-induced neutrophil activation and release of procoagulant neutrophil intracellular nets (“NETosis”),<sup>230,231</sup> constitute other potential procoagulant events. Endothelial injury with release of von Willebrand factor “strings” that bind PF4 helps to localize prothrombotic events to endothelium.<sup>232</sup>

Marked in vivo thrombin generation helps explain several clinical features of HIT, including its association with venous and arterial thrombosis (hypercoagulable state), the occurrence of decompensated disseminated intravascular coagulation (DIC) with low fibrinogen levels and/or elevated prothrombin time in

approximately 10–20% of patients, and the potential for deep vein thrombosis (DVT) to progress to venous limb gangrene, particularly in patients treated with warfarin.<sup>233</sup> This last syndrome results from impaired procoagulant–anticoagulant balance: Warfarin-induced protein C depletion leads to microvascular thrombosis caused by ongoing intense thrombin generation. Patients with warfarin-induced venous gangrene typically have a supratherapeutic international normalized ratio (INR), usually more than 3.5. The explanation is a concomitant severe decrease in factor VII that parallels the decrease in protein C. The importance of in vivo thrombin generation in HIT provides a rationale for consensus recommendations that an agent that reduces thrombin generation or directly inactivates thrombin be used for the management of this syndrome.<sup>234,235</sup>

The frequency of HIT varies among different patient populations. Medical patients appear to develop HIT less often than do surgical patients, and female gender is a minor risk factor for HIT.<sup>236</sup> The frequency of HIT can be as high as 5% when multiple risk factors are present (thromboprophylaxis with unfractionated heparin administered for two weeks to a female patient following major surgery), whereas the risk is very low or negligible in other settings (e.g., administration of LMW heparin during pregnancy).<sup>4,5,237</sup>

### **Iceberg model of HIT**

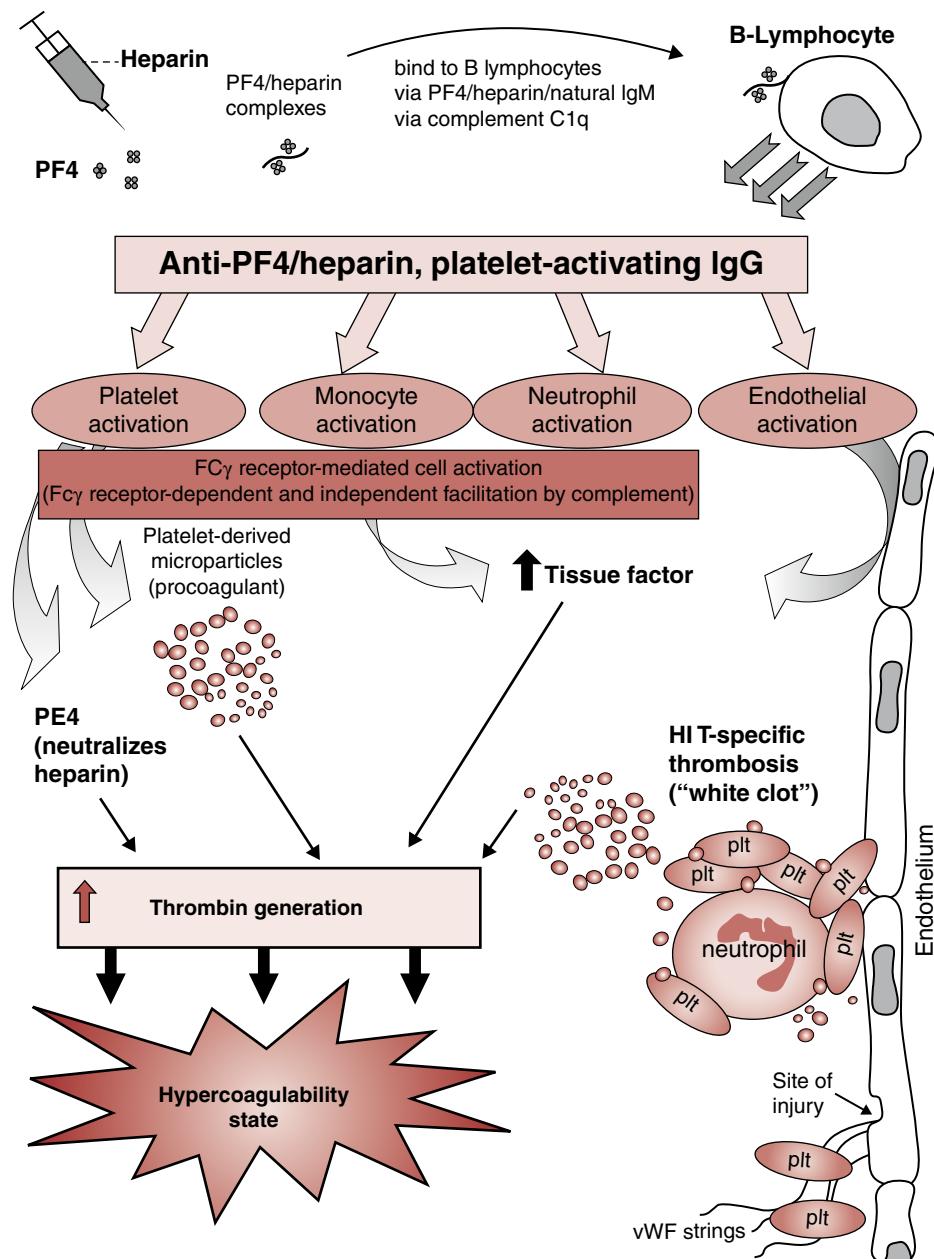
Figure 36.4 illustrates the relation between clinical HIT and detectability of anti-PF4/heparin antibodies by either washed platelet activation assay or PF4-dependent EIA.<sup>234</sup> The key concept is that whereas both types of assays have high sensitivity for diagnosis of clinical HIT, the former assay has greater diagnostic specificity. It is estimated that substantial overdiagnosis (~50%) of HIT will result<sup>238</sup> if a positive EIA of any magnitude is considered diagnostic of HIT irrespective of the patient’s clinical likelihood of HIT, as judged using a clinical scoring system (the 4Ts)<sup>239</sup> and the gold standard assay (platelet SRA).

### **Heparin-independent platelet-activating antibodies**

US reference laboratories that perform the SRA usually use the assay at low (0.1–0.5 U/mL heparin) and high heparin (100 U/mL heparin) concentrations;<sup>240</sup> a positive result is indicated when HIT patient serum produces serotonin-release at low, but not at high, heparin concentration.<sup>241</sup> However, by performing the SRA also in the absence of heparin (0 U/mL heparin, i.e., “buffer control”), the lab can determine if the HIT antibodies have heparin-“independent” platelet-activating properties, an important serological feature pointing to an aHIT disorder (delayed-onset HIT, persisting HIT, etc.).<sup>203,208,210,240</sup> As discussed in the following section, high-dose IVIG is an important treatment for patients who have a severe aHIT syndrome.

### **Management of heparin-induced thrombocytopenia-associated thrombosis**

*High-dose IVIG* is increasingly used as adjunctive treatment (along with anticoagulation) for HIT, especially severe aHIT.<sup>242</sup> The usual dose is 1 g/kg for two consecutive days (i.e., 2 g/kg total dose); the treatment appears to rapidly raise the platelet count and reduce hypercoagulability.<sup>242</sup> IVIG works through competitive inhibition of HIT antibody-induced platelet activation through the platelet Fc $\gamma$ IIa receptors; accordingly, there is no change in EIA reactivity, but blood samples obtained after IVIG treatment may show a negative SRA.<sup>243,244</sup> High-dose IVIG may also be effective in passivating the effect of HIT antibodies in situations of deliberate heparin rechallenge in patients who have heparin-dependent

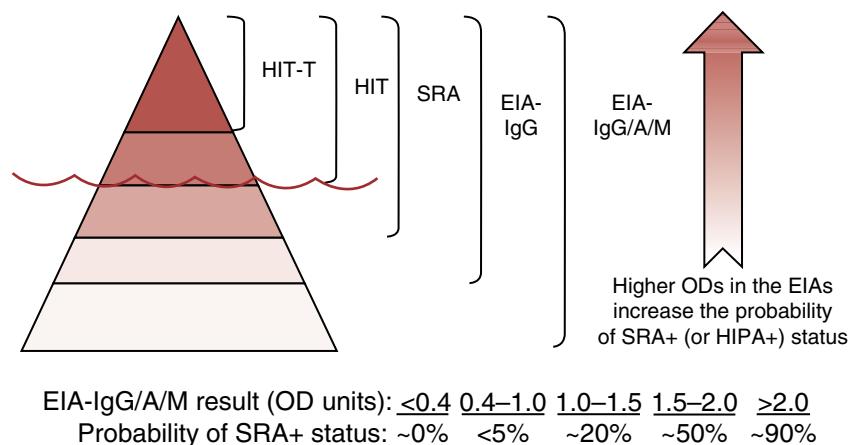


**Figure 36.3** Pathogenesis of heparin-induced thrombocytopenia. Ultralarge PF4/heparin complexes, interacting with natural IgM and complement, stimulate B-lymphocyte activation and subsequent formation of anti-PF4/heparin antibodies that, when platelet-activating, have the potential to cause HIT. Platelet activation by PF4/heparin/IgG complexes, mediated through platelet Fc $\gamma$ IIa receptors, results in thrombin generation, e.g., through platelet-derived procoagulant microparticles and PF4 neutralization of heparin's anticoagulant effects. "Pancellular" activation indicates activation of cells other than platelets, including monocytes, neutrophils, and endothelium. Recently, a role of the classic complement pathway in facilitating downstream cellular activation has been identified. Activated monocytes and endothelium express tissue factor; activated neutrophils release procoagulant "neutrophil extracellular traps" (NETs), a process known as "NETosis." PF4 binds to DNA within NETs, further promoting platelet activation. Release of von Willebrand factor (vWF) strings from injured endothelium binds PF4, further directing HIT-related prothrombotic effects to these vascular sites. These multifaceted pathological effects are responsible for the marked prothrombotic risk of HIT, which can involve large veins and arteries, as well as small vessels (microvascular thrombosis).

platelet-activating antibodies (e.g., because of recent HIT), e.g., for urgent vascular or cardiac surgery.<sup>245,246</sup>

Results of prospective and retrospective studies<sup>4,5,201</sup> indicate that approximately 50–75% of patients with HIT have new, progressive, or recurrent thrombosis (relative risk of thrombosis is ~12).<sup>247</sup> Thus, the need for an alternative anticoagulant is a common issue in the care of these patients.

**Argatroban** is a small-molecule DTI approved in the United States for the management and prevention of HIT-associated thrombosis.<sup>235,248</sup> Features of argatroban include its short half-life (40–50 minutes) as well as its hepatic route of metabolism. No dose adjustments are needed for patients with moderate renal failure, although dose reduction is needed in the presence of hepatic insufficiency. Anticoagulant monitoring is usually performed using the



**Figure 36.4** “Iceberg model” of HIT. Clinical HIT, comprising HIT with (HIT-T) or without thrombosis (HIT), is represented by the portion of the iceberg above the waterline; the portion below the waterline represents subclinical anti-PF4/H seroconversion. Three types of assays are highly sensitive for the diagnosis of HIT: the washed platelet activation assay (e.g., the serotonin-release assay [SRA], heparin-induced platelet activation [HIPA] test, or P-selectin expression assay [PEA; not shown]), the IgG-specific PF4-dependent EIAs (EIA-IgG), and the polyclonal EIAs that detect anti-PF4/H antibodies of the three major immunoglobulin classes (EIA-IgG/A/M). In contrast, diagnostic specificity greatly varies among these assays, being the highest for the SRA and lowest for the EIA-IgG/A/M. The approximate probability of SRA+ status in relation to a given EIA result, expressed in OD units, was obtained from the literature. With permission from Warkentin (2011).<sup>234</sup>

aPTT; although simple, aPTT monitoring can result in systematic underdosing of argatroban in patients with baseline (pretreatment) elevation in aPTT, for example because of severe HIT-associated DIC (“aPTT confounding”).<sup>249,250</sup> Argatroban also prolongs the INR,<sup>251,252</sup> which complicates the transition from argatroban to warfarin therapy. It is important to postpone introduction of warfarin until the thrombocytopenia has substantially recovered (platelet count at least 150,000/ $\mu$ L).<sup>235</sup>

**Bivalirudin** is an oligopeptide (20 amino acid) analog of hirudin, i.e., a DTI.<sup>252–254</sup> Bivalirudin is widely used for anticoagulation during percutaneous coronary intervention and is also approved for this indication in patients with HIT. It is sometimes used “off-label” for treatment of HIT, especially in critically ill patients. Its half-life is short (~25 min); bivalirudin is eliminated primarily through nonorgan metabolism (proteolysis), with minor renal elimination (~20%). As with argatroban, aPTT confounding can occur when treating patients with severe HIT-associated DIC.

**Danaparoid sodium** is a mixture of anticoagulant glycosaminoglycans with predominant antifactor Xa activity that decreases thrombin generation in patients with HIT.<sup>255</sup> A randomized trial showed a higher thrombosis resolution rate among patients treated with danaparoid and warfarin than among those treated with dextran and warfarin, especially for patients with severe thrombosis (92% vs. 33%;  $p < 0.001$ ).<sup>256</sup> Although some HIT sera show in vitro crossreactivity with danaparoid (i.e., enhanced platelet activation in the presence of danaparoid), this effect is usually weak; moreover, platelet-activating effects of many HIT sera are inhibited by danaparoid at therapeutic concentrations.<sup>257</sup> Because in vitro crossreactivity is not predictive of adverse outcome,<sup>255</sup> danaparoid should be given without preceding in vitro testing for crossreactivity. The success rate is high (approximately 85–90%), as defined by platelet count recovery without new thrombosis.<sup>255,258</sup> The anticoagulant effect of danaparoid is monitored with a chromogenic antifactor Xa assay, which must be performed using a standard curve prepared with danaparoid. The target range is generally 0.5–0.8 antifactor Xa U/mL, although levels of 1.0 U/mL or higher are used by some in the treatment of patients with severe thrombosis. Recommended

therapeutic dosing includes an initial intravenous bolus followed by continuous infusion (usually 200 U/hour with monitoring of antifactor Xa levels, if available). Danaparoid does not interfere with INR measurements. This is an advantage in the care of patients with venous thromboembolism in whom overlapping warfarin anticoagulation is usually performed after resolution of thrombocytopenia. Danaparoid is approved therapy for HIT in the European Union, Canada, and elsewhere, but it is neither approved for HIT management nor currently available in the United States.

**Fondaparinux** is a synthetic pentasaccharide anticoagulant modeled after the antithrombin-binding region of heparin; it has exclusive antifactor Xa activity. Although fondaparinux is associated with the formation of anti-PF4/H antibodies at a frequency similar to that seen with LMW heparin, it is uncommon for fondaparinux to increase platelet activation by HIT antibodies (i.e., it has a low frequency of in vivo “crossreactivity”).<sup>259</sup> Although not approved to treat HIT, fondaparinux is frequently used “off-label” for the treatment of HIT with or without thrombosis.<sup>260</sup> Indeed, observational studies of patients with well-characterized HIT suggest a high success rate (at least 90%) with fondaparinux.<sup>218,261–263</sup> Anticoagulant monitoring is not required, and thus the aforementioned problem of “aPTT confounding” seen with DTIs such as argatroban is not seen with fondaparinux.<sup>234</sup>

There are several important contraindications to therapy for acute HIT, including warfarin monotherapy and LMW heparin.<sup>235</sup> Warfarin therapy can lead to acute depletion of protein C, which can cause microvascular thrombosis in HIT and lead to venous limb gangrene.<sup>233</sup> However, once thrombocytopenia has resolved completely, it is reasonable to overlap warfarin cautiously with one of the agents that can reduce thrombin generation in HIT (danaparoid, fondaparinux, and argatroban). Alternatively, one can transition to a direct oral anticoagulant (DOAC; discussed subsequently). Use of LMW heparin is contraindicated because of a high chance of treatment failure (approximately 50% of patients have further thrombocytopenia or thrombosis). Prophylactic platelet transfusion is relatively contraindicated because even patients with severe thrombocytopenia do not usually have evidence of hemostatic

dysfunction, such as petechiae, and platelet transfusions theoretically may contribute to increased risk of thrombosis. It should be noted that one database study<sup>264</sup> that suggested greater risk of arterial thrombosis with platelet transfusion in HIT is methodologically flawed in that it could not account for severe thrombocytopenia per se as independent explanations for both greater risk of thrombosis as well as for platelet transfusion. Other observational studies have not found evidence to suggest that platelet transfusion in HIT is associated with thrombotic events.<sup>265,266</sup>

DOACs such as Xa inhibitors (rivaroxaban and apixaban) are increasingly used to treat HIT, either as primary therapy or by way of transition from initial therapy with a parenteral agent (e.g., DTI, fondaparinux, and danaparoid).<sup>235,267</sup> They may be especially helpful in patients with aHIT disorders as there is no risk of aPTT confounding.<sup>240,250</sup>

### **Management of isolated heparin-induced thrombocytopenia**

Isolated HIT is defined as HIT that is recognized because of thrombocytopenia without evidence of HIT-associated thrombosis.<sup>201</sup> Unfortunately, simply stopping administration of heparin or substituting warfarin for heparin is inadequate treatment for these patients. In a retrospective cohort study,<sup>201</sup> the risk of thrombosis among these patients was approximately 10% at two days, 40% at seven days, and 53% at 30-day follow-up evaluations. Other investigators<sup>268</sup> with a similar approach subsequently found a 38% rate of thrombotic events despite stopping administration of heparin. The frequency of thrombosis surprisingly was not any lower in the subgroup of patients for whom heparin was stopped fairly promptly (<48 hours) after the onset of thrombocytopenia than it was among patients with later cessation of heparin (45% vs. 34%;  $p = 0.26$ ).

For patients strongly suspected (or confirmed) to have isolated HIT, the authors discontinue heparin, start administration of an alternative rapidly acting anticoagulant, and screen for subclinical DVT by means of compression ultrasonography (approximately 50% of patients are shown to have DVT with this approach). Whether or not thrombosis is found, a therapeutic dose of anticoagulant is given to these patients. This is because prophylactic-dose anticoagulation appears to have a higher failure rate than does therapeutic-dose anticoagulation.<sup>258</sup> After platelet count recovery, the absence of venous thrombosis is confirmed before discharge. For patients found at initial or follow-up imaging to have venous thrombosis, overlapping warfarin anticoagulation is usually begun for longer term antithrombotic control, although as previously mentioned transition to a DOAC is increasingly performed.

### **Re-exposure to heparin in a patient with a history of heparin-induced thrombocytopenia**

Patients who have circulating HIT antibodies can have an abrupt decrease in platelet count if heparin is administered. However, the risk of such abrupt-onset HIT on re-exposure to heparin is restricted to the first few months after use of heparin. This is because HIT antibodies begin to decline after heparin is discontinued, and usually they are no longer detectable by the three-month follow-up evaluation.<sup>269</sup> Under exceptional circumstances (e.g., need to perform heart or vascular surgery), it is recommended to readminister heparin to a patient with previous HIT, provided that HIT antibodies are no longer detectable with a sensitive and reliable assay (e.g., SRA or PF4-dependent EIA).<sup>235,270,271</sup> Such patients often do not form HIT antibodies after the brief re-exposure to heparin, and if they do, these antibodies are not formed before Day 5. Nevertheless,

it seems prudent to limit the heparin re-exposure to the operation itself and to use an alternative anticoagulant for perioperative anti-coagulation, and also to perform platelet count monitoring for HIT for up to 10 days.

For patients who require heart or vascular surgery who still have circulating platelet-activating HIT antibodies, there is uncertainty about optimal management. Sometimes nonheparin anticoagulation is administered (e.g., bivalirudin),<sup>235</sup> but others prefer to remove HIT antibodies through plasma exchange and then administer heparin, and still others administer high-dose IVIG to passivate the effect of residual HIT antibodies prior to using heparin.<sup>245,246</sup>

### **Vaccine-induced immune thrombotic thrombocytopenia**

Rarely, adenoviral vector vaccines (ChAdOx1 nCoV-19 [Oxford-AstraZeneca]; Ad26.COV2 [Johnson & Johnson/Janssen]) used for the prevention of severe coronavirus disease 2019 (COVID-19) can cause a prothrombotic disorder characterized by oftentimes unusual, severe, or multiple thrombotic events (e.g., cerebral venous sinus thrombosis, splanchnic vein thrombosis, aortic and limb artery thrombosis, and venous thromboembolism), thrombocytopenia (often severe), and hypercoagulability (elevated d-dimer levels and hypofibrinogenemia), with high mortality.<sup>272–275</sup> Named “vaccine-induced immune thrombotic thrombocytopenia” (VITT) (an alternate designation is “thrombosis with thrombocytopenia syndrome” [TTS]), two features strongly implicate preceding vaccination as its trigger: (a) characteristic timing, with onset of symptomatic thrombosis between 5 and 30 days (occasionally later) postvaccination (day 0 = day of vaccination) and (b) (usually) strong-positive EIA for anti-PF4 antibodies plus positive platelet activation testing (especially when PF4 is supplemented in the assay), despite absence of proximate heparin exposure. Moreover, as seen in HIT, the VITT antibodies activate platelets through their FcγIIa receptors. Unlike in HIT, however, rapid HIT immunoassays (e.g., latex-enhanced and chemiluminescence) usually yield negative results.<sup>276,277</sup> It is not known how the vaccines trigger a strong platelet-activating immune response against PF4, but crossreactivity of antibodies reactive against anti-COVID-19 “spike protein” (the immune response triggered by COVID-19 vaccines) and PF4 is not likely;<sup>278</sup> rather, PF4-binding components within the vaccine are possible.<sup>279</sup>

Treatment of VITT includes upfront high-dose IVIG (1 g/kg for two consecutive days) and anticoagulation.<sup>280,281</sup> Although nonheparin anticoagulation (e.g., fondaparinux, DOAC, and DTI) is generally recommended (given the strong clinical and serological resemblance to severe aHIT), it seems likely that heparin is also effective, based upon (a) frequent in vitro inhibition of VITT serum-induced platelet activation by heparin, (b) platelet count improvement with heparin treatment seen anecdotally, and (c) the intriguing observation that VITT antibodies recognize the heparin binding site on PF4.<sup>282</sup>

### **Alloimmune thrombocytopenia**

Alloantigens are genetically determined molecular variations of proteins or carbohydrates that can be recognized immunologically by some healthy persons. Exposure to alloantigens occurs during pregnancy, transfusion, or transplantation. If alloantibodies form against platelet alloantigens, alloimmune thrombocytopenia can result from platelet clearance mediated by the reticuloendothelial system. Five alloimmune thrombocytopenic disorders have been described (Table 36.3),<sup>283</sup> the most common being neonatal alloimmune thrombocytopenia (NAIT).

**Table 36.3** Five Alloimmune Thrombocytopenic Syndromes**Classical Alloimmune Thrombocytopenic Syndromes**

- Neonatal alloimmune thrombocytopenia
- Post-transfusion purpura

**Other Alloimmune Thrombocytopenic Syndromes**

- Passive alloimmune thrombocytopenia
- Transplantation-associated alloimmune thrombocytopenia
- Platelet transfusion refractoriness

Source: Warkentin and Smith (1997).<sup>283</sup> Reproduced with permission of Elsevier.

**Alloantigens**

More than 30 platelet alloantigens have been identified.<sup>283–285</sup> Table 36.4 classifies the platelet alloantigens by glycoprotein localization and gene frequency, the latter divided into public and private (or low frequency, arbitrarily less than 0.02). A database of genetically confirmed alloantigens is maintained by the European Bioinformatics Institute with 29 antigens in the current list (<http://www.ebi.ac.uk>) and a database of 35 HPA polymorphisms and references is listed on the Versiti (Milwaukee, WI) website (<https://www.versiti.org/medical-professionals/precision-medicine-expertise/platelet-antigen-database/hpa-gene-database>).

More than one-half of the alloantigens that have been identified are located on one of the two glycoproteins that constitute the GPIIb/IIIa complex (platelet fibrinogen receptor). One of these alloantigens, HPA-1a (previously, Pl<sup>A1</sup>), is located on GPIIIa. It is responsible for most alloimmune thrombocytopenia in populations of European ancestry, including almost all patients with severe alloimmune thrombocytopenia. In contrast, the other major platelet glycoprotein complex (GPIb/IX, von Willebrand-factor-binding complex) is rarely implicated in alloimmune thrombocytopenia. However, the GPIa/IIa complex (platelet collagen receptor), which bears the HPA-5a/5b (Br<sup>a/b</sup>; Zav<sup>a/b</sup>) alloantigen system, is a relatively common cause of moderately severe alloimmune thrombocytopenia.<sup>286,287</sup> The HPA-15a/15b (Gov<sup>a/b</sup>) alloantigen system is expressed on CD109. It has been shown to be a relatively common cause of alloimmune thrombocytopenia that, like HPA-5a/5b, tends not to be severe.<sup>288</sup>

**Immunogenetics and frequency of alloimmune thrombocytopenia**

The HPA-1a alloantigen is far more immunogenic than its corresponding allele, HPA-1b. For example, consider the frequency of NAIT caused by either anti-HPA-1a or anti-HPA-1b alloantibodies

**Table 36.4** Platelet Antigens Classified According to Glycoprotein Location and Gene Frequency

Platelet-Specific Alloantigen (Alternative Nomenclature)	GP	Gene Frequency in Whites	NAIT	PTP	PAT	TAT	PTR
<b>GPIIb/IIIa: Public (Gene Frequency &gt;0.02)</b>							
HPA-1a (Pl <sup>A1</sup> , Zw <sup>a</sup> )	IIIa	0.85	++	++	+	+	(+)
HPA-1b (Pl <sup>A2</sup> , Zw <sup>b</sup> )	IIIa	0.15	+	+	–	–	(+)
HPA-3a (Bak <sup>a</sup> , Lek <sup>a</sup> )	IIb	0.61	+	+	–	–	(+)
HPA-3b (Bak <sup>b</sup> )	IIb	0.39	?	+	–	–	(+)
HPA-4a (Pen <sup>a</sup> , Yuk <sup>b</sup> )	IIIa	>0.99	+	+	–	–	(+)
<b>GPIIb/IIIa: Private/Low-Frequency (Gene Frequency &lt;0.02)</b>							
HPA-4b (Pen <sup>b</sup> , Yuk <sup>a</sup> )	IIIa	<0.01	+	–	–	–	–
HPA-6b (Tu <sup>a</sup> , Ca <sup>a</sup> )	IIIa	0.003	+	–	–	–	–
HPA-7b (Mo <sup>a</sup> )	IIIa	<0.01	++	–	–	–	–
HPA-7c (Hit <sup>a</sup> )	IIIa	<0.01	–	–	–	–	–
HPA-8b (Sr <sup>a</sup> )	IIIa	<0.003	+	–	–	–	–
HPA-9b (Max <sup>a</sup> )	IIb	0.002	+	–	–	–	–
HPA-10b (La <sup>a</sup> )	IIIa	<0.01	+	–	–	–	–
HPA-11b (Gro <sup>a</sup> )	IIIa	<0.001	+	–	–	–	–
HPA-14b (Oe <sup>a</sup> )	IIIa	<0.005	+	–	–	–	–
HPA-16b (Duv <sup>a</sup> )	IIIa	<0.01	+	–	–	–	–
Va <sup>a</sup>	IIb/IIIa	<0.002	+	–	–	–	–
<b>GP Ia/IIa: Public</b>							
HPA-5a (Br <sup>b</sup> , Zav <sup>a</sup> )	Ia	0.89	+	?	–	–	(+)
HPA-5b (Br <sup>a</sup> , Zav <sup>b</sup> , Hc <sup>a</sup> )	Ia	0.11	++	+	+	+	(+)
<b>GP Ia/IIa: Private</b>							
HPA-13b (Sit <sup>a</sup> )	Ia	0.0025	+	–	–	–	–
Swi <sup>a</sup>	Ia	<0.002	+	–	–	–	–
<b>GP Ib/IX: Public</b>							
HPA-2a (Ko <sup>b</sup> )	Ib <sup>α</sup>	0.89	–	–	–	–	?
HPA-2b (Ko <sup>a</sup> , Sib <sup>a</sup> )	Ib <sup>α</sup>	0.11	+	?	–	–	(+)
<b>GP Ib/IX: private</b>							
HPA-12b (Iy <sup>a</sup> )	Ib <sup>β</sup>	<0.01	+	–	–	–	–
<b>CD109: public</b>							
HPA-15a (Gov <sup>b</sup> )	CD109	0.53	+	?	–	–	–
HPA-15b (Gov <sup>a</sup> )	CD109	0.47	+	–	–	–	(+)
<b>GP38</b>							
Dy <sup>a</sup>	38 kD	<0.01	+	–	–	–	–
<b>Platelet Nonspecific Alloantigens</b>							
ABO			–	–	–	–	++
HLA			–	–	–	–	++

GP: glycoprotein; NAIT: neonatal alloimmune thrombocytopenia; PTP: post-transfusion purpura; PAT: passive alloimmune thrombocytopenia; TAT: transplantation-associated alloimmune thrombocytopenia; PTR: platelet transfusion refractoriness.

++, relatively common; +, established but rare; –, not reported; (+), probable association, but definitive link inconclusive; ?, possible association but not established. Also see the current list of polymorphisms on the European Bioinformatics Institute website (<http://www.ebi.ac.uk/ypd/hpa/table1.html>) and on the Versiti website (<https://www.versiti.org/medical-professionals/precision-medicine-expertise/platelet-antigen-database/hpa-gene-database>).

Source: Warkentin and Smith (1997).<sup>283</sup> Reproduced with permission of Elsevier.

in relation to the genotype frequency of HPA-1a (0.85) and HPA-1b (0.15). Thus, a homozygous HPA-1bb (Pl<sup>A1</sup>-negative) female, representing approximately 2% ( $0.15 \times 0.15$ ) of the population, would have an 85% probability of being exposed to the HPA-1a alloantigen during pregnancy. In contrast, a homozygous HPA-1aa female, representing approximately 72% ( $0.85 \times 0.85$ ) of the population, would have a 15% probability of being exposed to the HPA-1b alloantigen during pregnancy. Thus, if both alloantigens were equally immunogenic, one would expect anti-HPA-1b to occur approximately six times more often than anti-HPA-1a:  $(0.72 \times 0.15)/(0.02 \times 0.85) = 6.4$ . However, the *opposite* is actually observed: anti-HPA-1a is far more common than anti-HPA-1b (Table 36.5).<sup>286,289</sup> In a classic study of 348 cases of suspected NAIT,<sup>286</sup> only one case caused by anti-HPA-1b was found, compared with 144 cases of proven or suspected NAIT secondary to anti-HPA-1a. Thus, the observed ratio of NAIT caused by anti-HPA-1a/anti-HPA-1b (1:144 or 0.007) is almost 1000 times less than predicted by the theoretical ratio (6.4).

Immunogenetics is a major factor determining alloimmunization against HPA-1a. There is a strong association between formation of anti-HPA-1a and *HLA-DRB3\*0101* and *HLA-DQB1\*0201* (odds ratio, 25 and 40, respectively).<sup>290</sup> A recent meta-analysis<sup>291</sup> confirmed the strong role of *HLA-DRB3\*0101* in predicting for NAIT in HPA-1a negative women. Although the presence of *HLA-DRB3\*0101* predicts for immunization to HPA-1a, there is no association of gene dose with ICH and severity of the thrombocytopenia.<sup>292</sup> In contrast, no HLA association exists for immunization against HPA-1b.<sup>293</sup> Thus, it appears that persons with certain HLA genotypes are much more likely to generate an alloimmune response when GPIIa bears the leucine<sup>33</sup> substitution that determines the HPA-1a phenotype.

Overall, on the basis of the observed allelic frequencies, the expected theoretical ratio of NAIT for anti-HPA-5b, compared with anti-HPA-5a, should be approximately 8 (Table 36.5). A similar ratio (47:3 or 15.7) has been observed. However, although the expected and observed ratios are similar (contrasting the HPA-1a/1b system), a role for immunogenetics and alloimmunization also exists for the HPA-5a/5b system.<sup>294</sup>

**Table 36.5** Observed Frequencies of Neonatal Alloimmune Thrombocytopenia and Post-transfusion Purpura in Relation to Expected (Theoretic) Frequency of the HPA-1ab (Pl<sup>A1/A2</sup>), HPA-5ab (Br<sup>a/b</sup>), and HPA-3ab (Bak<sup>a/b</sup>) Alloantigen Systems

Target Alloantigen	Percentage of Pregnancies at Theoretic Risk of NAIT <sup>†</sup> (Descending Order)	Observed Cases of NAIT <sup>‡</sup>	Observed Cases of PTP <sup>§</sup>
HPA-3b	14.5	0	0
HPA-1b	10.8	0	29
HPA-3a	9.3	1	9
HPA-5b	8.7	6	11
HPA-1a	1.9	44	105
HPA-5a	1.1	0	7

<sup>†</sup> Percentage of pregnancies at theoretic risk of NAIT for a given target alloantigen is determined as follows:

$x(1 - x)^2 \times 100$ , where  $x$  is the gene frequency of the target alloantigen. Note the lack of correlation between the theoretical and observed risk for NAIT.

<sup>‡</sup> Data are from Mueller-Eckhardt et al.,<sup>192</sup> and represent serologic investigations using a defined protocol over an 18-month period ending 30 June 1988.

<sup>§</sup> For comparison, the serologic findings for cases of PTP are shown for which only one platelet alloantigen specificity was identified (from January 1990 to August 2006 at the Blood Center of Wisconsin).<sup>289</sup>

NAIT: neonatal alloimmune thrombocytopenia; PTP: post-transfusion purpura.

Source: Warkentin and Smith (1997).<sup>283</sup> Reproduced with permission of Elsevier.

## Severity of alloimmune thrombocytopenia

In general, the severity of thrombocytopenia is greater for alloimmune thrombocytopenia that involves the GPIIb/IIIa complex, compared with the GPIa/IIa complex (Table 36.6).<sup>283,286,289,295</sup> Because there are approximately 20 times more GPIIb/IIIa molecules compared with GPIa/IIa complexes on the platelet surface (40,000 vs. 2000), this suggests that greater numbers of alloantibodies binding to the more numerous GPIIb/IIIa receptors result in greater platelet destruction. Similarly, thrombocytopenia is less severe when alloimmunization is to HPA-15 on CD109 (2000 receptors).<sup>296</sup>

## Neonatal alloimmune thrombocytopenia

NAIT is a transient but potentially life-threatening thrombocytopenic disorder limited to fetal and neonatal life. It is caused by maternal IgG alloantibodies that cross the placenta and cause premature destruction of platelets bearing paternally derived platelet alloantigens (analogous to hemolytic disease of the fetus and newborn). NAIT occurs in approximately 1–1.5 per 1000 live births.<sup>297</sup>

Approximately 75% of cases in a population of European ancestry are caused by fetomaternal incompatibility for the platelet-specific alloantigen HPA-1a and 20% by HPA-5b.<sup>286</sup> Other alloantigens implicated in NAIT, including private alloantigens identified in only one or a few families (e.g., HPA6b [Tu<sup>a</sup>/Ca] and HPA-7b [Mo<sup>a</sup>]), are shown in Table 36.4. In East Asian populations, anti-HPA-4b (Pen<sup>b</sup>) is more common than anti-HPA-1a. Although HLA or ABO alloantibodies have been claimed to cause NAIT,<sup>298,299</sup> in most cases, undetected platelet-specific alloantibodies or another diagnosis caused the thrombocytopenia.<sup>300</sup> The role of anti-HLA was specifically examined in a study of 144 cases of NAIT, and it was found that the presence of maternal anti-HLA class I antibodies was not associated with neonatal platelet count or morbidity. The authors concluded that presence of anti-HLA antibodies is not a risk factor for NAIT.<sup>301</sup>

The typical clinical presentation of NAIT is isolated severe thrombocytopenia in an otherwise healthy neonate, especially if fetomaternal incompatibility involves an alloantigen on the GPIIb/IIIa complex (Table 36.6). Petechiae are found in 90%; gastrointestinal tract hemorrhage in 30%; and hemoptysis, hematuria, and retinal bleeding in fewer than 10% of patients. Isolated intraocular hemorrhage is rare.<sup>302</sup> Approximately 15% have intracranial hemorrhage.<sup>286</sup> The thrombocytopenia usually resolves within 1–3 weeks. Serious sequelae of fetal and neonatal intracranial bleeding include hydrocephalus, porencephalic cysts, and epilepsy. First-born offspring constitute approximately one-half of patients. This suggests that, unlike the situation for Rh-hemolytic disease of the fetus and newborn, sensitization can occur early during the first pregnancy.<sup>286</sup> Subsequently affected siblings usually have thrombocytopenia to a similar or greater extent, an observation used to emphasize preventive treatment in subsequent pregnancies. In the case of heterozygous fathers of affected children, an antigen risk assessment should be performed using fetal cells (obtained through amniocentesis) or cell-free fetal DNA (obtained from maternal blood)<sup>303</sup> in future pregnancies.

Laboratory investigation of suspected NAIT involves three steps. First, there must be a high index of suspicion: isolated thrombocytopenia in an otherwise well infant should be assumed to indicate NAIT until proved otherwise. The second step is to type maternal and paternal platelets to determine whether they are incompatible for a major platelet alloantigen. Commonly, the mother lacks

**Table 36.6** Severity of Thrombocytopenia by Platelet Count Nadirs ( $\times 1000/\mu\text{L}$ ) in Relation to Target Glycoprotein for Various Alloimmune Thrombocytopenic Syndromes

Glycoprotein	NAIT	PTP	PAT	TAT
<b>GPIIb/IIIa</b>				
HPA-1a (IIIa)	17 (n = 81)	6 (n = 43)	8 (n = 9)	8 (n = 4)
HPA-1b (IIIa)	9 (n = 2)	5 (n = 4)	—	—
HPA-3ab (IIb)	10 (n = 5)	3 (n = 4)	—	—
HPA-4ab (IIIa)	13 (n = 7)	6 (n = 1)	—	—
Mean	16	6	8	8
<b>GPIa/IIa</b>				
HPA-5b	44 (n = 48)	26 (n = 1)	35 (n = 1)	43 (n = 1)
HPA-5a	35 (n = 5)	—	—	—
Mean	43	26	35	43

Source: Data from Warkentin and Smith<sup>283</sup> and Brunner-Bolliger et al.<sup>295</sup>

The data show that alloimmune thrombocytopenic syndromes that involve GPIIb/IIIa are more likely to cause severe thrombocytopenia than are those involving GPIa/IIa. The data are combined for alloimmune thrombocytopenic syndromes involving either allele of the HPA-3ab and HPA-4ab alloantigen systems, whereas the data are shown separately for the alleles of the HPA-1ab and HPA-5ab systems.

NAIT: neonatal alloimmune thrombocytopenia; PTP: post-transfusion purpura; PAT: passive alloimmune thrombocytopenia; TAT: transplantation-associated alloimmune thrombocytopenia.

certain platelet alloantigens that are often associated with alloimmune thrombocytopenia; for example, maternal homozygous HPA-1bb (PI<sup>A1</sup>-negative) status confers risk of NAIT caused by anti-HPA-1a. The third step is to determine whether the mother has platelet alloantibodies in her serum. Sometimes, no alloantibodies can be detected in maternal serum despite severe neonatal thrombocytopenia. Indeed, for approximately one-fourth of HPA-1bb mothers with infants believed to have had NAIT, anti-HPA-1a cannot be detected.<sup>286</sup> Evidence for delayed appearance of anti-HPA antibodies until several weeks after birth suggests repeat testing for maternal antibodies 2–8 weeks postpartum.<sup>304</sup> The potential for low-incidence platelet-specific alloantigens to explain fetomaternal incompatibility means that maternal serum should be crossmatched with paternal platelets whenever possible. Although maternal immunization to low-frequency antigens can explain some cases of NAIT,<sup>305,306</sup> evidence from large studies indicates that low-frequency antigens account for only a minority of NAIT cases unresolved following investigations for the common HPA antigens.<sup>307</sup> Specific recommendations for the laboratory investigation of NAIT were recently presented by the Subcommittee on Platelet Immunology of the ISTH.<sup>308</sup>

Debate continues as to whether the titer of alloantibodies predicts severity of fetal thrombocytopenia. The maternal alloantibody is sometimes detectable for years following the birth of the child with NAIT. Patients with a history of NAIT must not donate blood because their plasma can trigger passive alloimmune thrombocytopenia. It is also possible that these patients may also be at risk for transfusion reactions and PTP should they receive incompatible blood products in future.

#### Neonatal treatment

The optimal treatment of a neonate in whom NAIT is suspected because of severe thrombocytopenia is to increase the platelet count urgently to safe levels, even before serologic confirmation of the diagnosis. In some centers (e.g., National Blood Service in the United Kingdom), HPA-1bb/HPA-5aa platelets can be obtained upon request; these should be effective for most patients.<sup>309</sup> When matched platelets are not available, washed and irradiated maternal platelets should be given to the neonate. These platelets are obtained by apheresis and are washed to remove the maternal alloantibodies.

Irradiation is performed to prevent graft-versus-host disease caused by maternal lymphocytes. In an emergency, immediate administration of whole-blood-derived platelets obtained from random donors may be of benefit to a bleeding infant.<sup>310,311</sup> It is uncertain if giving high-dose IVIG to the neonate increases the platelet count further beyond that of platelet transfusions.<sup>312</sup> Corticosteroids are not recommended.

#### Prenatal management

About one-half of the time, NAIT is suspected during the prenatal period, usually because the mother previously bore an affected infant, although the diagnosis sometimes is suggested in utero when fetal ultrasonography shows cerebral hemorrhage, hydrocephalus, or hydrops fetalis. One tenet of management is that thrombocytopenia in a subsequently affected offspring is generally as severe as, or more severe than, a previously affected sibling. Neonatal alloimmune thrombocytopenia caused by anti-HPA-1a is more likely to cause fetal morbidity and mortality than that caused by anti-HPA-5b and usually requires more aggressive treatment. When the father is known to be heterozygous for the implicated alloantigen (a situation that occurs approximately 25% and 20% of the time for NAIT involving the HPA-1a/b and HPA-5a/b systems, respectively), prenatal fetal typing is important, because it identifies the infant who is homozygous for the maternal antigen and is not at risk, obviating further treatment. For pregnancies at risk, general advice to the mother includes avoiding aspirin and nonsteroidal anti-inflammatory medications.

The general approaches that have been taken to manage pregnancies at high risk of severe NAIT<sup>313–315</sup> are regular administration of high-dose IVIG, repeated in-utero platelet transfusions, or both. The initial step is to obtain a fetal platelet count by means of percutaneous umbilical blood sampling, generally starting at 20–24 weeks of gestation. Because of the risk of fetal exsanguination, maternal platelets should be on hand for transfusion if the fetal platelet count is shown to be less than 50,000/ $\mu\text{L}$ . IVIG is given weekly at a dosage of 1 g/kg/week, starting within one week of documentation of fetal thrombocytopenia. Fetal blood sampling is repeated 4–6 weeks later; if no response is seen, glucocorticoid salvage treatment (prednisone, 60 mg/day) is started.<sup>313</sup> However, not all fetuses respond to this approach.

Another approach, which has been used in certain European centers, involves regular intrauterine platelet transfusions by means of percutaneous umbilical blood sampling, including a short time before delivery. This approach has led to good outcomes in situations in which previous siblings were severely affected.<sup>314</sup> However, each fetal platelet transfusion carries risk of hemorrhage and death<sup>315</sup> that likely depends on the experience of the fetomaternal unit. There is no consensus on which approach is preferred. A systematic review<sup>315</sup> concluded that noninvasive management with weekly maternal IVIG administration is generally effective without the relatively high rate of adverse outcomes seen with invasive strategies.

Regardless of the antenatal management, there is consensus that delivery should be by means of elective cesarean section, performed as soon as fetal maturity is documented. The major reason for this mode of delivery is that it allows an organized, multidisciplinary approach to the peripartum care of the newborn. This approach includes urgent determination of the cord platelet count; provision of washed, irradiated maternal platelets (or antigen-negative platelets); and, usually, the use of high-dose IVIG (1 g/kg/day for two consecutive days) to treat severe neonatal thrombocytopenia.

### **Post-transfusion purpura**

Post-transfusion purpura is a very rare disorder that typically manifests as severe thrombocytopenia and bleeding that begin 5–10 days after blood transfusion—usually red blood cells (RBCs), occasionally platelets or plasma—in a patient previously sensitized by pregnancy or transfusion.<sup>289,316</sup> In 85–95% of cases, women are affected; the median age is 52 years. The observation that previous blood transfusions can be sensitizing explains why, on occasion, males develop PTP. Sometimes the presumably sensitizing transfusion occurs only a few weeks earlier; consequently, PTP can present after just a few weeks of intermittent transfusions.<sup>289</sup>

Although thrombocytopenia usually lasts 1–4 weeks, the duration can be as short as three days<sup>317</sup> to as long as four months or more. The platelet count is usually less than 10,000/ $\mu$ L (Table 36.6). Mucocutaneous bleeding (wet purpura, petechiae, epistaxis, gastrointestinal, and urinary tract) is common, and approximately 5–10% of patients die, usually because of intracranial hemorrhage. Because effective treatments are available (discussed further in this chapter), it is important to diagnose PTP promptly to minimize morbidity and mortality. Diagnostic confusion with HIT can result because both syndromes can present 5–10 days following surgery, and sometimes PF4/heparin antibodies are present because of concomitant exposure to heparin.<sup>318–320</sup>

### **Pathogenesis**

Almost invariably, high-titer, platelet-specific alloantibodies are found in the patient's serum or plasma. Anti-HPA-1a is detected in 60% of cases, although several other platelet alloantigens have been implicated (HPA-1b, HPA-2a, HPA-2b, HPA-3a, HPA-3b, HPA-4b, HPA-5a, HPA-5b, and HPA-15b, and the isoantigen CD-36 [Nak<sup>a</sup>]).<sup>289,321</sup> Antibodies of more than one specificity (e.g., both anti-HPA-1a and HPA-2a alloantibodies)<sup>321</sup> are observed in approximately 15% of cases. As in NAIT, the HLA-DRB3\*0101 antigen is found in most HPA-1a-negative patients with PTP. As reported for NAIT, antibodies to HPA-3a may be difficult to detect in some patients with PTP.<sup>322</sup>

Although platelet-specific alloantibodies are usually identified, the pathogenesis of PTP remains obscure, and the quandary is that autologous platelets are also destroyed. The currently favored

hypothesis is that PTP represents a situation in which alloantibodies resulting from re-exposure to an incompatible platelet alloantigen have autospecificity (“pseudospecificity”). Although the platelet-specific alloantibodies are detectable for years following an episode of PTP, the autoreactive (or panreactive) antibodies are detectable only during the acute (thrombocytopenic) phase of PTP.<sup>321</sup> In keeping with this view, Taaning and Tonnesen<sup>323</sup> reported that panreactive GPIIb/IIIa antibodies are readily detected during, but not after, an episode of PTP. Kiefel *et al.*<sup>324</sup> reported that an antibody with allospecificity for HPA-1a, but not for HPA-1b, could be eluted from both autologous and donor HPA-1bb platelets that had been sensitized with acute-phase serum from a PTP patient, suggesting that use of adsorption and elution methods may help distinguish a reactivity profile of PTP sera from that seen with NAIT. One report<sup>325</sup> suggests that such alloantibodies with autoreactivity could arise spontaneously because a woman with HPA-1bb platelets and no history of blood transfusion developed “ITP” with antibodies showing specificity for HPA-1a. Cure of the thrombocytopenia by splenectomy was accompanied by disappearance of the HPA-1a-like antibodies. Studies by another group of investigators<sup>326</sup> indicate that two distinct types of antibodies—some with alloreactivity and others with autoreactivity—develop during the acute phase of PTP. Recently, an animal model was developed<sup>327</sup> in which cross-strain platelet immunization in mice commonly induces GPIIb/IIIa-specific alloantibodies combined with platelet-specific autoantibodies and varying degrees of thrombocytopenia, further supporting the view that PTP is a transient autoimmune disorder.

### **Treatment**

High-dose IVIG is the treatment of choice for PTP.<sup>316</sup> More than 90% of patients respond, attaining a platelet count greater than 100,000/ $\mu$ L in an average of four days.<sup>328</sup> Plasmapheresis may also be used. Although some physicians also give corticosteroids, this agent probably does not influence the course of disease and should be considered adjunctive rather than primary therapy.

Whole-blood-derived (unselected) platelets—which are likely to bear the HPA-1a antigen—are usually destroyed quickly, and can cause febrile or even anaphylactoid reactions. Antigen-negative platelets are the preferred component; however, the efficacy of HPA-1a-negative platelet transfusions (for patients with PTP caused by anti-HPA-1a) is also uncertain (but are appropriate to administer with life-threatening bleeding).<sup>316</sup>

For a patient who has recovered from PTP, future precautions usually include avoidance of incompatible blood components (i.e., only autologous, washed, or platelet alloantigen-compatible RBCs are given, or platelet alloantigen-compatible plasma or platelet products are given). However, PTP recurrence is uncommon even if incompatible blood is given,<sup>289</sup> possibly because residual high-titer platelet alloantibodies immediately clear the alloantigens. Patients with a history of PTP must not donate blood because their plasma can trigger passive alloimmune thrombocytopenia (PAT).

### **Passive alloimmune thrombocytopenia**

PAT is characterized by an abrupt onset of thrombocytopenia within a few hours after transfusion of a blood component, most often RBCs or plasma.<sup>283</sup> PAT is caused by the passive transfer of platelet-reactive alloantibodies in the component which rapidly clear the incompatible recipient platelets. In one study, glycoprotein-specific platelet–antibody studies confirmed that the alloantibodies were bound to the recipient's platelets *in vivo*.<sup>329</sup> Furthermore,

although the alloantibody can be detected in the blood donor's plasma, it may not be detectable in the recipient's plasma; this finding suggests that almost 100% of the transfused alloantibody binds to target platelets soon after transfusion.<sup>295,329</sup> Although anti-HPA-1a is most commonly implicated, antibodies to HPA-3a and HPA-5b have also been reported in this syndrome.<sup>283,295,329</sup> In general, the severity of bleeding parallels the degree of thrombocytopenia; thus, spontaneous mucocutaneous bleeding usually occurs only in patients with severe thrombocytopenia caused by anti-HPA-1a. The duration of thrombocytopenia is generally less than one week. It is important to investigate suspected passive alloimmune thrombocytopenia because the risk that numerous recipients can develop this syndrome means that the implicated blood donor must not donate blood in the future.

### Transplantation-associated alloimmune thrombocytopenia

In rare instances, alloimmune mechanisms explain thrombocytopenia that occurs in the setting of HSCT or transplantation of solid organs.

#### Hematopoietic transplantation

Panzer *et al.*<sup>330</sup> reported a 32-year-old man with chronic myeloid leukemia who had severe thrombocytopenia (platelet count, 17,000/ $\mu$ L) beginning 18 months after allogeneic marrow transplantation from his HLA-matched sister. High-dose IVIG produced transient increases in platelet count, and persisting remission followed splenectomy. Antibodies with HPA-1a specificity were eluted from the patient's platelets. This led to further investigations, which showed that a small number of residual, non-neoplastic lymphoid cells of host origin produced anti-HPA-1a against the HPA-1a-positive platelets formed by donor-derived megakaryocytes. Thus, host-versus-donor alloimmune thrombocytopenia resulted from mixed chimerism, in which residual host lymphoid cells derived from the HPA-1a-negative individual developed an alloimmune response against platelets derived from the engrafted HPA-1a-positive marrow.

A similar situation attributable to anti-HPA-5b after allogeneic marrow transplantation for chronic myeloid leukemia has been reported.<sup>331</sup> However, in this patient, HPA-5b alloantibodies were detectable both before and after transplantation, and the early post-transplantation thrombocytopenia gradually improved as elutable anti-HPA-5b became more difficult to detect.

#### Solid-organ transplantation

In rare instances, immunocompetent lymphoid cells within a transplanted solid organ cause alloimmune thrombocytopenia in the recipient of the organ. A dramatic scenario was reported by West *et al.*<sup>332</sup> Three organs obtained from a multiparous female donor with normal platelet counts (two kidneys and a liver) produced severe thrombocytopenia and bleeding within 5–8 days after transplantation into three separate recipients. The two recipients of renal transplants had thrombocytopenia refractory to high-dose IVIG and platelet transfusions. One of these patients died, but the other recovered after splenectomy performed 50 days after transplantation. The liver transplant recipient had organ rejection, which was accompanied by correction of the platelet count when he received a new liver allograft. HPA-1a alloantibodies were detected in the organ donor and post-transplant (but not pretransplant) recipient serum. These cases illustrate that passenger immunocompetent lymphoid cells occasionally induce severe

alloimmune thrombocytopenia when introduced into an alloincompatible recipient.

### Platelet transfusion refractoriness

Platelet transfusion refractoriness, which is failure to achieve the expected platelet increment after two consecutive platelet transfusion episodes, has several explanations (Table 36.7). Refractoriness is primarily due to anti-MHC class I antibodies produced by the recipient following multiple transfusions. However, nonimmune, patient-dependent factors are probably the most important, which means that poor platelet count recoveries can persist even when HLA alloimmunization is prevented with leukocyte-reduced blood components<sup>333</sup> and when HLA- or ABO-compatible platelets are given, especially in patients with marrow failure. High-titer anti-HLA antibodies can also account for transfusion refractoriness in perioperative settings.<sup>334</sup>

There is anecdotal evidence that platelet-specific alloantibodies sometimes cause refractoriness. However, prospective studies have shown that this is a relatively infrequent occurrence. For example, Novotny *et al.*<sup>335</sup> found that even when HLA alloantibody formation was largely prevented with blood components leukoreduced before storage, platelet-specific alloantibodies at most explained 4 of 79 (5%) cases of refractoriness. Similarly, while Wang and coworkers found anti-HLA alloantibodies in approximately 50% of patients with platelet refractoriness, only 2% had detectable platelet-specific alloantibodies.<sup>336</sup> There are occasions, however, on which the transfusion service needs to provide HLA- and platelet-specific antigen-compatible platelet products to manage some of these patients.<sup>337</sup> In one study, antibodies to HPA-15 were found in 2.3% of multiply transfused patients and were implicated in failure to obtain an adequate platelet count in one patient.<sup>338</sup> Anti-Nak<sup>a</sup> antibodies can also explain platelet transfusion refractoriness;<sup>339,340</sup> since anti-Nak<sup>a</sup> antibodies are formed by patients who are deficient in platelet GPIV (CD36), these are correctly termed *isoantibodies* rather than alloantibodies.

### Summary

A variety of platelet–antibody assays have improved the ability of the clinician to make an accurate diagnosis of immune thrombocytopenia in many diverse clinical settings that can involve pathogenic autoantibodies, alloantibodies, and drug-dependent antibodies. The treatment decisions that arise depend upon several relevant factors, including the nature of the specific diagnosis, the expected prognosis, and the presence of clinically evident bleeding or thrombosis.

**Table 36.7** General Causes of Platelet Transfusion Refractoriness, Listed in Probable Descending Order of Frequency

#### Nonimmune Mechanisms

- Septicemia, fever, disseminated intravascular coagulation, amphotericin B therapy, hypersplenism, and fixed platelet count requirements in severe thrombocytopenia

#### Platelet-Nonspecific Alloantibodies

- HLA alloantibodies
- ABO alloantibodies

#### Drug-Dependent Antibodies (e.g., vancomycin)

- Drug-dependent antibodies (e.g., vancomycin)
- Platelet-reactive antibodies

Source: Warkentin and Smith (1997).<sup>323</sup> Reproduced with permission of Elsevier.

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A full reference list for this chapter is available at: [www.wiley.com/go/simon/Rossi6](http://www.wiley.com/go/simon/Rossi6)

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# Coagulation concentrates for inherited bleeding disorders

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## Introduction

Congenital bleeding disorders can be categorized broadly into primary hemostatic defects (platelet disorders and von Willebrand disease) and secondary hemostatic defects (coagulation factor defects). Generally, mucocutaneous bleeding, including easy bruising, epistaxis, and heavy menstrual bleeding, is due to primary hemostatic defects, whereas secondary hemostatic defects are associated with deep, delayed bleeding especially into the joints and muscles.<sup>1</sup> Congenital bleeding disorders have historically led to significant disability in children, but advancements in the last 25 years have reduced both the morbidity and mortality associated with these conditions. Continuing research is underway and promising advancements in management modalities provide hope for ongoing improvements in the care of patients with congenital bleeding disorders. Nonetheless, there likely will always be a role for the use of coagulation concentrates in the care of this patient population.

underrepresented. Females can also be affected as a result of skewed lyonization, compound heterozygosity, or hemizygosity. In fact, up to 25% and 33% of patients with mild hemophilia A and B are women and girls.<sup>5</sup> Factor activity of <50% has been associated with increased bleeding in female carriers.<sup>6</sup>

Hemophilia A and B are clinically indistinguishable and are characterized by prolonged bleeding, hemarthroses, and soft tissue/muscle hematomas. While clinically they appear similar, FIX deficiency tends to be a milder disease with less severe orthopedic outcomes.<sup>2,7,8</sup> Laboratory screening results are similar in both disorders. Patients with hemophilia A and B will have a normal prothrombin time (PT), platelet count, and platelet function analyzer 100 (PFA-100), while their activated partial thromboplastin time (aPTT) will be variably prolonged (depending on the local reagents used) and will correct with a mixing study. Mild hemophilia cases, particularly those with levels >30%, may be missed due to aPTT sensitivity issues. Tests for FVIII and FIX activity will distinguish between the two diseases.

## Hemophilia

Hemophilia A and hemophilia B are the most common X-linked bleeding disorders and are the result of deficient or defective production of plasma clotting factors VIII (FVIII) and IX (FIX), respectively.<sup>2</sup> The gene for FVIII is located on the Xq28 band of the long arm of the X chromosome, and the FIX gene is located on the Xq27 band. Hemophilia A accounts for about 80% of all cases of hemophilia, with an incidence of 1:5000 live male births. Hemophilia B has an incidence of 1:20–30,000.<sup>3</sup> Hemophilia is present throughout the world and affects all races and ethnic groups. Approximately 1/3 of all new cases occur in families with no history of hemophilia and are due to spontaneous mutations.<sup>2,4</sup>

In affected males, FVIII and FIX activity levels generally correlate well with clinical bleeding severity and can help predict bleeding risk. Generally, all affected male members of a family will exhibit similar bleeding phenotypes and factor activity levels.<sup>2</sup> Severe disease is classified as FVIII or FIX activity of <1%, moderate is 1–5%, and mild disease is characterized by factor levels of 6–40%.<sup>2,4</sup> Approximately 20,000 individuals in the United States are affected by hemophilia, 43% of which are considered severe, 26% are moderate, and 31% are mild.<sup>3</sup> However, mild cases may be

## Genetics in hemophilia

As previously noted, the genes for FVIII and FIX are located on the long arm of the X chromosome. The FVIII gene is 186kb long and consists of 28 exons.<sup>1</sup> Over 2000 unique mutations have been described, with intron 22 inversion being the most common affecting almost 45% of individuals with severe hemophilia A.<sup>9–11</sup> FVIII is a complex glycoprotein containing 2351 amino acids separated into six domains: three A domains (A-1, A-2, and A-3), a connecting B domain, and two C terminal domains (C-1 and C-2).<sup>2,12,13</sup> While the B domain function is not well understood, it is not necessary for hemostatic activity.<sup>2</sup> Intracellular proteolytic cleavage by thrombin at the Arg1689 site results in the formation of a heterodimer with an N-terminal heavy chain (A-1, A-2, and partially proteolyzed B domain) bound to a C-terminal light chain (A-3, C-1, and C-2).<sup>12</sup> This heterodimeric FVIII circulates in the blood bound via the C2 domain with von Willebrand factor (vWF). vWF protects it from proteolytic degradation and concentrates FVIII at sites of injury.<sup>2</sup> Thrombin activates FVIII by cleaving sites in both the heavy and light chains to release FVIII from vWF.<sup>13</sup> The half-life ( $t_{1/2}$ ) of vWF-bound FVIII in the plasma is about 12 hours; low levels of vWF may reduce the  $t_{1/2}$  of infused FVIII.

FIX is shorter in length, only 34 kb long, and consists of eight exons.<sup>14,15</sup> Mature FIX is a serine protease that contains 415 amino acids, is synthesized in the liver, and requires a post-translational vitamin-K-dependent  $\gamma$ -carboxylation to become active. It has a  $t_{1/2}$  of about 24 hours.<sup>14,16</sup> Depending on the genetic defect, FIX deficiency may reflect a quantitative or qualitative abnormality in the FIX molecule. About 70% of FIX mutations are of the missense type.<sup>15</sup> Hemophilia B Lyden, a rare variant, occurs due to point mutations in the promoter region of FIX. These patients have severe deficiency in childhood; however, postpubertal androgen production or hormonal changes during pregnancy result in increased FIX promoter activity and thus increased FIX production, effectively correcting or significantly mitigating their disease.<sup>17</sup>

Hemophilia A and B are usually inherited in an X-linked recessive manner. Seventy percent of mild and moderate hemophilia cases have been shown to be familial on pedigree analysis, where only 57% of severe hemophilia B and 45% of severe hemophilia A are clearly familial. The remainder, designated as sporadic cases, are due to spontaneous mutations. Half of the patients with familial mild to moderate hemophilia or severe hemophilia B had an affected direct male ancestor and only 28% of familial severe hemophilia A had an affected direct male ancestor. In sporadic cases, 88% of mothers of affected individuals were found to carry the mutation, whereas only 19% of maternal grandmothers carried the mutation.<sup>18</sup> Many sporadic cases of the characteristic FVIII inversion originate from a de novo mutation found in the maternal grandfather's male germ cells.<sup>10</sup>

Hemophilia A can be diagnosed early in life by measuring FVIII levels on umbilical cord blood in individuals at risk for inheriting the disease, verifying the exact severity from the affected newborn. Prenatal diagnosis is now usually accomplished through molecular biological techniques analyzing various polymorphic markers from DNA obtained by chorionic villous sampling. These modern techniques, such as restriction fragment length polymorphism analysis, denaturing gradient gel electrophoresis, single-strand conformation polymorphism, and DNA sequencing, are accurate and can be obtained early in pregnancy.<sup>19</sup> The intron 22 inversion responsible for about half of the FVIII mutations can be detected by polymerase chain reaction in a potential carrier or using chorionic villous cells. This technique will diagnose almost all of the intron 22 inversions seen in severe hemophilia but has been shown to miss up to 4% of moderate and 12% of mild hemophilia A.<sup>20</sup> FIX levels are physiologically low at birth, and thus a low FIX level in the cord blood or from the affected newborn must be repeated at 6–12 months of age to confirm the severity of the hemophilia B diagnosis.<sup>1</sup>

### Clinical features

The clinical hallmark of hemophilia is bleeding, especially into the joints, muscles and soft tissue, but the site of bleeding and pattern of bleeding is widely varied based on disease severity and age.<sup>2,12</sup>

Individuals with mild hemophilia (activity 6–40%) usually have clinically mild disease and may only bleed after a hemostatic challenge such as surgery or trauma. These individuals are often diagnosed later in childhood if there is a lack of family history of hemophilia.<sup>4</sup> Those with moderate hemophilia have prolonged bleeding with surgery and trauma, but spontaneous bleeding is uncommon and variable. Severe hemophilia is associated with recurrent mucocutaneous bleeding, hemarthroses/deep soft tissue bleeds with minimal or no identified trauma, and severe postsurgical bleeding. Some patients with severe disease have a milder clinical disease; in contrast, some individuals with mild and moderate disease have a more severe

phenotype. It has been postulated that this considerable phenotypic heterogeneity might be related to the co-presence of a Factor V Leiden mutation and other prothrombotic conditions that counteract the bleeding tendency, differences in physical activity levels, and structural integrity of joints.<sup>12,21–24</sup> Additionally, some patients have a markedly different FVIII level if performed via one stage versus chromogenic assay.<sup>25</sup> If there is suspicion of a discrepancy in bleeding phenotype, both assays should be considered in both affected males and females.

The first hemostatic challenge experienced by a patient with hemophilia is birth. Up to 53% of patients with hemophilia experience a bleed by one month of age, with post-circumcision bleeding the most common (47.9%), followed by head bleeds (19.4%), and bleeding from heel sticks (10.4%).<sup>26</sup> The rate of intracranial hemorrhage (ICH) experienced in neonates with hemophilia is reported at 1–4%, much higher than the rate experienced in neonates without bleeding disorders. These hemorrhages have been associated with seizures, psychomotor retardation, and cerebral palsy.<sup>26–28</sup> Generally, prolonged labor and instrumentation during delivery (including forceps, vacuum assistance, and scalp electrodes) should be avoided in infants born to known hemophilia carriers to decrease the risk of ICH.<sup>27</sup> A screening cranial ultrasound to evaluate for ICH at birth is generally recommended.

Children become symptomatic more often after the newborn period and before two years of age.<sup>29</sup> The first symptomatic bleeding leading to the diagnosis of severe hemophilia has been shown to occur at a median of about 10 months of age (19 months with the presence of a prothrombotic risk factor). In patients with moderate hemophilia, the first symptomatic bleed may occur as late as 22 months, while mild hemophilia may go undetected for many years until a significant hemostatic challenge is encountered.<sup>21–23,30–32</sup> ICH is a serious complication in hemophilia at all ages, with 2% of patients older than two years facing this problem with an associated 20% mortality rate. Severe hemophilia and high titer inhibitors are independent risk factors for ICH development.<sup>33</sup>

Hemarthrosis or joint bleeding is pathognomonic of severe hemophilia. The age range for the first joint bleed in severe hemophilia varies significantly and has been reported as 0.2–5.8 years.<sup>34</sup> The factors that initiate the hemorrhage are often unknown and the onset can be random, occurring spontaneously or after minimal injury. Joint bleeds become more prevalent after individuals begin to bear weight. All synovial joints are at risk, but ankles are most commonly affected in children, and the knees, elbows, and ankles are more affected in adolescents and adults. Usually one joint is affected at a time, but a joint that experiences  $\geq 3$  bleeds in a six-month time period is referred to as a target joint.<sup>35</sup> Repeated hemorrhages into a joint leads to hemophilic arthropathy, which is associated with synovial hyperplasia, articular cartilage degradation, cystic changes in the subchondral bone, loss of joint space, and atrophy of the surrounding muscles, eventually leaving a deformed joint with contractures, pain, and decreased range of motion.<sup>12,36,37</sup>

Muscle hematomas are the second most common type of bleeding experienced in hemophilia, accounting for 10–25% of all bleeds.<sup>38</sup> Quadriceps, iliopsoas, and forearm muscles are commonly affected and, if the bleeds are large enough, can result in compartment syndrome leading to short- or long-term neurologic damage. Iliopsoas bleeding is a primary concern in hemophilia because significant blood loss can occur into this large muscle and nerve compression might follow.<sup>39</sup> Other bleeding encountered in hemophilia includes epistaxis, oral mucosal, gastrointestinal tract, and hematuria.

## Management of hemophilia

Hemophilia management is complex. It requires multidisciplinary comprehensive oversight focused on preventative care, factor replacement management, and treatment of disease complications.<sup>40</sup> Preventative care includes standard dental surveillance to reduce gingival disease; education on wearing medical alert devices and avoidance of aspirin, nonsteroidal anti-inflammatory drugs, and other platelet modifying medications; avoidance of contact sports; and vaccinations against blood borne pathogens.<sup>1</sup> Before the advent of factor replacement therapy, most patients with severe disease developed arthropathy prior to the age of 20 and the mortality rate due to ICH was significant. In the 1920s and 1930s, life expectancy of patients with hemophilia in developing countries, such as Finland and Sweden, was only 8–11 years and as recently as the 1960 life expectancy was still <30 years.<sup>41–43</sup> Many advancements in the medical management of hemophilia have been made since the 1960s (Figure 37.1). Unfortunately, as advancements were made in hemophilia management, significant setbacks were also encountered. Transmission of hepatitis C virus (HCV) and human immunodeficiency virus (HIV) complicated blood component therapy in the 1980s.<sup>44,45</sup> The implementation of scheduled factor replacement therapy led to the development of inhibitors and rising treatment costs, a major ongoing obstacle in developing countries.<sup>46</sup> Nonetheless, even with these setbacks, advancements in the last 30 years, including the emergence of comprehensive hemophilia treatment centers, recombinant factor concentrates, modified extended half-life concentrates, and a more widespread adoption of prophylactic home replacement therapy, have led to significantly improved outcomes in individuals with hemophilia.<sup>47</sup>

## Blood component therapy

Fresh-frozen plasma (FFP) and whole blood were the only preparations available to treat FVIII or FIX deficiency until 1964, when a method for concentrating FVIII into a cold-insoluble precipitate, or cryoprecipitate, was discovered.<sup>48</sup> Cryoprecipitate prepared from single plasma units contains about 50% of the FVIII, vWF, fibrinogen, and factor XIII (FXIII) of the original plasma unit, or about 100 IU of FVIII and 0.2 g of fibrinogen in 8–10 mL. Cryoprecipitate made elective surgery and outpatient treatment of bleeding episodes feasible for patients with hemophilia A. Cryoprecipitate is still used in many developing countries but has disadvantages over the newer commercially prepared concentrates, including increased risk of blood-borne pathogen transmission, marked variations

in FVIII and vWF content, larger volume, inadequate hemostatic protection, and required storage temperatures of <−20 to −30 °C.

In the 1970s, fractionation methods were developed that produced plasma-derived lyophilized concentrates of both intermediate-purity FVIII (containing vWF in addition to FVIII) and prothrombin complex concentrates (PCCs), which contain the vitamin-K-dependent clotting factors II, VII, IX, X, as well as proteins C and S. These new products allowed the adoption of home self-infusion replacement therapies for both hemophilia A and B and led to reduced morbidity compared to untreated patients. This even resulted in the pioneering of primary prophylaxis in Sweden, which achieved the goal of preventing bleeding episodes.<sup>49</sup> Hemophilia centers were able to focus on the development of comprehensive care programs since they were no longer overwhelmed with providing emergency treatments.<sup>50</sup> Subsequently, the discovery in 1977 of the effect of desmopressin on FVIII and vWF concentrations provided a relatively inexpensive and safe treatment option for many patients with mild to moderate hemophilia A (and mild vWD). This synthetic drug, which increases FVIII and vWF plasma levels by releasing them from Weibel Palade bodies in endothelial cells, reduced the use of plasma derived products in this population of nonsevere hemophilia A.<sup>4,51</sup>

The late 1970s and early 1980s were devastating in the care of hemophilia. Tragically, approximately half of the population with hemophilia in the United States, or 9300 individuals, became infected with HIV and 80% with hepatitis C.<sup>52</sup> As many as 90% of patients with hemophilia became seropositive for hepatitis B surface antigen. The first reported case of acquired immunodeficiency syndrome in individuals with hemophilia was reported in the early 1980s, and it became apparent that it was being transmitted via blood and blood components, including factor concentrates.<sup>53</sup> Approximately 90% of persons who received FVIII concentrates and 55% of those who received PCCs prepared in the United States between 1979 and 1984 became seropositive for HIV.<sup>54</sup> Fortunately, HIV proved to be heat labile, and by late 1984 almost all patients with hemophilia were receiving heat-treated concentrates. Additionally, in 1984, a solvent-detergent process for treating blood products was developed that drastically reduced the transmission of both hepatitis B and C.<sup>55</sup> By 1985, HIV seropositivity screening for blood and plasma donors was also in place. Shortly thereafter, methods to purify FVIII using murine monoclonal antibodies and immunoaffinity chromatography became commercially available in 1987. This purification process reduced viral contaminants by several logs and any residual viral risks were mitigated by pasteurization or solvent/detergent methods.<sup>56</sup> These measures greatly

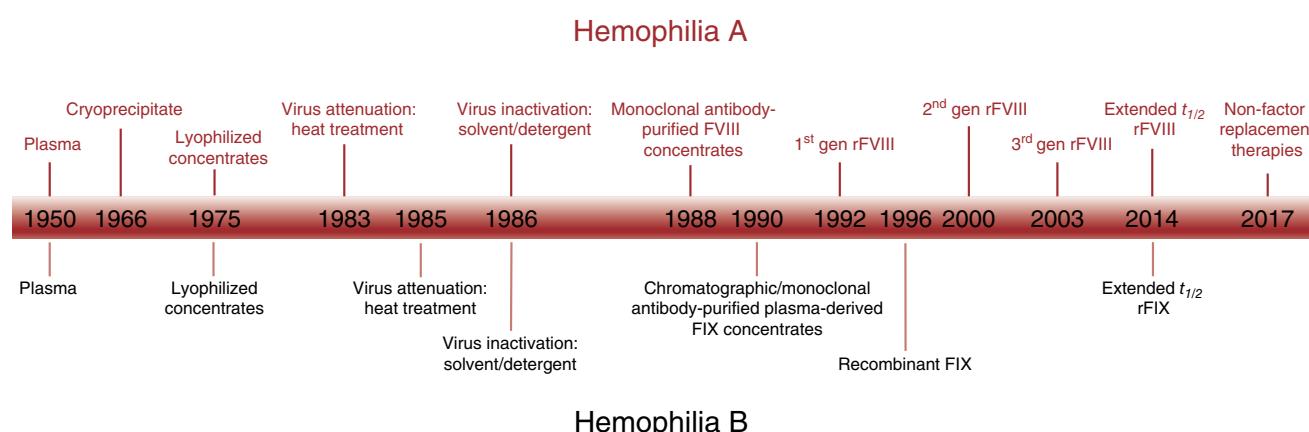


Figure 37.1 Evolution timeline of hemophilia A and hemophilia B treatment modalities.

improved the safety of plasma-derived FVIII and FIX products. Since 1987, HIV and hepatitis transmission by factor concentrates has not been reported in the North American population.<sup>54</sup>

Despite these advancements, the transmission of blood-borne viruses or novel infectious agents remained a concern. One of the most important innovations in hemophilia care came with the cloning of the FVIII gene in 1982 and the FIX gene in 1984, which allowed for the development and production of recombinant factor products, which were first shown to be clinically effective in 1989.<sup>57</sup>

### **Recombinant factor VIII**

Recombinant FVIII (rFVIII) was quickly developed after the molecular cloning of complementary DNA encoding human FVIII and the expression of human FVIII from recombinant DNA clones.<sup>58,59</sup> Three generations of standard half-life rFVIII products are commercially available with differences in their preparation. First-generation products utilized animal-derived proteins in cell culture and stabilized the final formulation of FVIII with human serum albumin. Second-generation products eliminated animal products during fermentation, and nearly all of the human albumin was eliminated in the final formulation as well. No human-derived proteins are used in the culture medium or final formulation of third-generation products.<sup>60</sup> Most patients in the United States and many other developed countries have been switched to rFVIII therapy for theoretical concerns over residual risks of blood-borne pathogen transmission. The commercially available rFVIII products are summarized in Table 37.1.

The only first-generation rFVIII product that remains on the market in the United States is Recombinate (Takeda, USA) and it has demonstrated the same pharmacokinetics and clinical effectiveness as plasma-derived FVIII products.<sup>61,62</sup>

Second-generation rFVIII products include Kogenate (Bayer, USA) and Helixate (CSL Behring, USA). Third-generation products include full length (Advate, Takeda, USA; Kovaltry, Bayer, USA; NovoEight, Novo Nordisk, Denmark) and truncated (Syntha, Pfizer, USA; Afystyla, CSL Behring, USA; Nuwiq, Octapharma, USA) preparations. The truncated preparations are B-domain-deleted (BDD) as the heavily glycosylated B domain is not needed for the hemostatic activity of FVIII.<sup>63</sup> BDD products have been shown to be safe, effective in the treatment of bleeding episodes, and well tolerated.<sup>64</sup> The important advancement with these products is that no human proteins are utilized in the initial preparation stages or in the final formulation. Nuwiq is a BDD product that was the second rFVIII product made in a human cell line with the rationale that it would potentially eliminate antigenic epitopes, and more closely resemble endogenous FVIII post-translational modifications, as compared to the use of nonhuman cell lines (hamster).<sup>65</sup> The Food and Drug Administration (FDA) has also approved the use of the extended  $t_{1/2}$  rFVIII products (Eloctate, Sanofi Genzyme, USA; Adynovate, Takeda, USA; Jivi, Bayer, USA). For Eloctate, BDD rFVIII is fused to the Fc portion of immunoglobulin G subclass 1 or IgG<sub>1</sub>.<sup>66,67</sup> This product has been proven to have prolonged circulation compared to other rFVIII products.<sup>68,69</sup> Adynovate and Jivi utilize PEGylation technology that has also been shown to prolong plasma circulation compared to standard half-life unmodified rFVIII products.<sup>70-72</sup>

### **Dosage and administration of factor VIII**

FVIII concentrates are routinely used to treat acute bleeding episodes in individuals with moderate or severe hemophilia and for those with mild hemophilia who show poor response to desmo-

pressin or are undergoing a major procedure. Factor replacement can be given “on demand” (after a bleeding episode), to treat a hemorrhage, or as “prophylaxis” (scheduled infusions to prevent or decrease the frequency or intensity of hemorrhages). Acute hemarthrosis, intramuscular bleeding, and surgical prophylaxis are common indications for factor replacement. Prompt treatment can prevent or reduce complications, like chronic joint disease, and can reduce the need for subsequent factor infusions (Table 37.2).<sup>73</sup> Usually, 1 U/kg of FVIII will increase the plasma FVIII concentration by 2% (0.02 IU/dL).<sup>12</sup> The  $t_{1/2}$  of FVIII is about 12 hours, but significant interpatient variability exists with ranges of 6–25 hours described.<sup>74</sup> The  $t_{1/2}$  may be shorter in patients that are younger, febrile, have ongoing extensive bleeding, or have an inhibitor.

Factor replacement can be performed using bolus dosing (more commonly) or administered as a continuous infusion. Continuous infusions are often utilized in operative and postoperative situations, as well as for managing central nervous system (CNS) hemorrhage and have the benefit of precision. Additionally, this is the only feasible way to maintain a level of 100% for a prolonged period of time.<sup>2</sup> A bolus dose of 50 U/kg of FVIII should be administered followed by a continuous infusion of 2–5 IU/kg/hour as initial dosing. FVIII activity levels should be monitored intermittently and infusion rates should be adjusted accordingly to ensure FVIII activity is in the desired hemostatic range, typically 80–120% for major procedures.<sup>2,75,76</sup>

### **Prophylaxis**

Primary prophylaxis refers to beginning factor replacement before a pattern of recurrent bleeding begins, particularly joint bleeding. Secondary prophylaxis refers to beginning the therapy after two or more joint bleeds but before the onset of joint disease.<sup>73</sup> The idea for prophylactic treatment started in Malmö, Sweden, after the observation that patients with moderate hemophilia had a significantly lower incidence of chronic arthropathy.<sup>77</sup> Patients with severe hemophilia A were monitored for 2–25 years on prophylactic factor infusions and were shown to have fewer bleeding events and less joint disease than those receiving on-demand treatment.<sup>78,79</sup> Randomized trials have demonstrated that prophylaxis administration improves outcomes and reduces joint disease in children with severe hemophilia when compared to episodic on-demand treatment.<sup>80</sup> Thus, prophylactic administration of factor to prevent bleeding episodes has become the standard of care for patients with severe hemophilia.

Prophylaxis in hemophilia A usually involves administration of FVIII every other day or three days a week at starting doses of 25–40 U/kg, targeting a trough level of >1% when feasible.<sup>81,82</sup> Since prophylaxis is now recommended to start at a young age, the frequent infusions may result in the need for a central venous access device (CVAD); although factor administration may be easier, CVAD placement requires careful consideration due to the risk of infections and thrombosis.<sup>82</sup> It is common to use an escalating dose/frequency schedule for prophylaxis in young children that entails starting with a once weekly infusion, escalating to twice weekly, and eventually thrice weekly. Some practitioners will increase to full dose prophylaxis regardless of bleeding phenotype, whereas others only increase dosing in those patients with a high incidence of bleeding.<sup>83</sup> Extended  $t_{1/2}$  products lessen the administrative burden by decreasing the frequency of prophylaxis dosing in some individuals with hemophilia or by providing a higher trough activity level in individuals with a high incidence of bleeding or shortened  $t_{1/2}$ .

**Table 37.1** Summary of the Recombinant Factor VIII Products

	Recombinant Factor VIII Product Features					
	Recombinate	Kogenate FS	Helixate FS	Advate	Xyntha	Kovaltry
<b>Host Cell</b>	CHO	BKH	BKH	CHO	CHO	BKH
<b>Purification and Virucidal Techniques</b>	ION Exchange Chromatography Mouse Monoclonal Antibody (Mab) Immunoaffinity Chromatography	ION Exchange Chromatography Mouse Monoclonal Antibody (Mab) Immunoaffinity Chromatography Solvent Detergent	ION Exchange Chromatography Mouse Monoclonal Antibody (Mab) Immunoaffinity Chromatography Solvent Detergent	ION Exchange Chromatography Mouse Monoclonal Antibody (Mab) Immunoaffinity Chromatography Solvent Detergent	Synthetic Peptide Affinity Chromatography Immunoaffinity Chromatography Solvent Detergent Nanofiltration	Chromatography Solvent Detergent Nanofiltration
<b>Stabilizer Available Vial Sizes (IU)</b>	Albumin 250 500 1000 1500 2000 3000	Sucrose 250 500 1000 2000 3000	Sucrose 250 375 500 750 1000 1500 1700 2000 2500 3000 4000	Mannitol Trehalose 250 500 1000 1500 2000 2500 3000	Sucrose 250 500 1000 2000 3000	Sucrose 250 500 1000 2000 3000
<b>Diluent Volume</b>	10ml	2.5ml up to 1000 units 5 ml for 2000 units+	2.5ml up to 1000 units 5 ml for 2000 units +	2ml up to 1700 units 5 ml for 2000 units+	4 ml	2.5 ml up to 1000 units 5 mL for 2000 units+
<b>Dosage Calculation t<sub>1/2</sub></b>	1IU/kg raises level by 2% 9.7–19.5 hours Room temperature, until expiration Not to exceed 86°F Can refrigerate at 36°F–46°F	1 IU/kg raises level by 2% 7.8–15.3 hours Room temperature, 12 months Not to exceed 77°F Should refrigerate at 36°F–46°F	1 IU/kg raises level by 2% 7.8–15.3 hours Room temperature, 12 months Not to exceed 77°F Should refrigerate at 36°F–46°F	8–14 hours Room temperature, 6 months Not to exceed 86°F Should refrigerate at 36°F–46°F	3.5–10.6 hours Room Temperature, 3 months Not to exceed 77°F Should refrigerate at 36°F–46°F	1 IU/kg raises level by 2% 12–14.4 hours Room Temperature, 12 months Not to exceed 77°F Can refrigerate at 36°F–46°F
<b>Storage</b>						(Continued)

**Table 37.1** (Continued)

	Recombinant Factor VIII Product Features					
	Afstyla	NovoEight	Eloctate	Adynovate	Jivi	Nuwiq
<b>Host Cell</b>	CHO	CHO	HEK	CHO	BHK	HEK
<b>Purification and Virucidal Techniques</b>	Multistep Chromatography process Solvent Detergent Nanofiltration	ION Exchange Chromatography Immunoaffinity Chromatography Chromatography Solvent Detergent Nanofiltration	Synthetic Peptide Affinity Chromatography Immunoaffinity Chromatography Solvent Detergent Nanofiltration	Immunoaffinity Chromatography Solvent Detergent	ION Exchange Chromatography Immunoaffinity Chromatography Nanofiltration Detergent	Multimodal cation chromatography ION Exchange Chromatography Affinity Chromatography Size exclusion Chromatography Solvent Detergent Nanofiltration
<b>Stabilizer Available Vial Sizes (IU)</b>	Sucrose 250 500	Sucrose 250 500	Sucrose 250 500 750	Mannitol Trehalose 250 500 750	Mannitol Trehalose 500	Sucrose 250 500
	1000	1000	1000	1000	1000	1000
	1500	1500	1500	1500		
	2000	2000	2000	2000	2000	2000
	2500					2500
	3000	3000	3000 4000		3000	3000 4000
<b>Diluent Volume</b>	2.5 ml up to 1000 units 5 mL for 1500 units+*	4 ml	3 ml	2 ml up to 1500 units 5 mL for 2000 units+	2 ml	2.5 ml
<b>Dosage Calculation</b>	1 IU/kg raises level by 2%*	1 IU/kg raises level by 2%	1 IU/kg raises level by 2%	1 IU/kg raises level by 2%	1 IU/kg raises level by 2%	1 IU/kg raises level by 2%
<b>t<sub>1/2</sub> Storage</b>	12.3–14.3 hours Room temperature, 3 months Not to exceed 77°F Should refrigerate at 36°F–46°F	7.7–10.8 hours Room temperature, 12 months Not to exceed 86°F Should refrigerate at 36°F–46°F	12–16.4 hours Room temperature, 6 months Not to exceed 86°F Should refrigerate at 36°F–46°F	11.8–14.69 hours Room temperature, 3 months Not to exceed 86°F Should refrigerate at 36°F–46°F	17.4–21.4 hours Room temperature, 6 months Not to exceed 77°F Should refrigerate at 36°F–46°F	11.9–17.1 hours Room temperature, 3 months Not to exceed 77°F Should refrigerate at 36°F–46°F

\* Multiplier needed to calculate FVIII activity level if monitoring factor activity with a one-stage clotting assay

BKH, baby hamster kidney; CHO, Chinese hamster Ovary; °F, degree Farenheit; HEK, human embryonic kidney; IU, international unit; kg, kilogram; ml, milliliter used

**Table 37.2** Suggested Plasma Factor Peak Level and Duration of Administration (When There Is No Significant Source Restraint)<sup>73</sup>

Type of hemorrhage	Hemophilia A				Hemophilia B			
	Peak factor level (IU/dL)	Treatment duration (d)	Peak factor level (IU/dL)	Treatment duration (d)	Peak factor level (IU/dL)	Treatment duration (d)	Peak factor level (IU/dL)	Treatment duration (d)
Joint	10–20	1–2 <sup>a</sup>	40–60	1–2 <sup>a</sup>	10–20	1–2 <sup>a</sup>	40–60	1–2 <sup>a</sup>
Superficial muscle/no NV compromise (except iliopsoas)	10–20	2–3 <sup>a</sup>	40–60	2–3 <sup>a</sup>	10–20	2–3 <sup>a</sup>	40–60	2–3 <sup>a</sup>
Iliopsoas or deep muscle with NV injury or substantial blood loss								
Initial	20–40	1–2	80–100	1–2	15–30	1–2	60–80	1–2
Maintenance	10–20	3–5 <sup>a</sup>	30–60	3–5 <sup>a</sup>	10–20	3–5 <sup>a</sup>	30–60	3–5 <sup>a</sup>
Intracranial								
Initial	50–80	1–3	80–100	1–7	50–80	1–3	60–80	1–7
Maintenance	20–40	8–14	50	8–21	20–40	8–14	30	8–21
Throat and neck								
Initial	30–50	1–3	80–100	1–7	30–50	1–3	60–80	1–7
Maintenance	10–20	4–7	50	8–14	10–20	4–7	30	8–14
Gastrointestinal								
Initial	30–50	1–3	80–100	7–14	30–50	1–3	60–80	7–14
Maintenance	10–20	4–7	50	—	10–20	4–7	30	—
Renal	20–40	3–5	50	3–5	15–30	3–5	40	3–5
Deep laceration	20–40	5–7	50	5–7	15–30	5–7	40	5–7
Surgery (major)								
Pre-op	60–80		80–100		50–70		60–80	
Post-op <sup>c</sup>	30–40	1–3	60–80	1–3	30–40	1–3	40–60	1–3
	20–30	4–6	40–60	4–6	20–30	4–6	30–50	4–6
	10–20	7–14	30–50	7–14	10–20	7–14	20–40	7–14
Surgery (minor)								
Pre-op	40–80		50–80		40–80		50–80	
Post-op <sup>d</sup>	20–05	1–5	30–80	1–5	20–50	1–5	30–80	1–5

Notes: In this table, the desired peak factor levels of CFC replacement shown for treatment of hemorrhages at different anatomical sites represent the ranges in global practice patterns depending on available resources. Importantly, it should be recognized that the goal of such treatment is effective control of bleeding and should be the same everywhere in the world. Lower CFC replacement levels require much closer observation for effectiveness of bleeding control, with a potentially greater chance of requiring additional CFC replacement to achieve the target plasma level as well as the hemostatic and musculoskeletal outcomes.

Abbreviations: CFC, clotting factor concentrate, NV, neurovascular.

<sup>a</sup> May be longer if response is inadequate.

<sup>b</sup> Sometimes longer as secondary prophylaxis during physical therapy.

<sup>c</sup> The duration of treatment refers to sequential days post-surgery. Type of CFC and patient's response to CFC should be taken into account.

<sup>d</sup> Depending on procedure; the number of doses would depend on the half-life of the CFC used.

## Recombinant factor IX

Until the late 1990s, intermediate-purity PCCs were the mainstay of treatment for hemophilia B. These products contained some inactivated as well as some activated coagulation factors and were associated with thromboembolic complications. Due to this thrombogenic potential, high-purity plasma-derived FIX concentrates (Mononine, CSL Behring, USA; Alphanine, Grifols, USA) were created and, although more costly, were less thrombogenic.<sup>84</sup> However, these products were plasma-derived and associated with the same theoretical risks seen with other plasma-derived products. Thus, recombinant FIX (rFIX) products were created.

Recombinant FIX products are made using no albumin, human plasma, or animal derived proteins, but are made in a mammalian cell line. The available rFIX products are summarized in Table 37.3. There are three standard acting products that have similar efficacies (Rixubis, Shire, USA; Benefix, Pfizer, USA; Ixinity, Aptev Biotherapeutics, USA), and three extended  $t_{1/2}$  rFIX products (Alprolix, Sanofi Genzyme, USA; Idelvion, CSL Behring, USA; and Rebinyn, Novo Nordisk, Denmark). Alprolix is similar to Eloctate in that rFIX is fused to the Fc portion of IgG<sub>1</sub>, which significantly prolongs the circulating plasma  $t_{1/2}$  of the FIX.<sup>85</sup> Rebinyn and

Idelvion use PEGylation technology to extend the circulating plasma  $t_{1/2}$ . Despite this prolonged circulating plasma  $t_{1/2}$ , there are reports of spontaneous bleeding and poor bleeding response in patients on extended  $t_{1/2}$  rFIX products, suggesting that the trough FIX activity levels alone may not predict bleeding phenotype.<sup>86</sup> This was corroborated in a phase IV study of rFIX-FP, in which arthralgia was the most commonly reported treatment emergent adverse event, and target joints developed in ~14% of study participants despite a trough FIX level of >7%.<sup>87</sup>

## Dosage and administration of factor IX

FIX requires a higher dose to achieve the same plasma concentrations as compared with FVIII replacement, although the extravascular distribution of FIX may provide additional hemostatic protection despite variable plasma levels. The recommended dosage for hemorrhage treatments varies based on the type and severity of bleed (Table 37.2).<sup>73</sup> The  $t_{1/2}$  of FIX is about 24 hours.<sup>74</sup> Plasma-derived FIX products will raise the FIX activity by 1% (0.01 IU/dL) when 1 U/kg is administered. Generally, 1 U/kg of rFIX will raise the plasma FIX activity by 0.8–1% (0.008 IU/dL).<sup>12</sup> Thus, to obtain 100% replacement, a patient with severe hemophilia B must

**Table 37.3** Summary of the Recombinant Factor IX Products.

Recombinant Factor IX Products Features			
	Benefix	Rixubis	Ixinity
<b>Host Cell</b>	CHO	CHO	CHO
<b>Purification and Virucidal Techniques</b>	Immunoaffinity Chromatography Nanofiltration	Ion Exchange Chromatography Nanofiltration Solvent Detergent	Chromatography Nanofiltration Solvent Detergent
<b>Stabilizer</b>	Sucrose	Sucrose	Trehalose Dihydrate Mannitol
<b>Available Vial Sizes (IU)</b>	250 500 1000  2000 3000	250 500 1000  2000 3000	250 500 1000 1500 2000 3000
<b>Diluent Volume</b>	5 ml	5 ml	5 ml
<b>Dosing Calculation</b>	1.2–1.4 IU/kg raises plasma level by 1%	1.1 IU/kg raises plasma level by 1%	1 IU/kg raises plasma level by 1%
<b><math>t_{1/2}</math></b>	14–28 hours	16–27 hours	17–31 hours
<b>Storage</b>	Room temperature not to exceed 86°F for 6 months Should refrigerate at 36°–46°F	Room temperature not to exceed 86°F for 6 months Should refrigerate at 36°–46°F	Refrigerate at 36°F–46°F (250 IU vials) Store at 36°–77°F (all other vials)
Recombinant Factor IX Products Features			
	Alprolix	Idelvion	Rebinyn
<b>Host Cell</b>	HEK	CHO	CHO
<b>Purification and Virucidal Techniques</b>	Column Chromatography Nanofiltration	Solvent Detergent	Monoclonal Affinity Chromatography Nanofiltration Solvent Detergent
<b>Stabilizer</b>	Sucrose	Sucrose Albumin 250	Sucrose Mannitol
<b>Available Vial Sizes (IU)</b>	500 1000 2000 3000	500 1000 2000	500 1000 2000
<b>Diluent Volume</b>	5 ml	2.5 ml up to 1000 IU vial 5 mL for 2000 IU vial	4 ml
<b>Dosing Calculation</b>	1 IU/kg raises plasma level by 1%	1 IU/kg raises plasma level by 1.08–1.65%	1 IU/kg raises plasma level by 1.82–1.92%
<b><math>t_{1/2}</math></b>	66.4–86.52 hours Room temperature not to exceed 86°F for 6 months Should refrigerate at 36°–46°F	90–104 hours Store at 36°F–77°F	69.6–89.4 hours Room temperature not to exceed 86°F for 6 months Should refrigerate at 36°–46°F

CHO, Chinese hamster Ovary; °F, degree Farenheit; HEK, human embryonic kidney; IU, international unit; kg, kilogram; ml, milliliter

receive 100–120 IU/kg. Factor IX concentrates may be given in bolus or continuous infusions, with continuous infusions usually utilized perioperatively and with CNS bleeding. A bolus dose of 100–120 U/kg should be infused followed by 4–8 U/kg/hour with periodic FIX activity measurements to maintain appropriate hemostatic levels, typically 80–120% for major procedures.<sup>2,75,76,88</sup>

### Ancillary therapeutic options

#### Desmopressin

Desmopressin is the treatment of choice for individuals with mild hemophilia A and some cases of moderate hemophilia A where a fourfold increase in FVIII activity 30–60 minutes after administration is predicted to be adequate for the type of bleeding episode.<sup>51,73,89</sup> Not all patients with mild hemophilia will respond to desmopressin, so a trial dose must be administered and FVIII activity levels subsequently monitored. Trial administrations are usually a weight-adjusted 0.3 µg/kg IV/subcutaneous (SC) dose, or a fixed dose of intranasal spray at 150 µg (one spray) or 300 µg (one spray in each nostril) for patients weighing <50 kg or >50 kg, respectively. Desmopressin may be dosed every 12–24 hours, but 3–4 repeated doses over a short period can result in tachyphylaxis (decreased responsiveness). Desmopressin side effects are generally mild and include tachycardia, headache, and flushing. Significant fluid retention resulting in hyponatremia and seizures can occur particularly in young children and the elderly, and all patients must be fluid restricted for at least 24 hours following administration.<sup>90</sup>

#### Antifibrinolytic agents

Antifibrinolytic agents, such as lysine analogs  $\epsilon$ -aminocaproic acid and tranexamic acid, inhibit plasminogen activation and are usually used as an adjuvant therapy to maintain hemostasis. These agents can be useful in preventing lysis of a formed clot, especially when bleeding occurs in areas of high fibrinolytic activity like the oral mucosa.<sup>73,91</sup> The recommended dose of  $\epsilon$ -aminocaproic acid is 50–100 mg/kg IV or orally every 6–8 hours, while the dose of tranexamic acid is 10 mg/kg IV every 6–8 hours or 15–25 mg/kg orally every 6–8 hours. For invasive dental procedures, therapy can be started as early as the evening prior to the procedure and continued for 7–10 days.<sup>91</sup> The use of tranexamic acid mouth wash has been shown to be effective at decreasing post-operative bleeding and transfusion requirements in patients with hemophilia following oral surgery when used in conjunction with systemic antifibrinolytic therapy.<sup>92</sup> The recommended dosing is 10 mL of 4.8% tranexamic acid solution for two minutes four times a day.

#### Vaccinations

Despite the current low risk of post-transfusion hepatitis, all persons with hemophilia should receive both hepatitis A and B immunizations. While all blood products are screened for hepatitis viruses, testing may not be 100% effective, particularly in donors with window phase infections. The risk of transmission can be further increased with plasma-derived products, in which plasma collections from 2500 to 20,000 donors are used to produce one lot of concentrate. However, in 2003 the Centers for Disease Control and Prevention investigated seroconversion to hepatitis A or B in people with bleeding disorders and found that no cases could be attributed to factor products.<sup>93</sup> Nonetheless, hepatitis vaccination is strongly recommended. Hepatitis A and B vaccines should be given subcutaneously due to the risk of intramuscular (IM) bleeding associated with IM vaccines.<sup>73</sup>

#### Avoidance of drugs that cause platelet dysfunction

Certain drugs can induce platelet dysfunction, which can exacerbate bleeding in patients with congenital bleeding disorders. Aspirin irreversibly inhibits platelet cyclooxygenase resulting in the inhibition of prostaglandin synthesis and thus should be avoided. Other medications known to induce platelet dysfunction include antihistamines, phenothiazines, and nonsteroidal anti-inflammatory agents (indomethacin and ibuprofen). Acetaminophen can be used to relieve mild pain in conjunction with rest, cooling, and elevation. Cyclooxygenase 2 (COX-2) inhibitors have also been shown to be effective in managing hemophilic arthropathy pain without interfering with platelet function and are thus considered a safe alternative for pain management in this population.<sup>94</sup>

#### Inhibitors in hemophilia

One of the most serious complications associated with hemophilia is the development of a neutralizing IgG alloantibody, or inhibitor, to the deficient factor.<sup>95</sup> Approximately 30% of patients with severe hemophilia A, 5–10% with mild and moderate hemophilia A, and 2–5% of patients with hemophilia B will develop an inhibitor.<sup>96–98</sup> The significant discrepancy in inhibitor rates may be partially explained by the presence of cross-reactive material (CRM). Many patients with hemophilia B will have circulating FIX antigen despite low activity because many FIX mutations are missense. The presence of this FIX CRM could potentially explain the lower risk of developing neutralizing antibodies when FIX medications are administered.<sup>99</sup> For hemophilia A, inhibitors usually develop in early childhood, within the first 50 exogenous factor exposure days; the risk declines after 150 treatment days.<sup>100</sup> For hemophilia B, the highest risk for inhibitor development is believed to be in the first 50–75 exposure days. The first 20 exposure days for patients with severe hemophilia B should be performed under close clinical supervision due to the risk for severe allergic reactions.<sup>101</sup>

Inhibitor formation in hemophilia is a complex multifactorial process. Associated risk factors include family history of inhibitors, hemophilia genotype, race, and immune regulatory polymorphisms (tumor necrosis factor- $\alpha$ , interleukin-10, and cytotoxic T-lymphocyte antigen-4). Inhibitor development rate is also dependent on the type of genetic mutation. In hemophilia A, inhibitors develop in 88% of patients with a large deletion, 25–41% with moderate deletions, 16–21% with minor deletions, 20% with intron 22 inversions, and 5% with missense mutations.<sup>9,102</sup> In hemophilia B, large deletions or minor deletions associated with a stop codon are associated with an inhibitor rate of 50–100%.<sup>103,104</sup> Immunogenicity differences between plasma-derived and recombinant factor products have been debated, but no conclusive evidence has shown a significant difference in inhibitor development between these products.<sup>105–108</sup>

Inhibitory antibodies are measured in Bethesda units. Given that FVIII is present at 1 unit/mL in normal plasma, a Bethesda unit represents the number of dilutions of patient plasma that reduces FVIII activity by 50%. Thus, higher titer inhibitors will require a higher number of dilutions to titrate their inhibitory effect down to 50%, resulting in a higher Bethesda unit titer. FVIII antibodies of <5 BU/mL are low titer or low responding inhibitor, whereas those >5 BU/mL represent a higher titer or high responding inhibitor. High responding inhibitors tend to be more persistent, and even though they may become undetectable after long periods of no treatment, they usually have a recurrent anamnestic response 3–5 days after a challenge with factor products.<sup>73</sup> Inhibitors complicate hemophilia treatment because of diminished responsiveness to exogenous

factor concentrates and reliance on less hemostatically effective bypassing agents. Progressive joint disease and significant mobility impairment are more prevalent in patients with inhibitors than those without.<sup>109</sup> Also, life-threatening anaphylaxis has been reported in up to 50% of patients with hemophilia B with FIX inhibitors upon exposure to exogenous factor concentrates.<sup>110</sup>

### Management of inhibitors in hemophilia

Management of patients with inhibitors is twofold: management/prevention of acute bleeding and attempting to eliminate the inhibitor. Bleeding in patients with low titer inhibitors can often be managed with FVIII concentrates in more frequent or higher doses if hemostatic levels of FVIII can be achieved. However, patients with high titer (>5 BU) inhibitors often require alternative therapies. Bypassing agents, like the activated prothrombin complex concentrate, FEIBA, and activated recombinant factor VII (rFVIIa), have been used and are equally efficacious.<sup>111</sup>

Activated prothrombin complex concentrates (aPCCs), a combination of the vitamin-K-dependent factors II, VII, IX, X and protein C and S at varying concentrations, have been used in the management of patients with both FVIII and FIX inhibitor for many years. The recommended dose of FEIBA, one type of aPCC, is 50–75 IU/kg every 12–24 hours, but repeated doses of more than 3–5 days should be monitored carefully due to thromboembolic risks.<sup>112</sup> FEIBA has been shown to be effective at treating acute bleeding, controlling perioperative bleeding, and for prophylaxis in hemophilia patients with inhibitors.<sup>112–114</sup>

The development of rFVIIa (Novo Seven, Novo Nordisk, Denmark; Sevenfact, Hema Biologics, USA) was an important landmark in the management of hemophilia patients with inhibitors. It has been demonstrated that rFVIIa at concentrations much higher than normal circulating levels mediate a tissue-factor-independent conversion of factor X to its activated form on a phospholipid surface.<sup>115</sup> In the absence of FVIII and FIX, rFVIIa induces hemostasis likely by enhancing thrombin generation on activated platelet surfaces. This leads to thrombin activation, which recruits more platelets and enhances platelet adhesion. This thrombin also recruits factor XIII leading to a tighter fibrin clot structure.<sup>116</sup>

The first successful use of rFVIIa in patients with hemophilia was during and following an open synovectomy.<sup>117,118</sup> Although the  $t_{1/2}$  is short (2–3 hours), rFVIIa has been used with clinical success in patients with inhibitors presenting with life/limb-threatening bleeds, as prophylaxis for joint bleeding, and as prophylaxis for elective surgical procedures.<sup>110,119–122</sup> Dosing for Novo Seven is typically 90 µg/kg with repeated dosing every 2–3 hours until hemostasis is achieved, followed by dosing at increasing intervals thereafter. Clearance rates may be higher in younger children. Despite its short  $t_{1/2}$ , daily prophylactic doses of 90 or 270 µg/kg have been shown to be effective in decreasing the frequency of joint bleeding.<sup>122,123</sup>

For Sevenfact, typical dosing for mild to moderate bleeding is 75 µg/kg with repeated dosing every three hours until hemostasis is achieved followed by increasing intervals thereafter. Another dosing strategy is an initial dose of 225 µg/kg with monitoring; if hemostasis is not achieved within nine hours of the first dose, administer additional 75 µg/kg doses every three hours until hemostasis is achieved. For severe bleeding, an initial dose of 225 µg/kg should be given. A 75 µg/kg dose can be given 6 hours later with subsequent dosing every 2 hours until hemostasis is achieved.<sup>124</sup> For the majority of patients with hemophilia B who have had severe allergic reactions to FIX products, rFVIIa is regarded as their treatment of choice.<sup>125</sup>

Another option for the management of patients with hemophilia and inhibitors is nonhuman (porcine) FVIII protein. Porcine FVIII has limited cross-reactivity with antihuman FVIII antibodies. A recombinant porcine FVIII concentrate (Obizur, Takeda, USA) was recently approved by the FDA to treat bleeding episodes in adults with acquired hemophilia A, but further studies are needed to evaluate its safety and efficacy in the treatment of patients with congenital hemophilia A and inhibitors.<sup>126,127</sup>

Staphylococcal protein A immunoabsorption columns are available outside of the United States to rapidly reduce levels of very high titer inhibitors to FVIII and FIX. Due to the availability of bypassing agents, these columns are rarely used but may have some benefit especially in reducing the incidence of nephrotic syndrome in patients with hemophilia B if used prior to immune tolerance therapy.<sup>128</sup>

Most recently, nonfactor replacement therapies have become available. The FDA has approved an FVIII mimetic bispecific antibody (emicizumab, Genentech, USA) that binds to FIXa and the FX zymogen in appropriate positions to promote tenase formation and FX activation.<sup>129</sup> Emicizumab prophylaxis has been shown to significantly reduce the annualized bleeding rate in patients with hemophilia A and an inhibitor compared to on-demand bypassing agent use.<sup>130</sup> Thrombotic microangiopathy and thrombosis were reported as complications when high-dose aPCC were utilized to treat an acute bleeding event while on emicizumab. Due to this complication, rFVIIa is considered first line for treatment of acute bleeding events in patients with hemophilia A and an inhibitor on emicizumab with low-dose (25–50 IU/kg) aPCCs reserved rarely for patients not responding to rFVIIa.<sup>130,131</sup> There are other nonfactor replacement therapies (antitissue factor pathway inhibitor [TFPI], RNA interference therapeutic that targets antithrombin, and other bispecific antibodies) currently in development.

### Immune tolerance therapy

The ultimate goal in the management of inhibitors is complete eradication, which is achieved between 60% and 80% of the time with immune tolerance induction (ITI).<sup>128</sup> ITI is costly and requires good venous access as well as patient adherence to be successful. Deferring ITI until the inhibitor titer is <10 BU can be considered, but if the titer does not decline after a 1–2-year period and/or the inhibitor is associated with a severe or life-threatening bleed then ITI should be considered earlier.<sup>132</sup> More recent guidance suggests that starting ITI immediately, regardless of the titer, is preferred.<sup>133</sup> Inhibitors are divided based on “good risk” and “poor risk” features. “Good risk” features include an inhibitor titer of <10 BU, a maximum inhibitor titer of <199 BU, and ITI started less than five years since inhibitor diagnosis. “Poor risk” inhibitors do not meet all three criteria.

Currently, there are no strong recommendations for the optimal dose and frequency for ITI, and protocols vary by institution.<sup>132</sup> The International Immune Tolerance Study showed that high-dose (200 IU/kg/day) and low-dose (50 IU/kg/3 times/week) FVIII infusions were equally efficacious in achieving tolerance in “good risk” patients with high titer inhibitors, but the high-dose group achieved a negative titer faster and had less bleeding.<sup>134</sup>

With the development of emicizumab addressing the limitations of bypassing agents, it was unclear if ITI would continue to have as important of a role in inhibitor management. The “Atlanta Protocol” investigated this concern and showed that patients can safely receive ITI (up to 100 IU/kg thrice weekly) with emicizumab and that it could effectively tolerize these individuals.<sup>135</sup> The ability to effectively tolerize patients while utilizing emicizumab would again allow for the use of FVIII products in the event of an acute bleeding

complication or in a surgical setting. There are ongoing studies investigating the safety and efficacy of this approach.

The success rate for ITI in patients with hemophilia B with inhibitors is much less than that for hemophilia A and may involve the use of a cumbersome immunosuppressive regimen that includes desensitization, rituximab, and mycophenolate.<sup>136</sup> The success rate is reduced by the development of nephrotic syndrome of unclear etiology about 8–9 months after the start of ITI. While nephrotic syndrome generally resolves after FIX product avoidance, its occurrence complicates any further ITI.<sup>137</sup>

### Gene therapy

Gene therapy seeks to replace the missing factor or protein by transfecting target tissue with a nucleic acid expression construct followed by endogenous factor synthesis. Hemophilia is a monogenic hereditary disorder with well-understood genetics, making it a potential target for gene therapy.<sup>138</sup> Gene therapy has been in clinical trials in hemophilia B for many years with limited success using adeno-associated viral vector serotype 2 (AAV2), but the response was not sustainable.<sup>139</sup>

More recent gene therapy clinical trials for severe hemophilia B used adeno-associated serotype 8 viral vector (AAV8), which encoded for a codon-optimized FIX. The AAV8 serotype was chosen since it transduces hepatocytes well, with minimal interactions with antigen presenting cells and cross-reactivity with preexisting anti-AAV2 antibodies. Six subjects received low-, intermediate-, or high-dose vector doses and all subjects expressed between 2% and 11% FIX activities many months after therapy. Some were able to stop prophylaxis therapy all together, while the others required less frequent FIX infusions.<sup>139,140</sup> Although sustained improvement in FIX levels were obtained, the levels achieved were not sufficient to prevent bleeding in the setting of a severe trauma or surgery. AAV5 has also been shown to be an effective vector in phase 1 and 2 studies for hemophilia B with a sustained FIX response in the mild to normal range.<sup>141</sup> Ongoing efforts are underway to further optimize gene therapy in hemophilia B.

Utilizing the AAV in patients with hemophilia A is more challenging due to the large size of the FVIII transgene; however, an AAV5 vector has produced a sustained FVIII activity in the mild hemophilia range with a clinically relevant (decreased annualized bleeding rate) response.<sup>142</sup> Also, a codon-optimized FVIII has been created using a BDD FVIII; injection into hemophilia A mice using a spleen focus forming virus promoter within a self-inactivating HIV-based lentiviral vector showed a sustained 29–44-fold increase in expression of FVIII levels.<sup>143</sup> The gene size used would be small enough to fit into an AAV vector, suggesting a potential gene delivery system. Ongoing efforts are also underway to optimize gene therapy in hemophilia A.

### von Willebrand disease

von Willebrand disease is the most common autosomally inherited bleeding disorder. The estimated prevalence of vWD is about 1%, but clinically significant vWD prevalence is about 1 in 1000.<sup>144,145</sup> vWD usually presents with mucocutaneous bleeding and postoperative hemorrhage, but the degree of bleeding is less severe than in hemophilia. No geographical or ethnic predilection exists, and females outnumber males almost 2:1 in most populations despite autosomal inheritance.<sup>146</sup> This likely represents a diagnosis bias due to the hemostatic challenge of menses. vWD occurs secondary to a quantitative or qualitative deficiency in vWF.

von Willebrand factor is a large multimeric glycoprotein that has many roles in both primary and secondary hemostasis. vWF mediates platelet adhesion to exposed endothelium by binding to GPIB/IX/V platelet surface glycoprotein, assists in platelet aggregation by binding to the GPIIb/IIIA platelet surface glycoprotein, and it binds to circulating FVIII to prevent protein C mediated proteolytic degradation.<sup>1</sup>

The gene for vWF is located on the short arm of chromosome 12, spanning 178 kb with 52 exons that code for a 2813 amino-acid-containing protein.<sup>1,147,148</sup> Megakaryocytes and vascular endothelial cells are responsible for synthesizing pro-vWF subunits that dimerize in the endoplasmic reticulum.<sup>149</sup> The pro-vWF dimers form multimers within the Golgi bodies, and the final product protein can be released directly into the plasma or stored either in the Weibel-Palade bodies or within platelet  $\alpha$ -granules. vWF in circulation ranges from 500 kDa up to 20 million Da (high molecular weight [HMW]), with the higher molecular weight vWF more physiologically active.<sup>1,149</sup> Under high shear stress, the larger multimers bound to platelets may stretch and expose Tyr<sup>1605</sup>–Met<sup>1606</sup> bond in the A2 domain of vWF, where ADAMTS13 (a disintegrin and metalloprotease with thrombospondin 1 motif, member 13) proteolyses vWF into its characteristic cleavage products.<sup>1,147,149</sup> vWF, irrespective of multimer size, has a circulating  $t_{1/2}$  of about 20 hours.<sup>149</sup>

vWF levels are influenced by many factors. As an acute phase reactant, vWF rises with stress, infection, and exercise. Chronic elevations in vWF are seen in hyperthyroidism as well as diseases with chronic endothelial damage, like diabetes. Increased estrogen levels, as seen with hormonal contraception, hormone replacement, and pregnancy, have been reported to increase vWF levels as high as 300–400%. Age is another significant factor as vWF levels naturally increase 1–2% per year; whether this diminishes the effect on bleeding in those with mildly reduced VWF is not clear.<sup>150</sup> Race also affects vWF levels, with African Americans having higher average levels compared to Caucasian populations.<sup>151,152</sup> Hypothyroidism has been shown to decrease circulating levels of vWF. ABO blood groups also influence vWF levels, with lowest levels found in blood type O and highest in blood type A. Certain variants, like the exon 28 D1472H polymorphism, have artificially low VWF:RCO without affecting GpIb $\alpha$  binding or leading to a bleeding phenotype.<sup>150,153–155</sup>

### Clinical manifestation

Bleeding symptoms are generally mild in type 1 disease and increase in severity with type 2 and 3 diseases. Mucocutaneous bleeding, including easy bruising, epistaxis, and menorrhagia, is the most common manifestation. Postoperative bleeding, especially following oral procedures, is frequently seen. Hemarthroses are uncommon in type 1, but can be seen in type 3 and 2N due to the associated reductions in FVIII activity.<sup>148</sup>

### Classification and diagnosis

von Willebrand disease has been classified into three main categories: type 1 (quantitative deficiency), type 2 (qualitative defects, including subtypes A, B, M, and N), and type 3 (complete vWF deficiency).<sup>149</sup> Laboratory evaluation for suspected vWD should initially include nonspecific hemostasis tests, including a complete blood count (CBC), PT, aPTT, thrombin time (TT), and fibrinogen levels to evaluate for other potential bleeding diathesis.<sup>1</sup> Platelet dysfunction has similar presentations and should also be considered. Specific vWF quantification and function testing includes circulating levels of VWF:Ag, measurement of vWF function through either ristocetin-based platelet

aggregation (VWF:RCO) or through von Willebrand collagen binding assays, ratio analysis of VWF:RCO/VWF:Ag, vWF multimer analysis, and FVIII levels (Figure 37.2).<sup>148,156,157</sup> Newer platelet-binding activities of vWF assays (VWF:GP1bM and VWF:GP1bR) have been developed which are ristocetin-independent and likely more reflective of the bleeding tendency. Recently, an international group published guidelines suggested the use of these newer vWF platelet-binding assays over VWF:RCO for the diagnosis of VWD.<sup>158</sup>

### Type 1 vWD

Type 1 vWD accounts for 75–80% of all vWD cases<sup>1,147,150</sup> and is inherited in an autosomal dominant fashion with incomplete penetrance.<sup>148</sup> It is characterized by equivalent mild to moderate reductions in both VWF:Ag and VWF:RCO, with a VWF:RCO/VWF:Ag ratio >0.6, reduced platelet-binding activity of vWF (VWF:GP1bM, VWF:GP1bR), normal or prolonged PFA-100 closure, normal vWF multimeric distribution analysis, and normal PT and CBC.<sup>1,148,149,158</sup> aPTT may be prolonged due to decreased FVIII activity when VWF:Ag is <0.35 IU/dL, but a normal aPTT does not rule out vWD and in fact the vast majority of cases have a normal aPTT.<sup>1</sup> Consensus guidelines released in 2021 recommend using a VWF:Ag or platelet-dependent VWF of <0.3 IU/mL regardless of bleeding phenotype or <0.5 IU/mL in patients with abnormal bleeding, for the diagnosis of type 1 vWD.<sup>158</sup>

Vicenza-type vWD is a type 1 vWD subtype (commonly referred to as type 1C) and is inherited in an autosomal dominant manner. It is characterized by low-plasma vWF levels, normal platelet vWF content, and supranormal multimers.<sup>159</sup> Although not usually present in the plasma, the larger multimers are observed after DDAVP administration. It is now recommended to utilize a desmopressin trial with one- and four-hour postinfusion testing to confirm increased vWF clearance for the diagnosis of type 1C.<sup>158</sup> Despite larger than normal multimers, the functional vWF activity and hemostatic function are decreased. It is unclear if the abnormal function is due to a quantitative or qualitative defect.<sup>159</sup>

### Type 2 vWD

Type 2 vWD accounts for 20–25% of all vWD cases and is associated with a qualitative defect in vWF. It is clinically similar to type 1vWD, and all subtypes were historically characterized by a VWF:RCO/VWF:Ag ratio <0.6, but new consensus guidelines suggest using a platelet-dependent vWF activity/VWF:Ag ratio of <0.7.<sup>1,147,158</sup> Either vWF multimer analysis or the ratio of vWF collagen binding (VWF:CB)/VWF:Ag should be utilized to confirm the diagnosis of certain subtypes.<sup>158</sup>

### Type 2A vWD

Type 2A vWD is mainly inherited in an autosomal dominant pattern and is associated with loss of the HMW and intermediate-weight vWF multimers.<sup>147–149</sup> The loss of multimers is either due to a defect in their synthesis or increased sensitivity to cleavage by ADAMTS13.<sup>1,149,150</sup> The bleeding diathesis described is usually mild to moderate, but can have significant gastrointestinal bleeding secondary to arteriovenous malformations, and generally these patients have a poor response to desmopressin.<sup>149,160–162</sup>

### Type 2B vWD

Type 2B vWD is characterized by mutations in the A1 domain that result in enhanced affinity of vWF for platelet receptor GpIb.<sup>149,163</sup> Many individuals will have mild to moderate thrombocytopenia due to the increased platelet aggregation that can be exacerbated by stress and desmopressin, and these individuals will have significantly increased RIPA at low concentrations of ristocetin.<sup>158,164,165</sup> Although vWF is synthesized normally, type 2B vWD shows decreased large molecular weight multimers because large multimers bind platelets spontaneously upon secretion and are subsequently cleaved by ADAMTS13.<sup>165,166</sup> The cleaved multimers are small and do not mediate platelet adhesion or connective tissue binding well.<sup>166</sup> Consensus guidelines released in 2021 suggest that targeted genetic testing is preferred to low-dose RIPA testing to distinguish between type 2A and type 2B vWD.<sup>158</sup>

	Quantitative Disorders			Qualitative Disorders				
	Type 1	Type 1C	Type 3	Type 2A	Type 2B	Type 2M	Type 2N	Platelet Type vWD
VWF:Ag	↓	↓	↓↓↓	Normal or ↓	Normal or ↓	Normal or ↓	Normal or ↓	Normal or ↓
VWF:RCO			↓↓↓	↓↓	↓↓	↓↓	↓↓	↓↓
VWF:GP1bM			↓↓↓	↓↓	↓↓	↓↓	↓↓	↓↓
VWF:GP1bM/VWF:Ag	≥0.7	≥0.7	≥0.7	<0.7	<0.7	<0.7	<0.7	<0.7
VWF Multimer Pattern	Normal	Normal	Absent	Loss of HMW & IMW	Loss of HMW	Normal	Normal	Loss of HMW
FVIII Activity	Normal or ↓	Normal or ↓	↓↓↓	Normal or ↓	Normal or ↓	Normal or ↓	↓↓	Normal or ↓
Platelet Count	Normal	Normal	Normal	Normal	Normal or ↓	Normal	Normal	↓ or ↓↓
Low-Dose RIPA	Absent	Absent	Absent	Absent	↑↑↑	Absent	Absent	↑
Other Testing								↓ FVIII:VWF binding

Ag, antigen; FVIII, Factor 8; HMW, high molecular weight; IMW, intermediate molecular weight; Rco, Ristocetin Cofactor; RIPA, ristocetin induced platelet aggregation; VWF, von Willebrand factor

**Figure 37.2** Expected laboratory values in von Willebrand disease based on subtype.

### Type 2M vWD

Type 2M vWD is characterized by decreased vWF affinity for platelets without the loss of HMW.<sup>149</sup> These patients usually have a mutation within the A1 domain that impairs vWF binding to the GpIb platelet receptor.<sup>167,168</sup> Recent evidence suggests that individuals with 2M subtypes experience a milder bleeding phenotype than other subtypes.<sup>161</sup>

### Type 2N vWD

Type 2N or Normandy variant vWD is associated with mutations in the region of the vWF gene (exons 18–21 or 24–27) that is involved in binding vWF to FVIII.<sup>169,170</sup> These mutations result in decreased or absent vWF binding to FVIII, which in turn results in premature degradation of FVIII.<sup>169</sup> VWF:Ag and VWF:RCo can be normal or borderline low, but the FVIII activity is disproportionately decreased (5–40%). Bleeding symptoms mimic those of hemophilia A and the decreased FVIII levels make the diagnosis difficult. Diagnosis is based on either genetic analysis of vWF gene or by enzyme-linked immunosorbent assay-based VWF:FVIII binding assay.<sup>1,158</sup>

### Type 3 vWD

Type 3 vWD has an almost complete deficiency of vWF. It is inherited in an autosomal recessive manner, and heterozygous relatives have mild or no bleeding symptoms.<sup>171–173</sup> This type is characterized by vWF:Ag of <5%, VWF:RCo of <5%, FVIII levels of <10%, and a prolonged aPTT.<sup>149</sup> These individuals may have a more severe bleeding tendency than the other vWD subtypes, including mucocutaneous hemorrhage as well as hemarthroses and hematomas, similar to that of moderate hemophilia.<sup>148</sup> Due to the complete deficiency of vWF synthesis, these patients do not respond to desmopressin.

### Platelet-type vWD

Platelet-type vWD is similar to type 2B in both clinical presentation and laboratory features. It is characterized by platelet hyperresponsiveness to RIPA, but it is associated with a gain-of-function mutation in the platelet GPIBA gene, which codes for the vWF receptor GPIba. This causes an enhanced affinity for vWF that forms vWF–platelet complexes that are cleared from circulation quickly.<sup>174</sup> Macrothrombocytopenia may also be seen.<sup>175</sup>

### Treatment

Treatment for vWD is dependent on the subtype. FVIII and vWF levels of >50% are generally considered to be sufficient for maintaining hemostasis except in the setting of severe trauma or invasive procedures.<sup>90</sup>

Desmopressin can be used to increase vWF and FVIII plasma concentrations because it promotes their release from endothelial stores.<sup>90</sup> Typically, a 2–4-fold increase above basal levels is seen in 30–60 minutes, and hemostatic levels of vWF and FVIII are present for 6–8 hours after administration.<sup>148</sup> Over 80% of patients with type 1 vWD will respond to desmopressin with the likelihood of response dependent on their initial VWF:Ag level. The 2021 vWD guidelines do recommend performing a desmopressin trial to ensure effectiveness prior to utilizing as a treatment option. An adequate response is described as an increase of at least 2× the baseline vWF activity with a sustained FVIII and vWF of 50 U/dL for at least four hours after administration.<sup>176</sup> Baseline VWF:Ag of <10 U/dL often do not have substantial responses.<sup>89,160,177,178</sup> Patients with Vicenza variant show a promising initial response to DDAVP, but the response is not sustained, with VWF:Ag levels

near baseline levels at about 240 minutes following administration.<sup>159</sup> Except for some patients with 2A disease, type 2 vWD typically does not respond to desmopressin and in fact can exacerbate thrombocytopenia in type 2B. Individuals with type 3 disease are considered unresponsive.<sup>89,178</sup> Desmopressin is administered using 0.3 µg/kg IV/SC dosing, or fixed doses of 150 µg or 300 µg by intranasal spray for weights of <50 kg or >50 kg, respectively. A desmopressin trial is needed to confirm adequate response in all patients. Repeated doses over a short period can result in decreased responsiveness, or tachyphylaxis, and fluid restriction is required for 24 hours following doses to avoid hyponatremia.<sup>90</sup> Of note there may be some hemostatic benefit in those with an incomplete response based on laboratory values; this benefit may allow use in specific situations such as epistaxis at home.<sup>176</sup>

Plasma-derived VWF:FVIII concentrates should be used for major bleeding events, prophylaxis for major surgeries, in patients where desmopressin is not beneficial (type 3 and nonresponsive patients with type 1 and 2 patients), and where desmopressin is contraindicated (type 2B, patients with seizure disorders, and infants).<sup>1</sup> There are three commercially available licensed plasma-derived products for vWF replacement in the United States that are FDA approved: Humate-P (CSL Behring, USA), Alphanate SD/HT (Grifols, USA), and Wilate (Octapharma, USA). As described below, these products are not interchangeable as they have differing ratios of vWF to FVIII.

Humate-P contains 50–100 IU/mL of VWF:RCo and 20–40 IU/mL of FVIII activity; the median  $t_{1/2}$  of the VWF:RCo activity is about 11 hours.<sup>179</sup> Alphanate SD/HT contains a higher FVIII activity of 40–180 IU/mL and ≥16 IU/mL of VWF:RCo activity. Its median VWF:RCo  $t_{1/2}$  is about seven hours. Wilate contains 90 IU/mL of VWF:RCo activity as well as 90 IU/mL of FVIII activity, with a median  $t_{1/2}$  of VWF:RCo of 13–16 hours.<sup>180</sup> Dosing is usually based on the VWF:RCo units. Minor procedures may only need 1–5 days of coverage, whereas major surgeries can require as many as 7–14 days hemostasis. Additionally, minor procedures and spontaneous bleeds may only require a single dose of 20–40 U/kg of VWF:RCo to adequately control bleeding, while major spontaneous bleeding or surgical procedures require more aggressive management. For example, major spontaneous bleeding or surgical prophylaxis may require doses of 50–60 IU/kg of VWF:RCo, with the goal of obtaining peak VWF:RCo levels of 80–100 IU/dL for up to three days and a nadir of 50 IU/dL for at least 5–7 days.<sup>148,156</sup> A loading dose of 50 IU/kg of VWF:RCo can be given 30–60 minutes before surgery with doses of 20–60 IU/kg of VWF:RCo every 8–24 hours afterward to maintain hemostasis depending on the severity of the procedure. Monitoring VWF:RCo and FVIII trough and peak levels will aid in determining the subsequent dosing schedule.<sup>148</sup> Continuous infusions after initial boluses have been shown to successfully achieve surgical hemostasis and may be required in certain situations.<sup>181</sup> Adverse reactions associated with these concentrates are rare, but include allergic/anaphylactic symptoms like rash, edema, chest tightness, and pruritus.<sup>182</sup> Venous thromboembolism has been reported with the use of these concentrates, so caution is warranted with their use in patients with known risk factors for thrombosis. In patients requiring ongoing replacement therapy, monitoring FVIII levels has been recommended to ensure FVIII levels remain in a safe range.<sup>183–185</sup> Patients with vWD who may require replacement therapy should be vaccinated against hepatitis A and B due to the risk of acquiring blood borne pathogens although there has not been a documented case of viral transmission with these products in many years.<sup>186</sup>

The vWD International Prophylaxis (VIP) study investigated the use of prophylactic vWF concentrates in patients with clinically severe vWD. This was a nonrandomized study with dose escalation based on bleeding symptoms; any of the vWF concentrates were allowed to be used. An interim retrospective review evaluating one year prior to initiating prophylaxis to at least six months afterward showed that prophylaxis resulted in a significant decrease in the number of annual bleeding events (12–3) in the 55 evaluated patients.<sup>187</sup> While only 11 patients completed the study, final analysis confirmed that prophylaxis with vWF concentrates can be highly effective in preventing some hemarthrosis and mucosal bleeding events in patients with clinically severe vWD (reduced median annualized bleeding rate from 25 to 6.1).<sup>188</sup> The 2021 consensus vWD management guidelines now recommend the use of long-term prophylaxis in patients with a history of clinically severe and frequent bleeding events (including heavy menstrual bleeding) regardless of the subtype of VWD.<sup>176</sup>

Recently, a recombinant vWF (rVWF) product has been approved by the FDA (Vonvendi, Takeda, USA). Phase 1 and 3 data showed that the mean plasma VWF:RCo  $t_{1/2}$  is 19.6 hours, which is longer than the  $t_{1/2}$  of plasma-derived vWF (7–16 hours).<sup>189–191</sup> However, in contrast to plasma-derived vWF concentrates, rVWF has no significant FVIII activity. This concern was investigated during the clinical trials. Interestingly, even in the absence of the co-administration of an rFVIII product, rVWF use in type 3 vWD still led to normalization of FVIII activity in the plasma that was sustained for 48 hours after the infusion. This was believed to be due to the stabilizing effect of rVWF on endogenously secreted FVIII.<sup>190,191</sup>

While cryoprecipitate has historically been used in the management of vWD, this product is rarely used today due to the risk of blood borne pathogens, fluid overload, and increased thrombotic risk. Among the few acceptable uses of cryoprecipitate in this population are the presence of life- or limb-threatening situations in which no vWF concentrates are available.

Antifibrinolytic monotherapy may be used to treat mild mucocutaneous bleeding, whereas combining antifibrinolytics with desmopressin or vWF concentrates may be useful in controlling bleeding, especially in the oral cavity, gastrointestinal tract, and genitourinary tract. Menorrhagia is a common complaint among women with vWD and can be severe enough to cause symptomatic anemia. Treatment may require a combination of hormonal contraception, desmopressin when indicated, as well as antifibrinolytic therapy.<sup>156,176</sup>

## Other rare congenital clotting protein disorders

### Prothrombin deficiency

Prothrombin (FII), a vitamin-K-dependent glycoprotein synthesized in the liver, is the inactive zymogen of thrombin, an enzyme that cleaves fibrinogen into fibrin and activates factor XIII (FXIII) to covalently crosslink fibrin into a stable sheath.<sup>192,193</sup> Congenital prothrombin deficiency is one of the rarest congenital bleeding disorders, with an estimated incidence of 1 in 2,000,000 births, with higher incidences in consanguineous marriages.<sup>193</sup> The inheritance pattern is autosomal recessive with more than 40 mutations described within the 20.3 kb prothrombin gene on chromosome 11p11–q12.12. These mutations are only clinically relevant in those that inherit abnormal alleles from both parents.<sup>193,194</sup> Defects are classified as quantitative, or hypoprothrombinemia (homozygotes and compound heterozygotes); qualitative, or dysprothrombinemia (homozygotes or heterozygotes); or combined.<sup>193</sup>

### Clinical manifestations

Bleeding severity seems to correlate with prothrombin levels in hypoprothrombinemia, but a discrepancy in bleeding severity and genotype exists in dysprothrombinemia. Prothrombin deficiency may be classified as severe if levels are <5%, moderate if 5–10%, and mild if >10%, respectively.<sup>195</sup> Patients with prothrombin activity levels of 20–40% are usually asymptomatic, and the  $t_{1/2}$  of prothrombin is about three days.<sup>196,197</sup> Patients with homozygous and compound heterozygous prothrombin gene defects can have moderate to severe bleeding. Bleeding manifestations include easy bruising, mucosal bleeding (epistaxis, gingival bleeding, and menorrhagia), hemarthroses, subcutaneous/muscle hematomas, and postoperative bleeding. Gastrointestinal bleeding is less common.<sup>193,196</sup> In the neonatal period, intracranial hemorrhage, severe umbilical cord bleeding, hematomas, and increased bleeding after circumcision have all been reported.<sup>196,198</sup> Heterozygotes are usually asymptomatic, but mucocutaneous bleeding and bleeding following tonsillectomy and tooth extraction have been described.<sup>193,196</sup>

### Diagnosis

Typically, prothrombin deficiency will result in the prolongation of both the prothrombin time (PT) and activated partial thromboplastin time (aPTT), but results are reagent-dependent and abnormalities could be minimal. Prothrombin antigenic and activity assays are required for diagnosis after vitamin K deficiency and liver disease have been excluded.<sup>193,198</sup>

### Management

Fresh-frozen plasma (FFP) and PCCs are the treatments of choice, as no pure prothrombin concentrates are available.<sup>193</sup> These modalities can be used individually or simultaneously. FFP at 15–20 mL/kg, which should raise prothrombin levels by 25%, can be used to treat acute bleeds. For more severe bleeding episodes and surgical prophylaxis, consider a loading dose of 10–20 mL/kg of FFP, followed by 3 mL/kg every 12–24 hours to maintain adequate hemostasis.<sup>193,196</sup> Dosing for PCCs is based on the amount of each factor in the specific product; it is important to note that the amount of factors vary not only between products, but between manufacturer-produced lots. Typical dosing for PCCs is 20–50 units/kg.<sup>193</sup> Monotherapy with antifibrinolytic agents ( $\epsilon$ -aminocaproic acid and tranexamic acid) have been used for mild bleeding. Due to the long  $t_{1/2}$  of prothrombin, prolonged courses of treatment should be dosed to maintain prothrombin activity >25%.<sup>196</sup>

### Factor V deficiency

Factor V (FV) is a large glycoprotein synthesized in the liver that is activated by thrombin to serve as a cofactor for activated factor X (FXa) in the prothrombinase complex (FVa/FXa) to convert prothrombin to thrombin.<sup>199,200</sup> Congenital FV deficiency is a rare autosomal recessive bleeding disorder, with a reported incidence of about 1 in 1,000,000.<sup>198</sup> The FV gene is located on the long arm of chromosome 1p23, and the inherited defect can be either homozygous, which is more prevalent in consanguineous families, compound heterozygote, or heterozygote.<sup>199,200</sup> Mutations can result in either quantitative (type 1) or qualitative (type 2) defects.<sup>199</sup>

### Clinical manifestations

Bleeding severity correlates poorly with FV activity levels, but patients that are severely affected (<5–10% activity) tend to be more predisposed to severe bleeding episodes.<sup>195,199,200</sup> This is likely due to the fact that 20–25% of circulating FV is stored within platelet  $\alpha$ -granules, which is released upon platelet activation, and seemingly binds

immediately to surface receptors forming the prothrombinase complex.<sup>195</sup> The bleeding phenotype in FV deficiency is clinically heterogeneous.<sup>195,199,200</sup> Severe deficiency can present at birth or in early childhood with easy bruising and mucosal membrane bleeding, such as epistaxis and oral cavity bleeding, but ICH and umbilical stump bleeding have also been reported. Other bleeding manifestations may include muscle hematomas, hemarthroses, menorrhagia, and postsurgical bleeding. Heterozygote individuals and those with activity levels >10–15% are generally asymptomatic but may have mild bleeding phenotypes.<sup>195,198–200</sup>

### Diagnosis

FV deficiency is associated with both a prolonged PT and aPTT. Measurement of FV activity and/or antigen is required to confirm the diagnosis after liver disease has been ruled out.<sup>199</sup>

### Management

There is no commercially available purified FV concentrate. Antifibrinolytic agents alone may be sufficient to control minor bleeding episodes.<sup>199</sup> Severe bleeding episodes or perioperative management should be managed with FFP, dosed at 15–20 mL/kg, to maintain FV activities >20%. The  $t_{1/2}$  of FV is 13–36 hours, so daily FFP dosing is generally sufficient.<sup>199,200</sup>

### Combined factor V and factor VIII deficiency

Combined FV and FVIII (FV/FVIII) deficiency is a rare autosomal recessive disorder with a reported incidence of less than 1 in 1,000,000 in the general population, with the highest prevalence in those of middle eastern Jewish and non-Jewish Iranian decent (1:100,000). There is a higher prevalence in consanguineous marriages.<sup>201,202</sup> The molecular basis for this disorder is a mutation in either the LMAN1 (lectin mannose binding 1) gene on chromosome 18q21 in 70% of the cases or MCFD2 (multiple coagulation factor deficiency gene 2) gene on chromosome 2p21 in 30% of the cases. These genes encode proteins essential for the intracellular transport of both FV and FVIII.<sup>203–205</sup>

### Clinical manifestations

No strong association between activity levels and clinical bleeding has been established, but individuals with <20% activity are more likely to have spontaneous bleeding and those >40% are generally asymptomatic.<sup>195</sup> The combined deficiency of FV and FVIII does not lead to more significant bleeding than a similar isolated deficiency of FV or FVIII alone.<sup>206</sup> FV/FVIII deficiency is usually associated with a mild bleeding phenotype, and common bleeding symptoms include epistaxis, easy bruising, oral cavity bleeding, menorrhagia, and postsurgical. Bleeding after circumcision has also been frequently reported. Less frequently, ICH, umbilical cord bleeding, hemarthroses, and gastrointestinal bleeding have been reported.<sup>206,207</sup>

### Diagnosis

Both PT and aPTT will be prolonged in FV/FVIII deficiency and correct with mixing studies. A decrease in the plasma activity of both FV and FVIII to between 5% and 30% is usually seen. Liver disease and systemic consumptive processes (i.e., DIC) need to be excluded.<sup>206</sup> Diagnosis is confirmed by genetic testing for LMAN1 or MCFD2 mutations.

### Management

Bleeding episode treatment requires a source of both FV and FVIII. FV replacement is achieved with FFP; FVIII replacement is achieved with FFP, desmopressin, and plasma-derived or recombinant FVIII. FFP should not be utilized solely for FVIII replacement due to its

shorter  $t_{1/2}$  as compared to FV.<sup>206,207</sup> Recombinant FVIIa has been successfully used to control severe bleeds, but the safety of this off label use has not been established.<sup>208</sup> Monitoring of replacement treatment can be done with FV and FVIII assays.

### Factor VII deficiency

Factor VII (FVII) is a vitamin-K-dependent serine protease that, when activated, binds to exposed tissue factor (TF) to form the tissue factor complex, which activates FIX and FX and initiates the formation of a stable fibrin clot.<sup>209</sup> Congenital FVII deficiency is an autosomal recessive condition and is considered the most common rare congenital bleeding disorder, with an incidence of 1 in 500,000.<sup>197,210</sup> To date, over 130 mutations have been described in the FVII gene, located on chromosome 13q34.<sup>209,210</sup>

### Clinical manifestations

Bleeding symptoms do not correlate well with FVII activity levels, and there is a poor association between laboratory phenotype and clinical severity.<sup>195,197,210</sup> Heterozygotes are typically asymptomatic, while compound heterozygotes and homozygotes have bleeding symptoms ranging from asymptomatic to severe hemorrhagic diathesis.<sup>209,210</sup> Individuals with FVII activities >25% usually remain asymptomatic, while FVII activity of <5–10% may be associated with more severe spontaneous bleeding.<sup>195,210</sup> Commonly reported bleeding symptoms include epistaxis, easy bruising, oral cavity bleeding, and postoperative bleeding.<sup>209</sup> Menorrhagia is also a frequent complication and can be quite severe.<sup>210</sup> Muscle hematomas and hemarthrosis have less frequently been reported, and CNS and GI bleeding, while rarely encountered, can be severe.<sup>209</sup>

### Diagnosis

An isolated prolongation of the PT that corrects with a mixing study is characteristic of FVII deficiency. A FVII activity assay is required to confirm the diagnosis after vitamin K deficiency and liver disease have been excluded.<sup>209</sup>

### Management

Many interventions are available to manage FVII deficiency, and therapies should be individualized based on clinical situations due to the short *in vivo*  $t_{1/2}$  (6–8 hours) of FVII. The recombinant FVIIa product Novo Seven (Novo Nordisk, Denmark) is the treatment of choice. Sevenfact (Hema Biologics, USA) currently does not have an indication for the management of bleeding in congenital FVII deficiency. Novo Seven has a short *in vivo*  $t_{1/2}$  and increased clearance in children which requires a frequent dosing schedule of 15–30 µg/kg every 4–6 hours in certain clinical situations. It is notable that much lower dosing and decreased frequency of infusions are needed for FVII deficiency as compared to patients with hemophilia and inhibitors.<sup>209–211</sup> Single intermediate Novo Seven doses (60 µg/kg) have been shown to be effective in many mild to moderate bleeding episodes.<sup>212</sup> Although it has been associated with an increased thrombosis risk, recent studies have shown that the use of a minimally effective dose of Novo Seven is both safe and efficacious at obtaining and maintaining hemostasis in bleeding episodes and as surgical prophylaxis.<sup>211,212</sup>

Plasma-derived FVII (pdFVII) concentrates are similar to PCCs except for the higher content of FVII. Although effective in obtaining FVII activity sufficient for adequate hemostasis, the concentrations of the other vitamin-K-dependent factors are higher than FVII, conferring an increased thrombosis risk.<sup>209</sup> FFP, 10–15 mL/kg, is an inexpensive and readily available treatment modality and is often the only treatment available in many developing countries.

Challenges with using FFP, including increased risk of blood-borne pathogens, limited effectiveness, fluid overload, and the need for repeated administrations every 6–8 hours, have limited its use in clinical practice. aPCCs/PCCs can also be effective, but their use is limited in practice due to concerns similar to the use of FPP.<sup>209,210</sup>

Prophylaxis in FVII deficiency is generally limited to certain clinical situations. Menorrhagia and associated iron deficiency are common complications in women with FVII deficiency. If menorrhagia is not well controlled with antifibrinolytics and hormonal contraception, rFVIIa prophylaxis with Novo Seven could be considered.<sup>209</sup> Prophylaxis should also be considered in patients with a severe bleeding phenotype and recurrent bleeding episodes.<sup>210</sup>

Monitoring replacement therapy is accomplished by following FVII activity levels. FVII activity of 50% is associated with a normal PT.<sup>209</sup>

### **Factor X deficiency**

Factor X (FX) is a liver synthesized vitamin-K-dependent plasma glycoprotein that is essential for thrombin formation as it binds with FVa to form the prothrombinase complex.<sup>213,214</sup> Congenital FX deficiency is a rare autosomal recessive disorder with a reported incidence of 1 in 1,000,000; 1 in 500 are carriers. There is a higher prevalence in consanguineous marriages.<sup>201,214</sup> The FX gene is located on chromosome 13q34, and 105 distinct mutations have been described in individuals with FX deficiency resulting in either a quantitative (type 1) or qualitative (type 2) defect.<sup>213</sup>

### **Clinical manifestations**

FX deficiency has been classified into three groups: severe (<1% activity), moderate (1–5% activity), and mild (6–10% activity).<sup>214</sup> In patients with FX deficiency, bleeding symptoms correlate with activity levels. Patients with severe deficiencies tend to have more significant bleeding histories, while activity levels of <10% have been associated with spontaneous bleeding. Individuals with activities of 10–40% only have minor spontaneous or triggered bleeding if they have any symptoms at all, and those with activity >40% are generally asymptomatic.<sup>195</sup> Compared to other rare congenital bleeding disorders, severe FX deficiency tends to be associated with more severe bleeding symptoms. Easy bruising, epistaxis, oral cavity bleeding, menorrhagia, GI bleeding, hematomas, and hemarthroses are commonly reported. Intracranial hemorrhage, bleeding after circumcision, an umbilical cord bleeding have been reported in the neonatal period.<sup>213,214</sup>

### **Diagnosis**

The PT and aPTT are typically both prolonged in FX deficiency since it serves as the first enzyme in the common pathway. They both correct on mixing studies, and vitamin K-deficiency and liver disease need to be excluded. Both immunologic and functional assays are required to classify FX deficiency. Reductions in both FX activity and antigen suggest a type 1, or quantitative, defect, whereas a reduction in activity with normal antigen concentrations suggests a type 2, or qualitative, defect.<sup>213</sup>

### **Management**

A FX activity of 10–40% is generally accepted as hemostatic and should be the goal for replacement therapy, keeping in mind the relatively long FX  $t_{1/2}$  of 20–40 hours.<sup>214</sup> There is a plasma-derived FX (pdFX) concentrate available in Europe and the United States (Coagadex, Bioproduts Laboratory, United Kingdom). The safety and efficacy of pdFX has been established for on-demand, perioperative, and prophylactic therapies.<sup>215–221</sup> Dosing for on-demand

management is 30 IU/kg for patients aged <12 years and 25 IU/kg for patients ≥12 years of age with repeated doses every 24 hours until hemostasis has been achieved. For perioperative management, the initial dose is calculated based on the patient's baseline FX level and the desired level (usual goal is 70–90 IU/dL) with subsequent doses as needed to maintain an activity >50 IU/dL until the risk for bleeding has subsided. While data does exist to direct dosing in younger cohorts, it is more limited.

FX replacement therapy can also be accomplished with FFP or aPCCs/PCCs. Single doses of FFP (15–20 mL/kg) or aPCCs/PCCs are usually sufficient to control most bleeding symptoms, but daily doses may be required if prolonged hemostasis is required. Due to thrombosis concerns with these factor replacement therapies, individuals should be monitored clinically for symptoms of thrombosis and monitoring with FX levels and D-dimers should be considered in those receiving prolonged treatments.<sup>213</sup> Minor bleeding symptoms may be controlled with just topical therapies and/or antifibrinolytics.<sup>214</sup>

Prophylaxis is generally limited to certain situations. Menorrhagia that is uncontrolled with hormonal therapy and antifibrinolytics may benefit from prophylaxis with FFP or aPCCs/PCCs. Prophylaxis should be considered in patients with severe deficiency and repeated bleeding episodes, especially CNS bleeds, hemarthrosis, and hematomas.<sup>222</sup> Prophylaxis for a child born to family already with one severely affected child may prevent complications, like ICH.<sup>213</sup> For prophylaxis using pdFX, the dosing is 40 IU/kg twice weekly for patients aged <12 years and 25 IU/kg twice weekly for patients ≥12 years of age with a goal trough FX level of ≥5 IU/dL with a max peak not to exceed 120 IU/dL.

### **Factor XI deficiency**

Factor XI (FXI) is a plasma glycoprotein synthesized in the liver that circulates as the zymogen of a serine protease (FXIa) bound in a complex with high-molecular-weight kininogen (HMWK). Once activated by FXIIa, FXIa activates FIX, which maintains thrombin production at vascular injury sites.<sup>223</sup> Congenital FXI deficiency is generally inherited as an autosomal recessive trait, but the structure of circulating FIX could result in a dominant negative effect, leading to a dominant inheritance pattern. More than 220 mutations have been described in the FXI gene, located on the long arm of chromosome 4 (4q35.2).<sup>224</sup> The estimated prevalence is about 1 in 1,000,000 in most populations but is substantially higher at 1 in 450 among the Ashkenazi Jewish population.<sup>224</sup>

### **Clinical manifestations**

FXI activity levels do not correlate well with bleeding tendencies.<sup>195,224</sup> Severe FXI deficiency is defined as activity levels <20%, while activities between 20% and the lower limit of the normal activity (65–80%) are considered mildly deficient.<sup>224</sup> Overall, FXI deficiency is associated with a mild bleeding diathesis, but bleeding may occur at the time of the injury or hours later. Spontaneous bleeding is rare in even with severe FXI deficiency.<sup>195,224,225</sup> Epistaxis and menorrhagia may occur. Postoperative and post-trauma bleeding is common, especially in areas with high fibrinolytic activity such as the oral cavity, nose, and genitourinary tract.<sup>224,225</sup> Bleeding is less common at other trauma sites including circumcisions, cutaneous lacerations, orthopedic surgery, and appendectomy.<sup>226</sup> Mild deficiency is generally associated with an asymptomatic state, but a low risk of postoperative bleeding still exists.<sup>195</sup>

Inhibitor development has been described in congenital FXI deficiency following exposure to FFP or FXI concentrates. Although

these individuals still rarely have spontaneous bleeds, they may suffer prolonged bleeding during surgery even with appropriate therapy. FXI inhibitors should be suspected in individuals receiving appropriate therapy that continue to bleed and continue to have prolonged aPTTs.<sup>224</sup> Interestingly, FXI deficiency likely conveys a decreased ischemic stroke risk as well, as recent evidence showed FXI activity >95% was a potential risk factor for ischemic strokes but does not protect against myocardial infarctions, as postulated in Hemophilia A and B.<sup>224,225</sup>

### Diagnosis

Factor XI deficiency is characterized by an isolated prolonged aPTT. The aPTT will be greater than two standard deviations above normal values in homozygotes and may be normal or only slightly prolonged in heterozygotes; however, this depends upon the sensitivity of the PTT reagent utilized. After liver disease is excluded, confirmation of FXI deficiency is achieved by demonstration of reduced FXI activity. Since FXI deficiency can remain asymptomatic until an injury occurs, patients of Ashkenazi Jewish descent undergoing surgery or invasive procedures should consider screening with an aPTT; FXI activity should be measured if the screening test is abnormal.<sup>226</sup>

### Management

Perioperative replacement therapy should be utilized for higher risk surgical procedures in areas of high fibrinolytic activity. FXI replacement is achieved with the use of FFP or FXI concentrates. FFP is typically dosed at 15–20 mL/kg, but again caution must be used as the potential for fluid overload, transmission of infectious agents, and allergic reactions are all present.<sup>224,226</sup> Plasma-derived FXI concentrates have been available since the 1980s.<sup>226,227</sup> Each FXI concentrate also contains antithrombin so caution must be applied with use in the elderly, those with cardiovascular disease, and those with other thrombotic risk factors due to the 10% risk of arterial and venous thrombosis after FXI concentrate administration.<sup>224,226,227</sup> The goal of FXI replacement therapy for severe FXI deficiency is to attain approximately 40–45% activity for seven days with major surgery and approximately 30% for five days for minor procedures.<sup>224,226</sup> Since the  $t_{1/2}$  of FXI is about 45 hours, prolonged courses of therapy can be managed with bolus dosing on alternating days.<sup>227</sup>

In individuals with FXI inhibitors, rFVIIa (Novo Seven, Novo Nordisk, Denmark) has been used successfully off label in small single and repeated doses (15–30 µg/kg) with concurrent antifibrinolytic therapy to manage major surgeries, including open heart for dissecting aortic repair. Caution must be again applied as both of these agents do have an associated thrombotic risk. Fibrin sealants may be an appropriate alternative to antifibrinolytic therapy in some situations.<sup>224</sup>

Minor procedures (i.e., tooth extraction, colonoscopy with biopsy, and skin biopsy) do not usually require factor replacement therapy. These situations can typically be managed with antifibrinolytic therapy. Fibrin sealants may also be used in some situations.<sup>224,226,227</sup>

### Factor XIII deficiency

Factor XIII (FXIII) is a transglutaminase heterotetramer consisting of two catalytic subunits (A) and two carrier subunits (B). Thrombin, along with cofactors fibrinogen and calcium, cleaves the activation peptide from the A subunit of FXIII to initiate its activation. The major function of FXIIIa is the crosslinking of fibrin chains, resulting in a mechanically stronger thrombus. Congenital FXIII deficiency is a rare autosomal recessive bleeding

disorder with a reported incidence of about 1 in 2,000,000, with severe disease present in individuals who are homozygotes or compound heterozygotes. Congenital FXIII deficiency is more commonly seen in consanguineous marriages. FXIII-A deficiency is much more common than FXIII-B deficiency, for which there are only a few reported cases in the literature.<sup>228–230</sup> The gene coding for FXIII-A is on chromosome 6p24–25 and over 100 mutations have been described so far.<sup>229</sup> FXIII deficiency type I results from decreased synthesis of the protein and type II describes a qualitative defect of a normal concentration of defective FXIII-A.<sup>231</sup>

### Clinical manifestations

Factor XIII activity levels correlate well with bleeding symptoms experienced.<sup>195</sup> The deficiency is considered severe when activity levels are <5%, moderate if 5–10%, and mild when >10%.<sup>228</sup> The first and most clinically characteristic bleeding symptom experienced is bleeding from the umbilical cord days after birth.<sup>228–230</sup> Easy bruising/subcutaneous hematomas, intramuscular bleeds, hemarthroses, delayed bleeding after surgery or trauma, and ICH (the most common cause of disability or death) have all been reported. FXIII deficiency also has associated nonbleeding symptoms including recurrent spontaneous abortions and impaired wound healing/scar formation.<sup>228,229</sup> Individuals with mild and moderate deficiency may only have mucocutaneous bleeding or might be completely asymptomatic.<sup>195</sup>

### Diagnosis

Standard coagulation screening labs of PT, aPTT, thrombin time (TT), and fibrinogen are all normal in FXIII deficiency. The following algorithm has been recommended for the diagnosis of FXIII deficiency:<sup>231</sup>

- 1 First-line screening to detect all forms of FXIII deficiency should be obtained with a quantitative function FXIII activity assay, either by the measurement of ammonia released during the transglutaminase reaction or by the measurement of labeled amine incorporated into a protein substrate.
- 2 A. If the FXIII quantitative screen is abnormal, then
  - measure FXIII-A<sub>2</sub>B<sub>2</sub> antigen—if decreased, measure FXIII-A and FXIII-B antigens;
  - measure FXIII activity and FXIII-A antigen in the platelet lysate.
- B. If FXIII quantitative screen is normal, then
  - perform mixing study to evaluate for neutralizing antibodies;
  - if high suspicion of FXIII deficiency, consider type II defect.
- 3 Perform molecular genetic studies and use SDS-PAGE to evaluate fibrin crosslinking.

The urea clot solubility test has been traditionally used to screen for FXIII deficiency, but it can only detect individuals with severe deficiency so this screening test is no longer routinely recommended.<sup>231</sup>

### Management

Due to the clinical severity of the bleeding in FXIII deficiency, once factor activity of <1% is confirmed, prophylactic factor replacement therapy is the standard of care.<sup>231</sup> Prophylactic therapy should also be considered in individuals with factor activity <4–5% with severe bleeding phenotypes.<sup>232</sup> Treatment decisions should take into account that activity levels of 3–5% are usually sufficient to prevent spontaneous bleeding and the  $t_{1/2}$  of plasma FXIII is 11–14 days, meaning a monthly dosing schedule should be sufficient.<sup>228</sup>

FFP and cryoprecipitate are easily available sources of FXIII, providing 1 and 3 units/mL of FXIII, respectively, that can be used in the management of FXIII deficiency. FFP is dosed at 15–20 mL/kg and cryoprecipitate is 1 unit per 10 kg of body weight every 20–30 days. Plasma-derived pasteurized FXIII (pdFXIII) concentrate (Corifact, CSL Behring, USA) is preferred over FFP and cryoprecipitate because of the higher concentration of FXIII and lower risk of blood born virus transmission.<sup>228</sup> Prophylactic dosing of pdFXIII is recommended at 10–35 units/kg every 4–6 weeks.<sup>228,229,231</sup> For major surgery, FXIII activity levels should be maintained at >5% until wound healing is complete. Prophylactic FXIII therapy should be ideally started before 5–6 weeks of gestation in pregnancy to prevent spontaneous abortions and miscarriages.<sup>228</sup>

A recombinant FXIII (rFXIII) product (Tretten, Novo Nordisk, Denmark) has been approved for prophylactic therapy in the United States. It has been shown to be both safe and effective at preventing bleeding episodes in children and adults with congenital FXIII-A deficiency. Although about 10% of patients developed non-neutralizing antibodies to FXIII, none of these patients had bleeds that required treatment and these antibodies eventually became undetectable despite further rFXIII exposure.<sup>233</sup> It is important to note that this product is not effective for the rare case of FXIII subunit B deficiency.

### Fibrinogen disorders

Fibrinogen is the soluble glycoprotein precursor to insoluble fibrin. It is synthesized in the liver and consists of two sets of three polypeptide chains.<sup>234</sup> The three chains ( $\alpha$ ,  $\beta$ , and  $\gamma$ ) of fibrinogen are coded by different genes located in a 50-kb region on chromosome 4.<sup>235,236</sup> Normal circulating plasma fibrinogen concentration is about 150–350 mg/dL and it is constitutively secreted into circulation where it has a  $t_{1/2}$  of about four days. Fibrinogen levels of about 100 mg/dL are associated with adequate hemostasis.<sup>210,234</sup> Fibrinogen is converted into fibrin in three phases: (1) thrombin cleaves fibrinogen to produce fibrin monomers; (2) an organized polymeric structure forms from self-assembly of fibrin units; (3) FXIIIa covalently crosslinks fibrin.<sup>234</sup> Along with fibrin clot formation, fibrinogen also has a role in nonsubstrate thrombin binding, platelet aggregation, and fibrinolysis.<sup>235,236</sup>

Congenital fibrinogen deficiency is a rare bleeding disorder that affects the quantity of fibrinogen (afibrinogenemia or hypofibrinogenemia), the quality/activity (dysfibrinogenemia), or both (hypodysfibrinogenemia).<sup>210,234,236</sup> Clinical manifestations vary according to the type of deficiency and a strong association exists between clinical bleeding severity and fibrinogen levels.<sup>195,236</sup>

### Afibrinogenemia

Afibrinogenemia is a rare autosomal recessive disorder with a reported prevalence of 1 in 1,000,000 and a higher incidence in consanguineous marriages.<sup>234</sup> Over 80 distinct mutations, with a majority in the  $\alpha$  chain, have been identified in afibrinogenemia and individuals who are homozygotes or compound heterozygotes.<sup>195</sup> It is characterized by an undetectable plasma fibrinogen level.

Many individuals are diagnosed in the neonatal period as up to 85% of cases of afibrinogenemia present with umbilical stump bleeding. Some present later in life following a significant bleeding challenge. Mucocutaneous bleeding, muscle hematomas, oral cavity bleeding, prolonged bleeding after venous puncture, GI bleeding, and hemarthroses have all been reported in afibrinogenemia. ICH is a major cause of death in this population.<sup>234</sup> Afibrinogenemic women have gynecologic complications including menorrhagia, spontaneous recurrent abortions, as well as ante and postpartum

hemorrhage.<sup>210,234</sup> Issues with wound healing and wound dehiscence have also been reported.<sup>235</sup> Importantly, both arterial and venous thromboembolic events have been reported in afibrinogenemia both before and after replacement therapy, so close monitoring and high clinical suspicion are warranted.<sup>210,234,235</sup>

### Hypofibrinogenemia

Hypofibrinogenemia is inherited in an autosomal fashion and is associated with heterozygous fibrinogen gene mutations, thus is more prevalent than afibrinogenemia.<sup>234,236</sup> Hypofibrinogenemia is characterized by a fibrinogen level <100 mg/dL. Patients are often asymptomatic, but may have a mild bleeding phenotype. Many are only diagnosed after a major hemostatic challenge. Bleeding after trauma, hematomas, menorrhagia, and GI bleeding are the most frequently reported bleeding symptoms and wound healing can also be an issue.<sup>195,234,235</sup>

### Dysfibrinogenemia and hypodysfibrinogenemia

Dysfibrinogenemia and hypodysfibrinogenemia are rare autosomal dominant disorders caused by heterozygous missense mutations in one of the three fibrinogen genes, making them more prevalent than type 1 disorders.<sup>234</sup> Patients are frequently asymptomatic, but can present with a bleeding diathesis (usually bleeding after trauma/surgery or postpartum) or thrombosis.<sup>210,234,235</sup> Women can suffer from spontaneous abortions, stillbirths, and postpartum thrombosis.<sup>234,235</sup>

### Diagnosis

Any test that depends on fibrin as the end point (PT, aPTT, and TT) will be prolonged in hypofibrinogenemia or afibrinogenemia.<sup>234,235</sup> Erythrocyte sedimentation rates are determined mainly by fibrinogen and thus will be low. Absence of immunoreactive fibrinogen must be demonstrated to diagnose afibrinogenemia, while a proportional decrease in functional and immunoreactive fibrinogen is required to diagnose hypofibrinogenemia.<sup>234</sup>

For dysfibrinogenemia, screening should include a TT, reptilase time, as well as an immunologic and functional fibrinogen assay. Classically, TT will be prolonged while the immunologic fibrinogen assay will be normal and the functional fibrinogen assay will be low.<sup>234,235</sup> With normal functional fibrinogen present, a prolonged reptilase time is consistent with dysfibrinogenemia. Hypodysfibrinogenemia will show both a qualitative defect as well as a quantitative defect with fibrinogen levels of 50–120 mg/dL.<sup>235</sup> Mutation analysis should be obtained if possible as it can provide useful information for carrier testing and prenatal diagnosis.

### Management

Fibrinogen levels of 100–150 mg/dL are generally considered to be sufficient for hemostasis, so <100 mg/dL is typically used as the trigger for fibrinogen replacement.<sup>237</sup> FFP, cryoprecipitate, and fibrinogen concentrates are all available for fibrinogen replacement. Since the  $t_{1/2}$  of fibrinogen is so long, every other day replacement is usually sufficient for prolonged courses of treatment.

Plasma-derived fibrinogen concentrates have been approved for the management of acute bleeding events in adults and children with hypofibrinogenemia and afibrinogenemia only (RiaSTAP, CSL Behring, USA; Fibryga, Octapharma, USA).<sup>238,239</sup> Therapeutic doses can be administered in relatively small volumes with a minimal risk of bloodborne viral transmission. The risk of thrombosis with fibrinogen concentrates is low, and these agents have been shown to have a better clinical efficacy profile than FFP.<sup>237</sup> Initial dosing should be 70 mg/kg with a goal fibrinogen level of 100 mg/dL.<sup>237,240</sup>

Life-threatening bleeds and surgical procedures may require higher initial doses as well as more frequent dosing to maintain higher fibrinogen levels.<sup>240</sup>

FFP dosed at ~15 mL/kg can be used for fibrinogen replacement, noting an average fibrinogen concentration of 2.5 g/L although with significant variations.<sup>237</sup> Cryoprecipitate contains about 15 g/L of fibrinogen, and one unit of cryoprecipitate per 5–10 kg of body weight should increase the fibrinogen concentration by 50–100 mg/dL. Again, these products do share similar limitations due to risks of fluid overload, exposure to blood born viruses, and thrombosis.

Weekly prophylactic fibrinogen replacement may be appropriate in certain situations. Primary prophylaxis to prevent bleeding episodes should be considered in patients with afibrinogenemia. Secondary prophylaxis could be considered in individuals with recurrent severe bleeding, such as hemarthroses and hematomas, or after a life-threatening bleed (ICH). Prophylaxis plans should be individualized due to the associated potential thrombotic risk.<sup>236</sup>

Antifibrinolytic therapy may also be helpful in certain situations. Particularly, it may be useful following dental procedures or to treat mucosal bleeding. Fibrin glue is another option to aid in the treatment of superficial wounds.<sup>234</sup>

Women with congenital afibrinogenemia can conceive, but pregnancies usually end in spontaneous abortion by 5–8 weeks.<sup>234</sup>

It is recommended that fibrinogen levels should be maintained at >50 mg/dL during the first two trimesters, >100 mg/dL during the third trimester, and >150 mg/dL during labor.<sup>241</sup>

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## CHAPTER 38

# Coagulation factor concentrates and pharmacologic therapies for acquired bleeding disorders

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## Introduction

Acquired bleeding disorders are much more common than congenital bleeding disorders and may occur in association with specific clinical conditions or may arise spontaneously in otherwise healthy patients. Unlike inherited bleeding disorders, acquired bleeding disorders are often diagnosed in adults, where they may present at any age. Bleeding symptoms can vary from mild bruising and mucosal bleeding to prolonged postoperative bleeding and severe hemorrhage. Management of bleeding in patients with acquired coagulation disorders requires prompt recognition of the underlying disorder to guide therapy.

Under physiologic conditions, hemostasis is achieved through the constriction of damaged vessels, activation of primary hemostasis leading to the formation of a platelet plug, and activation of secondary hemostasis, resulting in a stabilized fibrin clot at the site of bleeding. Parallel to this, the fibrinolytic system is activated to control clot formation and propagation and dissolve the unnecessary (and potentially dangerous) clots after hemostasis is achieved. Specific and nonspecific hemostatic and prothrombotic agents exploit these physiologic pathways to tip the balance toward forming and maintaining clots, thereby reducing bleeding. In this chapter, the coagulation factor concentrates, both recombinant and human-derived, and the pharmacologic agents utilized to manage acquired bleeding disorders are reviewed.

## Prothrombin complex concentrate

### Nonactivated prothrombin complex concentrate

#### Overview

Prothrombin complex concentrates (PCCs) contain vitamin-K-dependent coagulation factors (VKDFs) and are available as activated and nonactivated concentrates. Nonactivated three-factor PCC (3F-PCC) contains variable concentrations of factors II, IX, and X, while four-factor PCC (4F-PCC) also contains a sufficient amount of factor VII (and proteins C and S) (Table 38.1).

### Manufacturing and storage

PCCs are plasma derivatives prepared from pooled human plasma purified from either DEAE cellulose adsorption (e.g., Profilnine SD) or ion exchange chromatography (e.g., Kcentra). Viral inactivation is performed with either vapor heat (e.g., Kcentra) or solvent detergent (e.g., Profilnine SD). Clotting factors remain inactivated through the addition of heparin or antithrombin, or adjusting the pH. PCCs are stored at room temperature as a lyophilized powder and can be reconstituted rapidly with a small diluent volume, which allows for rapid administration.

### Indications

Most PCCs are approved for prophylaxis and treatment of factor IX deficiency in patients with hemophilia B. However, with the advent of high purity plasma-derived factor IX concentrates and recombinant factor IX products, PCCs are no longer used for this indication.

The main approved indication for 4F-PCC is the urgent reversal of vitamin K antagonists (VKA), where they constitute the first line of treatment (Table 38.2).<sup>1,2,3</sup> PCCs contain concentrated VKDFs that are approximately 25 times higher than in normal plasma, which allows for a smaller infusion volume compared to FFP and decreased risk of transfusion-associated circulatory overload.<sup>4</sup> Advantages of PCC over plasma also include rapid administration, lack of required ABO blood typing, and lower risks of allergic reactions, transfusion-associated lung injury, and viral transmission. Additionally, vitamin K should be administered along with PCCs due to the relatively short half-lives of VKDFs in the PCCs.

In a large phase IIIb study comparing 4F-PCC and plasma for urgent VKA reversal in patients with major bleeding, 4F-PCC achieved rapid INR reduction in 62.2% of patients compared with 9.6% of patients receiving plasma (difference 52.6%; 95% confidence interval [CI], 39.4–65.9%). Additionally, 4F-PCC was non-inferior in achieving effective hemostasis in 72.4% of treated patients versus 65.4% of patients transfused with plasma (difference 7.1%; 95% CI, 5.8–19.9%).<sup>5</sup> However, for visible bleeding,

**Table 38.1** Prothrombin Complex Concentrates

Products	Factor Levels (IU/mL)			
	II	VII	IX	X
Available in the USA				
<b>Four-factor PCC</b>				
Kcentra (CSL Behring)	17–40	10–25	20–31	25–51
<b>Three-factor PCC</b>				
Profilnine SD (Grifols)	≤150	≤35	≤100	≤100
Bebulin VH (Baxter)	24–38	<5	24–38	24–38
Available outside the USA				
<b>Four-factor PCC</b>				
Beriplex (CSL Behring) <sup>a</sup>	20–48	10–25	20–31	22–60
Octaplex (Octapharma) <sup>b</sup>	14–38	9–24	25	18–30
Cofact (Sanquin) <sup>c</sup>	14–35	7–20	25	14–35
Prothromplex T (Baxter) <sup>d</sup>	30	25	30	30
PPSB-Th <sup>e</sup>	20	20	20	20
<b>Three-factor PCC</b>				
Prothromplex HT (Baxter) <sup>f</sup>	30	–	30	130

The values given are the number of units per 100 factor IX units (IU/mL) in each 20 mL vial.

<sup>a</sup> UK and EU.

<sup>b</sup> UK, Canada, and EU.

<sup>c</sup> EU.

<sup>d</sup> Austria.

<sup>e</sup> Japan.

<sup>f</sup> Australia.

Source: Goodnough and Shander (2011).<sup>4</sup> Adapted with permission from the American Society of Hematology.

**Table 38.2** Guidelines for Management of Elevated INR in Patients on Vitamin K Antagonists. Based on Recommendations from the 2012 ACCP 9th Edition Guidelines<sup>17</sup> and ASH 2018 Guidelines<sup>18</sup>

Clinical Setting	INR	Management
Absent bleeding	>4.5 but <10	Temporary cessation of VKA alone without the use of vitamin K
Absent bleeding	>10	Temporary cessation of VKA with or without the addition of oral vitamin K
Life-threatening bleeding	Elevated INR	4F-PCC and vitamin K (5–10 mg by slow intravenous infusion) in addition to cessation of VKA

4F-PCC was superior to plasma.<sup>6</sup> In another phase IIIb study comparing PCC with plasma for urgent surgery, 4F-PCC was superior to plasma for rapid INR correction, shorter wait time for surgery, and blood loss.<sup>7</sup>

4F-PCCs have not been compared head to head with 3F-PCCs for the treatment of VKAs, but 3F-PCCs are thought to be inferior due to nontherapeutic levels of factor VII and lack of proteins C and S. If 4F-PCC is unavailable, a 3F-PCC can be given, often with a small amount of plasma and intravenous vitamin K to provide factor VII.

These PCCs have also been used off-label for the reversal of factor Xa inhibitors, apixaban and rivaroxaban, although there is limited data to support their use since evaluations have been limited to single-arm observational studies. A recent systematic review of a case series including 340 patients who received PCC to treat direct FXa inhibitor-related major bleeding showed a pooled proportion of patients with effective hemostasis of 69% (95% CI, 61–76%) or 77% (95% CI, 63–92%), depending on the criteria used, and a thromboembolism rate of 4% (95% CI, 1–8%).<sup>8</sup> Given the low certainty of this evidence, PCC is generally considered second-line therapy if Andexanet alfa (see later) is not available for the reversal of factor Xa inhibitors.<sup>9,10</sup>

## Dosing

PCCs are standardized according to factor IX content, and dosing is often based on units of factor IX per kg of body weight. The concentrations of VKDF factors in PCCs vary by the manufacturers and lot numbers and are listed on the vial. In patients with INR prolongation due to VKA, dosing is either based on body weight and degree of INR prolongation (as per package insert) or fixed dosing based on real-life experience publications. If weight-based dosing is given, repeated dosing is not recommended because it does not increase efficacy and may inadvertently increase the risk of thromboembolic complications. To date, fixed versus weight-based dosing has not been compared in a randomized trial. Vitamin K should be given in addition to PCC for the treatment of VKA reversal.

## Adverse reactions

The most common serious adverse reactions are thromboembolic events (TEEs), although the overall risk is low. Most TEEs are due to delay in re-anticoagulation following a major bleed in patients with an underlying hypercoagulable state. In a meta-analysis of 27 studies, including 1032 patients, the incidence of TEE was 1.4% (95% CI, 0.8–2.1).<sup>11</sup> Recognized adverse effects also include allergic reactions. Additionally, there is a theoretical risk of heparin-induced thrombocytopenia in heparin-containing PCC products. However, since there are no reported HIT-related cases from PCC therapy, this risk is considered theoretical, especially in a bleeding patient.

## Activated prothrombin complex concentrate Overview

Activated PCCs (APCCs) are PCCs that contain some activated factors. The only available APCC in the United States is FEIBA (factor VIII inhibitor bypassing agent), which consists of mainly activated factor VII, likely activated FX (because FEIBA is given every 8–12 hours and FVIIa has a  $t_{1/2}$  of 2 hours), and nonactivated II and IX.

## Manufacturing and storage

FEIBA is purified from pooled human plasma by DEAE-Sephadex adsorption, and viral transmission is reduced through nanofiltration and vapor heat treatment. FEIBA is stored as a lyophilized powder in single-use vials containing 500, 1000, or 2500 units per vial.

## Indications

APCC is approved in patients with hemophilia A or B with inhibitors to control and prevent bleeding, as well as to manage perioperative bleeding.<sup>12</sup> It is not indicated in hemophilia A or B patients without inhibitors. It is often the first line of therapy for patients with acquired hemophilia A due to autoantibodies who present with bleeding. Acquired hemophilia is a rare condition with a bimodal presentation affecting young women during the postpartum period and the elderly (>70 years). Most bleeds are in soft or fatty tissues (abdominal wall, buttocks, breast, etc.) rather than joint bleeding.

Generally, nonactivated 4F-PCC is preferred over APCC for the urgent reversal of VKAs due to the theoretical prothrombotic risks associated with the presence of activated FVII/Xa in APCC. APCC has also been used off-label for direct Xa anticoagulant reversal, although no high-quality randomized studies support its use. If idarucizumab is not available, APCC may be considered for reversing the dabigatran effect in life-threatening bleeding, although only a few small retrospective case series and observational studies support its use.<sup>13–16</sup>

## Dosing

For the treatment of bleeding in patients with acquired hemophilia, dosage and frequency depends on the type and extent of bleeding but is typically dosed as 50–100 IU/kg intravenously every 8–12 hours until clinical response, with a maximum of 100 IU/kg for a single dose and a maximum daily dose of 200 IU/kg. In the event that idarucizumab is unavailable, a single dose of APCC at 50 IU/kg may be given to manage bleeding due to dabigatran.

## Adverse reactions

Due to the presence of activated factor VII (and FXa), APCC increases the risk of both arterial and venous thromboembolic events and should be used with caution in patients with high thrombotic risk. Furthermore, APCC is contraindicated in the setting of disseminated intravascular coagulation or acute thrombosis. All acquired hemophilia patients receiving more than a few days of APCC should be monitored for DIC development.

## Recombinant activated factor VII

### Overview

Recombinant activated factor VII (rFVIIa) is a vitamin-K-dependent glycoprotein that initiates the tissue factor pathway of the coagulation cascade. At physiologic concentration, rFVIIa complexes with tissue factor (TF) at the site of vascular injury, forming the TF-FVIIa complex (a tenase). This complex is a key initiator of the coagulation cascade. It activates FX, which in turn combines with factor Va to form a prothrombinase complex, and it is this complex that activates prothrombin to thrombin. The resulting small burst of thrombin catalyzes a series of reactions on the platelet membrane essential for a larger thrombin burst, but also activates tissue factor pathway inhibitor (TFPI) to shut down the tissue factor pathway. At pharmacologic doses, rFVIIa can bind to activated platelets, which concentrates factor X activation to tissue injury sites and subsequently activates thrombin (bypassing the intrinsic pathway factors), or may also generate more thrombin by overwhelming TFPI.

### Manufacturing and storage

There are two FDA-approved recombinant factor VIIa products in the United States, NovoSeven RT and SevenFact, which have different properties, dosing, and approved indications. NovoSeven is a recombinant glycoprotein that is cloned and expressed in baby hamster kidney cells (BHK cells) followed by a chromatographic purification process during which the single-chain protein is proteolytically converted by autocatalysis to its active two-chain form. NovoSeven is available as a lyophilized powder in single-use vials of 1, 2, 5, or 8 mg which are stored at room temperature and need to be reconstituted before administration. After reconstitution, the solution may be stored at room temperature or refrigerated for up to three hours.

SevenFact is produced using genetically modified transgenic rabbits. Human factor VII is expressed in the rabbit mammary gland, secreted into the milk, and converted to its activated form during purification. SevenFact is stored as a lyophilized powder in single-use vials containing 1 or 5 mg, and is reconstituted in single-dose prefilled glass syringes. After reconstitution, the dose must be given within four hours.

### Indications

NovoSeven and SevenFact have different approved indications. Both products are approved in the United States to manage hemophilia A or B with inhibitors (SevenFact is only approved for

individuals ≥12 years of age). NovoSeven is also approved for the treatment of patients with acquired hemophilia, congenital factor VII deficiency, and Glanzmann's thrombasthenia with refractoriness to platelet transfusions.

There is growing use of rFVIIa for off-label indications. In fact, the vast majority of patients given rFVIIa in the United States receive it for off-label indications despite the questionable clinical benefit and lack of established dosing in this context.<sup>19,20</sup> The most common off-label uses of rFVIIa include cardiovascular surgery, trauma, and liver disease. Some clinical studies have shown reduced blood product utilization, but have not demonstrated improvements in more clinically relevant outcomes like reduced morbidity or mortality when rFVIIa is used for cardiovascular surgery,<sup>21,22</sup> traumatic coagulopathy,<sup>23,24</sup> or intracerebral hemorrhage.<sup>25</sup> There have also been reports of rFVIIa usage in the treatment of patients receiving anticoagulants, although specific reversal agents are preferred, with recent neurocritical guidelines recommending against the use of rFVIIa for VKA reversal in patients with intracranial hemorrhage.<sup>26–28</sup> The use of rFVIIa in off-label indications has also raised safety concerns with increased TEE.<sup>29,30</sup> Therefore, off-label uses of rFVIIa should be approached with caution, especially in patients at high risk for venous thromboembolism, and only given in life-threatening bleeding that has failed to respond to conventional therapies.

### Dosing

In patients with congenital hemophilia A or B with inhibitors, the typical dose of NovoSeven is 90–120 µg/kg administered as an intravenous bolus. Due to the short half-life of NovoSeven ( $t_{1/2}$  is about 2–3 hours), NovoSeven is typically given every 2–3 hours based on clinical response. SevenFact is dosed either as an initial bolus of 75 or 225 µg/kg, depending on the bleed severity. Since the half-life of SevenFact is similarly short ( $t_{1/2}$  is about 1.4–1.7 hours), the timing of subsequent doses depends on the initial bolus dose and the severity of the bleed.

Slightly lower doses of NovoSeven are given in acquired hemophilia, typically 70–90 µg/kg every 2–3 hours until hemostasis is achieved. In Glanzmann's thrombasthenia, dosing is typically 90 µg/kg every 2–6 hours until hemostasis is achieved. In patients with congenital factor VII deficiency, lower doses are administered, typically 15–30 µg/kg/dose every 4–6 hours, since the goal is to maintain hemostatic levels of FVII rather than bypass the intrinsic pathway factors. There is no established laboratory monitoring to guide the response to rFVIIa, so repeated administration should be based on clinical responses.<sup>31</sup> Dosing for off-label indications is not well established.

### Adverse reactions

rFVIIa carries a US boxed warning for arterial and venous thrombotic events. However, the rate of serious arterial and venous thrombotic events is low when rFVIIa is used for the four approved indications: hemophilia A or B with inhibitors, acquired hemophilia, congenital Factor VII deficiency, and Glanzmann's thrombasthenia.<sup>32,33</sup> Risk factors for thrombosis in these populations include older age, cardiac and cardiovascular disease, and concomitant use of APCC. rFVIIa should also be used with caution in patients with increased tissue factor expression, such as those with crush injury, septicemia, and disseminated intravascular coagulation.

As noted above, safety concerns have arisen when rFVIIa is used for off-label indications with increased TEE reported.<sup>34–36</sup> A large comprehensive review of 35 placebo-controlled trials of rFVIIa

demonstrated a high overall increased risk of arterial but not venous thromboembolic rates, especially among the elderly.<sup>37</sup>

Neutralizing alloantibodies to endogenous factor VII following exposure to rFVIIa have been reported in patients with severe congenital factor VII deficiency<sup>38</sup> but have not been reported in patients receiving rFVIIa for other indications.<sup>39</sup> rFVIIa is also associated with hypersensitivity reactions related to the manufacturing process. SevenFact is contraindicated in patients with an allergy to rabbit or rabbit proteins due to the risk of severe hypersensitivity reactions.

## Vitamin K

### Overview

Vitamin K is a fat-soluble vitamin that occurs in two bioactive forms. Vitamin K<sub>1</sub> (phylloquinones) is synthesized by plants and is the major dietary source of vitamin K. Vitamin K<sub>2</sub> (menaquinones) is produced by intestinal bacteria, and is also found in animal and soy protein products, but has limited bioavailability. Vitamin K has a major role in hemostasis as it is a required cofactor in hepatic cells for making coagulation factors II, VII, IX, and X, and the natural anticoagulant proteins C, S, and Z functional. The vitamin-K-dependent  $\gamma$ -carboxylation of glutamic acid residues to  $\gamma$ -carboxylated glutamyl at the N-terminal regions of the VKDF provides Ca<sup>2+</sup>-binding sites that are necessary for binding to anionic phospholipids at the sites of tissue injury. Vitamin K is a cofactor in the  $\gamma$ -carboxylation reactions and is subsequently reduced to an inactive vitamin K epoxide. Vitamin K epoxide reductase (VKOR) recycles vitamin K epoxide to reduce vitamin K to be reused. Warfarin and other VKAs produce their antithrombotic effects by blocking the action of VKOR and thus inhibiting the cyclic regeneration of active vitamin K. In conditions of vitamin K deficiency, there is diminished functionality of noncarboxylated proteins (also known as “proteins induced in vitamin K absence” or PIVKAs) that cannot bind to the phospholipid membrane and Ca<sup>2+</sup>, impairing hemostasis.

The daily dietary requirement of vitamin K is small (100–200  $\mu$ g/day), and vitamin K can easily be recycled, so vitamin K deficiency is rare in an otherwise healthy child or adult. However, acquired vitamin K deficiency can occur from inadequate intake or in patients with fat malabsorption syndromes, including celiac disease, sprue, inflammatory bowel disease, pancreatic insufficiency, cystic fibrosis, extensive small bowel resection, and severe biliary stasis, as well as ingestion of overdoses of vitamin E or salicylates. Antibiotic therapy contributes to vitamin K deficiency by inhibiting the synthetic capacity of vitamin-K-producing bacteria.<sup>40</sup> Certain antibiotics, such as cephalosporins, contain an N-methylthiotetrazol ring that directly interferes with VKOR and prevents the recycling of vitamin K. The combination of inadequate dietary intake of vitamin K and the use of broad-spectrum antibiotics is an insidious cause of vitamin K deficiency in hospitalized patients.

### Manufacturing and storage

Both vitamin K<sub>1</sub> and K<sub>2</sub> are biologically active, but only vitamin K<sub>1</sub> is available for use. It is available as an oral capsule or tablet, and also as an injectable emulsion that can be administered intravenously, intramuscularly, or subcutaneously. Due to the poor water solubility of vitamin K, a small amount of solvent or emulsifier (e.g., polyoxyethylated fatty acid derivative) is added to dissolve the vita-

min K into liquid form. The solution has a concentration of 1 mg/0.5 mL or 10 mg/mL. If a patient cannot swallow tablets, the parenteral form can also be administered in small oral doses.<sup>41</sup>

### Indications

The FDA-approved indications of vitamin K<sub>1</sub> include the treatment of coagulation disorders due to vitamin K deficiency, management of excessive anticoagulation due to VKAs, and VKDF deficiency secondary to other drugs or malabsorption factors limiting absorption, synthesis, or metabolism of vitamin K.

Vitamin K<sub>1</sub> is important in the treatment of accidental or surreptitious ingestion of superwarfarin rodenticides. After growing resistance to warfarin rodenticide among rodent populations, superwarfarin rodenticides, most commonly brodifacoum, were developed which are remarkably potent VKOR antagonists with exceedingly long half-lives (weeks to months) relative to warfarin.<sup>42</sup> Ingestion of these superwarfarin rodenticides can result in life-threatening bleeding, and treatment requires large doses of vitamin K<sub>1</sub> for up to 6–9 months.

Vitamin K<sub>1</sub> is also FDA approved for the prevention and treatment of vitamin K deficiency bleeding (VKDB), previously known as hemorrhagic disease of the newborn, which is a rare hemorrhagic disease in newborns that can cause life-threatening bleeding. Newborns have a physiologic deficiency of vitamin K due to several factors: the immature liver that does not effectively utilize vitamin K, the sterile gut, the poor placental transfer of vitamin K, and the low concentration of vitamin K in breast milk. The risk of VKDB can be further increased by prematurity, intestinal malabsorption defects, liver disease, and maternal exposure to warfarin, anticonvulsant drugs, or antituberculosis drugs. Prophylactic administration of vitamin K<sub>1</sub> to newborns diminishes the transient decrease in vitamin-K-dependent factors and prevents VKDB.<sup>43</sup> This practice is mandated by law in many countries, which accounts for the rarity of the disorder in the developed world.

The off-label use of vitamin K<sub>1</sub> in liver disease is common practice, but there is a lack of high-quality evidence to support this indication. There is an impaired synthesis of clotting factors from hepatic dysfunction in liver disease, leading to a prolonged prothrombin time (PT). Vitamin K<sub>1</sub> supplementation rarely improves coagulation parameters in patients with liver disease,<sup>44</sup> and there is a lack of randomized studies to support the use of vitamin K<sub>1</sub> in this population.<sup>45</sup>

### Dosing

The dose and route of vitamin K<sub>1</sub> depends on the severity of the clinical manifestations and the underlying cause. The intravenous route results in a more rapid reduction of the INR compared to subcutaneous or oral administration, but bears the risk of extremely rare anaphylactic reactions.<sup>46,47</sup> The rate of intravenous administration should not exceed 1 mg/minute to minimize the risk of this and other infusion reactions. Subcutaneous administration of vitamin K<sub>1</sub> is associated with an unpredictable response, particularly in patients with anasarca, and is, therefore, generally not recommended.<sup>48,49</sup> Intramuscular administration can cause hemorrhage, so this route should also be avoided aside from prophylaxis against VKDB.

Management of excessive anticoagulation from VKA should be guided by the degree of INR prolongation and the presence or absence of clinical bleeding (Table 38.2).<sup>50,51</sup> Minor or moderate elevation of the INR (above the therapeutic range but less than 10) in the absence of bleeding can generally be managed safely by

decreasing or omitting several warfarin doses or giving low-dose vitamin K<sub>1</sub>. Significant elevation of the INR (greater than 10), even in the absence of clinically evident bleeding, is generally treated by temporary discontinuation of warfarin and administration of vitamin K<sub>1</sub> (5–10 mg orally). If there is major or life-threatening bleeding from warfarin, then 10 mg of intravenous vitamin K<sub>1</sub> should be administered due to the faster onset of action in addition to cessation of VKA and administration of 4F-PCC. In cases of ingestion of superwarfarin rodenticides, vitamin K<sub>1</sub> doses in excess of 100 mg orally daily may be required because of the potency and extremely long biological half-lives of these poisons.<sup>52</sup>

For the treatment of vitamin K deficiency, typically 10 mg of vitamin K<sub>1</sub> restores adequate levels of vitamin-K-dependent coagulation factors within 24 hours. The dose can be repeated in 48–72 hours if the PT remains prolonged.

To prevent VKDB, all newborns should receive a single, intramuscular dose of 0.5–1 mg of vitamin K<sub>1</sub>.<sup>53</sup> If parents decline the intramuscular route, an alternative option is repeated doses of 2 mg oral vitamin K<sub>1</sub>.<sup>54</sup>

### Adverse reactions

Parental vitamin K<sub>1</sub> has a boxed warning for hypersensitivity reactions at an incidence of 3 per 10,000 doses of intravenous vitamin K<sub>1</sub>.<sup>55</sup> The reactions are consistent with an anaphylactoid reaction, may be related to the solubilizer, and can lead to severe hypotension, bradycardia or tachycardia, bronchospasm, and death. Risk factors may include faster administration rate, higher doses, and inadequate dilution. Less severe reactions may occur, including local site reaction, flushing, dizziness, and dysgeusia.

## Antifibrinolytic agents

### Overview

Fibrinolysis is responsible for clot resorption and requires the conversion of plasminogen to active plasmin on the surface of the fibrin clot by tissue plasminogen activator or urokinase. Plasmin cleaves fibrin into fibrin degradation products. The antifibrinolytic agents tranexamic acid (TXA) and  $\epsilon$ -aminocaproic acid (EACA) are synthetic lysine analogs that competitively block the lysine binding sites on plasminogen, inhibiting its ability to interact with the lysine residues on the fibrin surface and subsequent activation to plasmin. The main difference between TXA and EACA is that TXA is 7–10 times more potent, so lower doses are required to achieve the same effect as EACA.<sup>56</sup>

### Manufacturing and storage

TXA is available in intravenous and oral tablet form. An oral solution can also be compounded for topical applications. EACA is available as an intravenous solution, oral tablet, and oral solution.

### Indications

Antifibrinolytic agents are useful in the management of mucosal bleeding where high fibrinolytic activity can occur. EACA is FDA approved for the treatment of bleeding due to high fibrinolytic activity and the treatment of traumatic hyphema. TXA is only FDA approved for menstrual bleeding<sup>57</sup> and short-term treatment and prevention of bleeding in hemophilia during and following tooth extractions.<sup>58</sup> Both TXA and EACA have been successfully employed in numerous off-label clinical settings and have generally been safe and effective, as detailed below.

Antifibrinolytic agents have demonstrated efficacy with reduced blood loss and transfusion requirement in numerous elective and emergency surgical settings. The most widely studied are cardiothoracic<sup>59</sup> and orthopedic surgeries<sup>60–62</sup> with smaller studies in other surgical fields such as urology, vascular, thoracic, neurosurgery and otorhinolaryngology. A large meta-analysis showed that TXA reduces blood loss in surgical patients by one-third compared to placebo.<sup>63</sup> A 2011 Cochrane systemic review similarly showed that TXA decreases the need for a perioperative blood transfusion by 39% compared to placebo (RR 0.61, 95% CI, 0.53–0.7).<sup>64</sup> EACA also reduced the need for a perioperative blood transfusion by 19% (RR 0.81; 95% CI, 0.67–0.99). Neither agent increased the risk of deep vein thrombosis, pulmonary embolism, myocardial infarction, or stroke.

Antifibrinolytics are also safe and effective in reducing bleeding and mortality in trauma, with TXA being the most well-studied antifibrinolytic agent in this setting. The CRASH-2 trial randomized over 20,000 trauma patients to receive TXA or placebo and showed reduced mortality with early administration of TXA.<sup>65</sup> Empiric TXA reduced both all-cause mortality (14.5% vs. 16%;  $p = 0.004$ ) and bleeding-related mortality (4.9% vs. 5.7%;  $p = 0.0077$ ) without increasing thromboembolic events. There have been numerous criticisms of the CRASH-2 trial, including (1) the small difference in mortality of less than 1% that may not be clinically relevant, (2) the greatest efficacy occurred if TXA was administered within three hours of injury (with increased risk of death observed if TXA was administered after three hours), and (3) only half of the patients received a blood transfusion or emergent surgery. The MATTERs study, a retrospective review of the military experience, similarly showed improved survival with the use of TXA following combat injury but raised the concern of increased thromboembolic events (although this was the only study that showed increased thrombosis rates with TXA).<sup>66</sup> The CRASH-3 trial randomized over 12,000 adults with traumatic brain injury and found early administration of TXA within three hours of injury reduces head-injury-related death.<sup>67</sup> The use of early administration of antifibrinolytics is likely to be the most beneficial in trauma patients with hyperfibrinolysis guided by viscoelastic measurements.

TXA also reduces blood loss during cesarean section<sup>68</sup> and vaginal delivery. The WOMAN study showed early administration of TXA decreased bleeding-related mortality among women with postpartum hemorrhage (PPH) (1.5% vs. 1.9%,  $p = 0.045$ ) with no increase in thromboembolic events.<sup>69</sup> Accordingly, the 2012 WHO recommendations include the use of TXA for the treatment of PPH.<sup>70</sup> Evidence to support prophylactic administration of TXA in vaginal deliveries to prevent postpartum hemorrhage is less robust, with a large randomized trial showing no effect.<sup>71</sup>

Antifibrinolytics are commonly used prophylactically as an adjunct therapy in patients with hematologic malignancy, but there is a lack of clearly established benefit. A 2016 Cochrane review included only 86 patients with hematological disorders and severe thrombocytopenia and showed no significant reduction of bleeding with antifibrinolytics.<sup>72</sup> The a-Treat trial randomized over 300 patients with hematologic malignancy and therapy-induced thrombocytopenia to TXA or placebo in addition to routine platelet transfusions; this study found no difference in WHO Grade 2+ bleeding.<sup>73</sup>

Antifibrinolytics have also been used in off-label settings including dental procedures for patients on oral anticoagulation,<sup>74</sup> gastrointestinal bleeding,<sup>75</sup> epistaxis, hemoptysis, and surgical-related bleeding in patients with hereditary bleeding disorders like von

Willebrand disease or hereditary thrombocytopathies.<sup>76,77</sup> In some cases, it may be beneficial to reconstitute TXA as an oral solution and apply it topically to improve hemostasis.<sup>78</sup>

### Dosing

Dosing regimens and routes of TXA and EACA vary significantly in individual studies and depend on the clinical indication. TXA is typically administered at 10 mg/kg intravenously three times daily for 2–8 days. Oral TXA is available as 650 mg tablets and can be given as 1300 mg three times daily for a maximum of five days during menses. EACA is usually dosed as 50–100 mg/kg followed by 50 mg/kg every six hours with a maximum of 24 g in 24 hours. Because of high bioavailability, the same dose of EACA is given intravenously or orally. Given the predominant renal excretion (95% for TXA and 65% for EACA), dose adjustments should be made in the setting of renal insufficiency. Generally, EACA is preferred over TXA in the setting of renal insufficiency.

### Adverse reactions

It must be remembered that antifibrinolytic agents stabilize a pre-formed clot—they do not promote thrombosis per se. Thus, despite the theoretical risk of thrombosis associated with antifibrinolytics, numerous studies have shown that these agents are generally safe without an increase in TEE. Nonetheless, antifibrinolytics are generally contraindicated in patients with active thrombosis. In disseminated intravascular coagulation (DIC), antifibrinolytics should be used with caution due to the risk of impairing fibrinolysis and potentiating severe thrombosis. However, antifibrinolytics may have a role in patients with hyperfibrinolytic DIC. Antifibrinolytics are contraindicated in patients with upper urinary tract bleeding due to the risk of glomerular capillary thrombosis and intrarenal and intraureteral obstruction.

Both EACA and TXA are generally well tolerated; however, the long-term use of EACA has been associated with skeletal muscle weakness and rhabdomyolysis.<sup>79</sup> TXA can cause mild headaches, muscle weakness, and visual disturbances. It has also been associated with seizures, particularly with higher doses or in patients with underlying renal dysfunction, which is thought to be from dose-dependent CNS hyperexcitability. Rapid infusions of TXA can cause hypotension, so infusion rates should not exceed 1 mL/minute.

## Desmopressin

### Overview

Desmopressin (1-deamino-8-d-arginine vasopressin, DDAVP) is a synthetic analog of vasopressin that induces the release of von Willebrand factor (VWF) and factor VIII from storage sites within the endothelial cells. Compared to the natural hormone vasopressin, which activates both V<sub>1</sub> and V<sub>2</sub> receptors, DDAVP has no appreciable pressor activity due to decreased affinity for V<sub>2</sub> receptors and has enhanced diuretic potency due to increased affinity for the V<sub>1</sub> receptors.<sup>80</sup> Tissue plasminogen activator (tPA) is also released from Weibel-Palade bodies by DDAVP, but the excess plasmin is mostly complexed to α<sub>2</sub>-antiplasmin and does not contribute to fibrinolysis.<sup>81</sup> The mechanism of improved hemostasis following DDAVP administration in patients with platelet defects is unclear; it is likely independent of the increase of high-molecular-weight VWF multimers,<sup>82</sup> and may be mediated by enhanced interaction with the vascular subendothelium<sup>83</sup> and possibly increased platelet membrane glycoprotein expression.<sup>84</sup>

### Manufacturing and storage

DDAVP is available in intravenous, subcutaneous, and intranasal formulations. The intravenous formulation is available as a 4 µg/mL solution stored at 2–8 °C. In July 2020, there was a worldwide recall of DDAVP nasal sprays because of superpotency, and it is expected to remain off the market until mid-2023.

### Indications

The response to DDAVP varies depending on the underlying hemostatic abnormality, so it is generally useful to perform a DDAVP challenge to confirm responsiveness before clinical use. DDAVP is typically effective short term in patients with mild to moderate hemophilia A and type 1 von Willebrand disease (VWD) for minor bleeding, as well as for brief minor invasive procedures or dental work. Off-label use in type 2A VWD can be considered, although the response is variable. DDAVP is contraindicated in type 3 VWD due to a lack of efficacy and in type 2B VWD due to increased platelet binding leading to worsening thrombocytopenia and possibly bleeding. In patients with acquired von Willebrand syndrome (AVWS), DDAVP has about a 30% response rate, and the half-life of released VWF tends to be shorter.<sup>85</sup>

DDAVP is efficacious in certain conditions with normal factor VIII and VWF levels, including some patients with inherited platelet dysfunction,<sup>86</sup> antiplatelet-induced thrombocytopeny, uremic thrombocytopeny,<sup>87,88</sup> and cardiopulmonary bypass.<sup>89,90</sup> With the exception of perioperative blood loss after aspirin use, most of the studies evaluating DDAVP in these indications have only observed shortened bleeding times, and it remains unclear whether there is a reduction in more clinically significant bleeding. Additionally, with the increasing use of erythropoietin and resultant correction of the hemostatic defect in patients with chronic renal insufficiency,<sup>91</sup> there is a decreased frequency of uremic bleeding and the need for DDAVP in this population.

DDAVP does not seem to be effective in patients with liver cirrhosis. Early studies did demonstrate shortened bleeding time with DDAVP<sup>92,93</sup> but subsequent studies showed a lack of improvement in laboratory indices of primary hemostasis<sup>94</sup> and no benefit of DDAVP in variceal bleeding<sup>95</sup> or hepatectomy.<sup>96</sup>

### Dosing

Although DDAVP is available subcutaneously, it is predominantly administered intravenously or intranasally. Peak activity levels occur about 30 minutes after intravenous administration and 90–120 minutes after intranasal or subcutaneous administration. The half-life is 2–4 hours, although the half-life can be prolonged to nine hours in severe renal impairment. Typical intravenous dosing is 0.3 µg/kg diluted in 50–100 mL saline infused slowly over 30 minutes to avoid hypotension. The maximum single dose is 20–30 µg. Intranasal dosing is 150 or 300 µg depending on if the patient's weight is less than or greater than 50 kg, respectively. Doses may be repeated after 8–12 hours and then once daily thereafter. Tachyphylaxis can occur with more frequent dosing or after several days of DDAVP, so use should be limited to 2–3 days.

### Adverse reactions

Due to the antidiuretic effect, symptomatic hyponatremia and volume overload can occur. To minimize this risk, fluid restriction and electrolyte monitoring is recommended for 24 hours after use. Administration of DDAVP in children under the age of two years is contraindicated because of the risk of hyponatremic seizures. DDAVP has vasodilator properties caused by release of nitric oxide, leading to hypotension, tachycardia, and facial flushing.<sup>97</sup> The

intranasal formulation can also cause abnormal lacrimation, conjunctivitis, ocular edema, and rhinitis. Tachyphylaxis occurs with repeated dosing, possibly from depleted factor VIII and VWF stores. Mannucci *et al.*<sup>98</sup> showed an average decrease of 30% with the second dose of DDAVP compared with the initial dose, but there were no further reductions with the third or fourth dose. Given the predominant renal excretion, DDAVP is generally contraindicated in renal impairment with a creatinine clearance less than 50 mL/min, although it has been used off-label in patients with renal failure experiencing uremic bleeding.<sup>99</sup>

DDAVP should be used cautiously in patients with active cardiovascular disease because of reports of increased arterial thrombotic events, especially acute cerebrovascular thrombosis and myocardial infarction. A systemic review of 16 randomized trials evaluating the use of DDAVP to prevent perioperative blood loss in cardiac surgery showed a 2.4-fold increased risk of perioperative myocardial infarction with DDAVP compared to placebo (2.39; 95% CI, 1.02–5.6%).<sup>100</sup> However, a separate systemic review<sup>101</sup> and a meta-analysis<sup>102</sup> failed to show a statistically significant increased risk of arterial thrombosis with perioperative DDAVP.

## Protamine

### Overview

Protamine sulfate is a strongly basic, arginine-rich protein that binds to the negatively charged heparin molecules forming an inactive salt, neutralizing heparin's anticoagulant effect within minutes. The electrostatic binding of the cationic protamine sulfate with the anionic heparin results in a stable complex and displaces the antithrombin/heparin complex, allowing antithrombin to recover its activity.<sup>103</sup> Protamine also has intrinsic anticoagulant effects in the absence of heparin, including reducing platelet activity and aggregation, inhibition of coagulation factors (Va and Xa), and enhanced fibrinolysis. This activity can produce paradoxical increases in bleeding if protamine is overdosed. These anticoagulant effects are not detected by routine coagulation tests except in rotational elastometry (ROTEM). Heptem clotting time is typically more prolonged than Intem clotting time after cardiac bypass surgery, where a higher amount of protamine is given than needed for heparin neutralization.

### Manufacturing and storage

Protamine sulfate was initially derived from salmon fish sperm, but it is now increasingly produced with recombinant biotechnology. It is supplied as a solution with a concentration of 10 mg/mL.

### Indications

Protamine is indicated for the reversal of unfractionated heparin (UFH). Protamine can also be used in clinically significant bleeding with low-molecular-weight heparin (LMWH), but the anticoagulation effect is not fully reversed by protamine due to the smaller molecular size and reduced sulfate charge density of LMWH.<sup>104</sup> The low sulfate charge density of LMWH results in decreased protamine binding capacity with neutralization of anti-IIa activity, but variable neutralization of the anti-Xa activity of LMWH.<sup>105</sup>

### Dosing

Protamine sulfate has an immediate onset of action within minutes with a very short half-life (~7 minutes). Dosing is generally based on protamine-to-heparin ratios, with 1 mg of protamine sulfate neutralizing 80–100 units of heparin with a maximum single dose of protamine of 50 mg and a maximum infusion rate of 5 mg/minute. Protamine is dosed based on the amount of UFH received over the preceding three hours, taking into account the half-life of intravenous UFH of about one hour (e.g., if a patient has been receiving UFH heparin at a rate of 1000 units/hour, then 17.5 mg of protamine sulfate will be needed to neutralize a total of 1750 units of UFH).

For the reversal of LMWH administered within the preceding eight hours, 1 mg of protamine is given for every 1 mg of enoxaparin. If clinically significant bleeding persists (or if enoxaparin is administered greater than eight hours prior), an additional 0.5 mg of protamine can be administered for every 1 mg of enoxaparin.<sup>106</sup> Given the anticoagulant properties of excessive protamine, overdosing should be avoided as excess protamine can lead to increased bleeding.

### Adverse reactions

Protamine can cause rare but serious hypersensitivity reactions with hypotension, pulmonary vasoconstriction, pulmonary hypertension, and bradycardia.<sup>107</sup> Risk factors for the anaphylactic reaction include allergies for fish proteins, history of vasectomy, and prior exposure to protamine-containing insulin, all of which can lead to preformed antibodies against protamine sulfate. Infusing protamine slowly can mitigate the risk of severe allergic reactions.<sup>108</sup>

## Andexanet alfa

### Overview

Andexanet alfa is a recombinant-modified inactive form of factor Xa approved for reversing the factor Xa inhibitors, apixaban and rivaroxaban (Table 38.3). It acts as a “decoy” to bind and

**Table 38.3** Specific Reversal Agents for Direct Oral Anticoagulants

Pharmacologic Agent	Andexanet alfa	Idarucizumab
Mechanism of action	Recombinant-modified factor Xa protein that binds and sequesters direct Xa inhibitors	Humanized monoclonal antibody fragment that binds dabigatran with high affinity
Dose	Low versus high dose depending on the dose and timing of the last direct Xa inhibitor*	5 g total dose (given as two separate 2.5 g doses) administered as an intravenous bolus
Onset	Rapid within minutes	Rapid within minutes
Half-life	~1 hour	~45 minutes
FDA-approved indications	Reversal of the anticoagulant effect of apixaban and rivaroxaban in patients with life-threatening or uncontrolled bleeding	Reversal of the anticoagulant effect of dabigatran in patients with life-threatening or uncontrolled bleeding or for emergency surgery or procedures
Serious adverse reactions	Arterial and venous thromboembolic events	Mostly venous thromboembolic events

\* High-dose of andexanet alfa should be given if the rivaroxaban or apixaban dose is 10 or 5 mg, respectively (or unknown dose), and less than eight hours has elapsed since the last dose was given; otherwise, the low-dose andexanet alfa should be given.

sequester factor Xa inhibitors. Additionally, andexanet alfa causes off-target inhibition of tissue factor pathway inhibitor (TFPI), which can lead to increased thrombin generation and thromboembolic events.

### Manufacturing and storage

Andexanet alfa is stored as a lyophilized powder in single-use vials of 100 or 200 mg of recombinant factor Xa.

### Indications

Andexanet alfa is FDA approved for the reversal of anticoagulation with rivaroxaban or apixaban in patients with life-threatening or uncontrolled bleeding. While andexanet alfa is currently only FDA approved for the reversal of apixaban and rivaroxaban, it also has neutralizing activity against other direct and indirect factor Xa inhibitors. Approval for andexanet alfa was based on a single-arm open-labeled trial (ANNEXA-4) of 352 patients with acute major bleeding within 18 hours of receiving a factor Xa inhibitor (although nearly 30% of patients were excluded from the efficacy analysis due to levels less than 75 ng/mL).<sup>109,110</sup> Excellent or good hemostatic efficacy at 12 hours was obtained in 82% of patients (204 of 249 patients). During the 30-day follow-up period, thrombotic events occurred in 10% of patients (34 of 352 patients), which were mainly arterial. Because of its very short  $t_{1/2}$ , it is not approved for patients requiring emergent surgery while on factor Xa inhibitors. There are no randomized studies comparing andexanet alfa with PCC and no compelling data to choose one over the other for the reversal of factor Xa inhibitors for bleeding patients. However, for urgent surgery, PCC is preferred due to its longer duration of effect.

### Dosing

Dosing of andexanet alfa is based on the specific factor Xa inhibitor (e.g., apixaban or rivaroxaban) and the dose and time of last administration of that inhibitor. Andexanet alfa is administered as an initial bolus over 15–30 minutes, followed by a two-hour infusion. The initial bolus is either 400 or 800 mg followed by an infusion of 4 mg or 8 mg/minute for the low versus high dose of andexanet alfa, respectively. Given the high thromboembolic risks associated with andexanet alfa, it is essential to resume anticoagulation as soon as hemostasis is achieved and clinically appropriate.

### Adverse reactions

The most serious adverse events associated with andexanet alfa include arterial and venous thromboembolic events, ischemic events including myocardial infarction and ischemic strokes, cardiac arrest, and sudden death. Other adverse drug reactions include infusion reactions, urinary tract infections, and pneumonia.

## Idarucizumab

### Overview

Idarucizumab is a humanized monoclonal Fab antibody fragment that binds dabigatran with a 350-fold higher affinity than thrombin, neutralizing its activity and reversing the anticoagulant effect within minutes (Table 38.3).<sup>111</sup>

### Manufacturing and storage

Idarucizumab is packaged in ready-to-use vials of 2.5 g.

### Indications

Idarucizumab is indicated for the reversal of the anticoagulant effect of dabigatran in patients with life-threatening or uncontrolled bleeding or for emergency surgeries. FDA approval was based on the single-arm prospective cohort study, Reversal Effects of Idarucizumab on Active Dabigatran (RE-VERSE AD).<sup>112,113</sup> Idarucizumab was administered in 503 patients taking dabigatran with either uncontrolled bleeding (301 patients) or patients requiring an urgent procedure (202 patients), with nearly all patients achieving normalization of the diluted thrombin time within four hours. Of the 203 evaluable patients with uncontrolled bleeding, 134 patients (67.7%) had confirmed bleeding cessation. Among the patients receiving idarucizumab before a procedure, periprocedural hemostasis was normal in 93.4% of patients. The thrombotic event rate was 4.8% at 30 days and 6.8% at 90 days. Notably, all of the thrombotic events that occurred within 72 hours of idarucizumab administration failed to reinitiate anticoagulation.

### Dosing

Idarucizumab is dosed as 5 g intravenously and administered as two separate 2.5 g doses no more than 15 minutes apart. Repeated doses are generally not given but can be considered if there is persistent bleeding with evidence of the dabigatran effect. Case reports have shown incomplete reversal of dabigatran with idarucizumab; repeat doses of idarucizumab may be required in patients with massive dabigatran accumulation with evidence of re-elevation of coagulation parameters.<sup>114</sup>

### Adverse reactions

The most common side effects include headaches, nausea, and constipation. Since patients on anticoagulation therapy have underlying hypercoagulable states, the cessation of dabigatran and administration of idarucizumab may increase the thrombotic risk, so anticoagulation should be resumed as soon as clinically indicated.

## Clinical settings

### Disseminated intravascular coagulation

Disseminated intravascular coagulation (DIC) is always secondary to an underlying condition, most commonly infection/sepsis, followed by obstetrical catastrophes, trauma, malignancy, and intravascular hemolysis. Increased expression of tissue factor on various cells (monocytes/macrophages, tumor cells, etc.) is characteristic of DIC and leads to pathologic activation of the coagulation system, resulting in widespread microvascular thromboses with ischemic tissue injury. Life-threatening hemorrhage can also occur from consumption of coagulation factors and platelets, as well as accelerated fibrinolysis. DIC is a clinicopathological syndrome, with a highly variable presentation that may manifest as acute or chronic, localized or systemic, and hemorrhagic or thrombotic (and sometimes both). There is no single diagnostic test for DIC, and, therefore, a careful assessment of hemostasis tests is essential. Because of the consumption of coagulation factors, various tests become abnormal depending upon the severity of DIC and synthetic function of the liver and bone marrow. Thus, in an acute DIC scenario, generally, there is a prolongation of PT and aPTT, decreased fibrinogen, increased d-dimers, and moderate to severe thrombocytopenia. These are accompanied by reduced levels of procoagulant factors (e.g., factors II, VII, X, and V) and natural

anticoagulants (e.g., antithrombin, protein C, and protein S). The International Society on Thrombosis and Hemostasis developed a simple scoring system based on the underlying condition associated with DIC and routine coagulation tests that include platelet count and fibrin-related markers (e.g., D-dimer), PT, and fibrinogen.<sup>115</sup> Prospective validation demonstrated a sensitivity of 91% and a specificity of 97%.<sup>116</sup>

Identification and treatment of the underlying cause is essential in the management of DIC. Supportive management should be tailored to the individual patient, underlying condition, laboratory studies, and presence and severity of bleeding and thrombotic complications. It is important to remember that many patients who have definitive laboratory evidence of DIC may not have clinically significant thrombosis or hemorrhage. Such patients typically do not require any specific therapy other than the treatment of the underlying process. In contrast, patients with active bleeding or impending procedures, as well as laboratory evidence of severe consumption of fibrinogen (fibrinogen level less than 100 mg/dL), coagulation factors (PT greater than 1.5 × control value), or thrombocytopenia (platelets <50,000/µL), should be treated by transfusion of cryoprecipitate, plasma (10–15 mL/kg), and/or platelets. In the absence of bleeding or a planned procedure, platelet transfusions should be avoided as long as the platelet count is maintained above 10,000–20,000/µL.<sup>117–119</sup> A restrictive blood component transfusion approach is based on the fact that there is no clear evidence that a liberal transfusion approach improves bleeding complications and may increase thrombotic complications because of adding fuel to the fire effect.

Low-dose unfractionated heparin may help inhibit the activation of coagulation in DIC and slow the consumptive process.<sup>120</sup> However, the use of heparin in DIC remains controversial, and the overall impact on clinically relevant outcomes has not been definitively demonstrated in clinical trials.<sup>121,122</sup> Low doses of heparin can be considered in patients without thrombosis, typically at a continuous infusion rate of 5–10 U/kg/hour (~300 U/hour), and therapeutic anticoagulation is indicated in patients with overt thrombosis.

When bleeding associated with DIC is refractory to heparin and transfusion therapy, antifibrinolytic agents are occasionally used, although they are generally contraindicated. In addition to stabilizing clots, antifibrinolytic agents also reduce FDP production. However, inhibition of fibrinolysis has the potential to exacerbate fibrin deposition, resulting in severe thrombotic complications. Therefore, antifibrinolytic agents should be reserved for life-threatening hemorrhage and should be considered for use only in conjunction with heparin in the setting of DIC.

There is conflicting evidence on the use of antithrombin in patients with DIC. A large randomized controlled trial, KyberSept, failed to show a survival benefit with the use of high-dose antithrombin in patients with sepsis.<sup>123</sup> Additionally, there was increased bleeding when antithrombin was administered with heparin. While a post hoc analysis showed a significant mortality reduction in septic patients with DIC,<sup>124</sup> this benefit was not replicated in a meta-analysis<sup>125</sup> and has not been validated prospectively. Despite initial promising data with activated protein C in patients with severe sepsis and DIC, subsequent evaluation likewise failed to demonstrate improved survival. Furthermore, this therapy showed an increased risk of bleeding, prompting the withdrawal of activated protein C (drotrecogin alfa) from the market.<sup>126–128</sup>

## Chronic liver disease

Chronic liver disease results in complex changes in the hemostatic system. Alterations in pro- and antihemostatic pathways lead to a delicately “rebalanced” hemostasis that may predispose to both bleeding and thrombotic complications. Primary hemostasis is affected by decreased platelet number (from splenic sequestration, decreased thrombopoietin, and bone marrow suppression) and function.<sup>129,130</sup> However, the effect of thrombocytopenia is rebalanced by very high von Willebrand factor (VWF) levels (endothelium-derived) and increased function due to reduced levels of the VWF cleaving protease ADAMTS-13.<sup>131</sup> Secondary hemostasis is altered, with reduced levels of all procoagulant factors synthesized by the liver; however, thrombin generation is rebalanced by a simultaneous reduction in natural anticoagulants and elevated FVIII (from endothelium).<sup>132</sup> Enhanced fibrinolysis can result from increased tissue-type plasminogen-activator (t-PA) relative to increased levels of plasminogen activator inhibitor 1 (PAI-1) and decreased levels of alpha-2-antiplasmin.<sup>133</sup> Therefore, significant liver dysfunction can lead to both bleeding and thrombosis from multiple factors, including impaired biosynthesis of multiple hemostatic factors, failed clearance of inhibitory factors, and fibrinolysis.

Conventional coagulation tests may be abnormal in cirrhosis with prolonged PT, and sometimes aPTT, and decreased fibrinogen levels (in approximately 30–50%). However, these levels are misleading and do not reflect the complex “rebalanced” hemostasis or predict the risk for bleeding.

Given that functional hemostasis remains intact despite abnormal laboratory studies of hemostasis, routine prophylactic transfusions or administration of prohemostatic agents should generally be avoided in patients with cirrhosis. The exception is vitamin K, which can be administered to patients with liver disease who may be deficient, but it is often poorly effective, and bleeding may persist even if the PT is shortened.<sup>134,135</sup> There is a lack of high-quality evidence to show the clinical benefit of prophylactic plasma or platelet transfusions prior to procedures in cirrhotics.<sup>136,137</sup> Moreover, variceal bleeding, which commonly occurs in patients with cirrhosis, is due to elevated portal pressures rather than hemostatic failure; the transfusion of blood products further increases portal and central venous pressures and, thus, promotes further bleeding.<sup>138</sup> Thrombopoietin receptor antagonists, avatrombopag and lusutrombopag, are approved to raise platelet count in patients with chronic liver disease undergoing intermediate or high-risk procedures; however, the clinical benefit is unknown due to concurrent elevation of VWF.<sup>139,140</sup> Fibrinogen levels are seldom decreased enough from liver dysfunction to cause major bleeding. However, severe hypofibrinogenemia (fibrinogen level <100 mg/mL) in a patient with active bleeding or requiring surgery should be treated with cryoprecipitate (VWF levels are already too high for the use of DDAVP). Large epidemiological studies have shown that cirrhotics have twice the incidence of venous thromboembolism than the general population. Therefore, thromboprophylaxis should not be withheld based on the prolongation of PT and aPTT in hospitalized patients with cirrhosis.<sup>141</sup>

## Acquired von Willebrand syndrome

Acquired von Willebrand syndrome (AVWS) is a rare bleeding disorder that results in structural and functional alterations of VWF. Generally, it occurs in association with an underlying disorder, including myeloproliferative neoplasms, lymphoproliferative disorders, solid malignancies, autoimmune disorders, and cardiovascular

disorders with increased shear stress.<sup>142</sup> The pathophysiology of AVWS may involve several different mechanisms, such as autoantibodies that cause functional interference or rapid clearance of VWF from the plasma, adsorption of VWF to malignant cells, or high shear stress causing increased proteolysis of high molecular weight multimers. The clinical features and laboratory abnormalities are similar to congenital VWD. However, unlike congenital VWD, AVWS occurs in patients with no personal or family history of bleeding disorders. Symptoms can vary from mild mucocutaneous bleeding (ecchymosis, epistaxis, gingival hemorrhage, or menorrhagia) to severe, life-threatening hemorrhage.

Laboratory studies may be notable for a prolonged closure time on platelet function analyzer (PFA-100). aPTT may also be prolonged. Levels of VWF activity (ristocetin cofactor activity and collagen-binding activity), VWF antigen, and factor VIII are often depressed. Selective loss of high-molecular-weight multimers of VWF can be seen in AVWS associated with cardiovascular diseases with increased shear stress, and in this setting it may resemble type 2A VWD. The inhibitors cause rapid clearance of VWF from the circulation, so inhibitory activity often cannot be demonstrated in the plasma by mixing studies.

If possible, the underlying disorder associated with AVWS should be treated, which may lead to long-term remission (e.g., treatment with cytoreductive therapy in myeloproliferative neoplasms or corrective cardiac surgery in aortic valve stenosis). In patients with acute bleeding, therapeutic options include DDAVP, infusion of intermediate purity factor VIII concentrates containing large quantities of VWF or recombinant VWF. Cryoprecipitate may be given as a source of VWF if the above products are unavailable, but one must remember to give a very high dose of cryoprecipitate (30–50 units). Responses to DDAVP and replacement therapy depend on the cause of AVWF and may be only transiently effective because of the short half-life of VWF caused by excessive clearance from the circulation.<sup>143</sup> Antifibrinolytic agents may be beneficial, particularly in patients with mucosal bleeding. In therapy-resistant life-threatening bleeding, rFVIIa can be considered, although the safety has not been well established.<sup>144</sup> Administration of high-dose intravenous immune globulin (1 g/kg for two days or 0.4 g/kg for five days) can sometimes be effective in patients with AVWF, and repeated administration of intravenous

immune globulin every three weeks may control chronic bleeding. AVWS can also be seen in patients with myeloproliferative disorders who manifest extreme thrombocytosis ( $>1000 \times 10^9/L$ ). In this setting, large VWFs are adsorbed to GPIb on platelets leading to decreased VWF in the plasma and bleeding symptoms. Such patients often benefit from plateletpheresis to lower platelet count of  $<500 \times 10^9/L$ .

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## CHAPTER 39

# Perioperative transfusion practice

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## Background

Anemia, thrombocytopenia, and derangements in coagulation parameters are common in the perioperative setting.<sup>1–4</sup> Historically, concerns for surgical hemorrhage associated with adverse patient outcomes have driven liberal transfusion practices in these surgical populations. However, a growing body of evidence supports more conservative transfusion practices.<sup>5,6</sup> These sentiments are largely based upon accumulating evidence suggesting a lack of efficacy with liberal transfusion practices,<sup>6,7</sup> increased awareness regarding the risks of transfusion therapies,<sup>8–10</sup> and an increased appreciation of blood components as a valuable health-care resource—both in terms of supply limitations and associated costs.<sup>11,12</sup> In light of these findings, dedicated patient blood management (PBM) programs have been implemented, aiming to cultivate more evidence-based transfusion practices.<sup>13</sup> Despite these advances, the literature continues to describe the liberal and highly variable utilization of transfusion therapies in surgical populations.<sup>14,15</sup> Examples include red blood cell (RBC) administration for the correction of stable asymptomatic anemia and plasma administration for the correction of abnormal coagulation parameters in the absence of bleeding.<sup>16–19</sup>

Although the risk for hemorrhage must be considered in all patients undergoing invasive procedures, it is important to recognize that our ability to accurately predict bleeding risk remains disappointingly limited.<sup>15,19,20</sup> Likewise, the determinants of when to transfuse RBCs to a bleeding surgical patient remain poorly defined. Moreover, it must also be acknowledged that the practice of the prophylactic administration of either plasma products or platelet components with the intention of modifying bleeding risk is largely devoid of evidence and may be potentially harmful.<sup>21–23</sup>

This leaves us with challenging questions. What is the best way to assess a patient's risk of perioperative bleeding? What is the best approach to mitigating this bleeding risk? How should perioperative anemia be managed? What options are there to avoid or minimize allogeneic blood transfusions? What clinical and laboratory information helps to guide transfusion decision-making? What is the role of transfusion therapy in special circumstance for surgical patients? In this chapter, we discuss anemia, its impact on surgical outcomes, and preoperative anemia optimization; the impact of RBC transfusions on surgical patient outcomes; the utility of preoperative hemostatic

testing; the management of perioperative anticoagulation and antiplatelet therapies; conservative versus liberal transfusion practices; blood avoidance techniques; topical hemostatic therapies; perioperative transfusion guidelines; the role of plasma transfusion in special circumstances; and the role of platelet transfusion in special circumstances.

## Anemia and surgery

In a physiological sense, anemia is a state of inadequate red cell mass to support tissue oxygen requirements. However, given the difficulties of accurately assessing red cell mass or tissue oxygen needs at the patient level, anemia is more commonly defined by hemoglobin (Hb) concentrations (i.e., mass of hemoglobin per given blood volume) or hematocrit (Hct) values (i.e., proportion of whole blood volume comprised by red cells) falling below normative reference values. In the mid-1900s, the World Health Organization (WHO) assigned reference Hb ranges to define anemia, including: <11.0 g/dL for children 0.5–5 years, <11.5 g/dL for children 5–12 years, <12.0 g/dL for children 12–15 years and nonpregnant women ≥15 years, <11.0 g/dL for pregnant women, and <13.0 g/dL for men ≥15 years.<sup>24</sup> It should be noted that these definitions were assigned based upon observed distributions of hemoglobin in a limited number of population-based studies rather than by the physiological significance of Hb concentrations themselves. In any case, employing these definitions, anemia is extraordinarily common, affecting approximately 25% of the world's population,<sup>25</sup> though the prevalence and burden of anemia are highest in low- and middle-income countries. Not surprisingly, anemia, as defined by these criteria, is the most common preoperative hematologic abnormality encountered in surgical patients.<sup>26</sup> Estimates of the prevalence of preoperative anemia generally range from 25% to 40%,<sup>1,27–31</sup> though prevalence varies substantially by surgery type, approaching 75% in those with advanced colon cancer.<sup>32</sup> Rather than representing a distinct disease process itself, anemia is often a sign of underlying diseases or conditions that could negatively impact surgical outcomes. Such negative consequences are not restricted to those with severe anemia, which is typically defined by Hb concentrations <8–9 g/dL. Adverse outcomes such as renal injury, stroke, and even death have been shown

to be associated with surgical patients categorized as having mild or moderate anemia.<sup>27,28,33,34</sup> Further, recent research in cardiac surgical patients suggests increasing risk for adverse perioperative outcomes for every 1 g/dL decrease in preoperative Hb below 13 g/dL in females and 14 g/dL in males.<sup>35</sup> Importantly, anemia represents a potentially modifiable risk factor for adverse perioperative outcomes, and there is growing recognition of the importance of appropriate optimization throughout the spectrum of perioperative care.<sup>36</sup>

### **Impact of anemia on surgical outcomes**

There is now abundant literature regarding the relationships between preoperative anemia and surgical outcomes. For example, in a study of more than 200,000 adults undergoing major noncardiac surgery, with data obtained from the American College of Surgeons' National Surgical Quality Improvement Program (ACS NSQIP) database derived from 211 hospitals worldwide, more than 30% of patients presented to surgery with mild (Hct >29% but <39% in men and <36% in women) or moderate-to-severe (<29% in men and women) preoperative anemia.<sup>27</sup> In multivariable regression analyses, the odds of 30-day postoperative mortality were higher in patients with anemia compared to those without (odds ratio [OR] 1.42, 95% confidence interval [CI], 1.31–1.54), with similar relationships noted for mild (OR 1.41, 95% CI, 1.30–1.53) and moderate-to-severe anemia (OR 1.44, 95% CI, 1.29–1.60). Similar findings were observed for 30-day composite postoperative morbidity (cardiac, central nervous system, respiratory, sepsis, urinary tract, venous thromboembolism, and wound complications; OR 1.35, 95% CI, 1.30–1.40 for any anemia, OR 1.31, 95% CI, 1.26–1.36 for mild anemia, and OR 1.56, 95% CI, 1.47–1.66 for moderate-to-severe anemia).

Numerous additional investigations have also noted similar relationships between preoperative anemia status and adverse perioperative outcomes in noncardiac surgery,<sup>37–40</sup> such that there is no longer any uncertainty regarding the relationship between preoperative anemia and surgical outcomes. For example, in an analysis of more than 300,000 veterans >65 years of age undergoing noncardiac surgery, preoperative Hct was inversely correlated with both cardiac events and mortality.<sup>37</sup> Specifically, there was a 10% increase in adverse events with mild anemia (Hct 36.0–38.9) and a greater than 50% increase with more severe anemia (Hct 18.0–20.9). In the European Surgical Outcomes Study including data from nearly 40,000 patients, preoperative anemia was associated with both postoperative morbidity and mortality.<sup>41</sup> Outcomes were progressively worse with greater severity of preoperative anemia. Similar relationships between anemia and adverse outcomes, including mortality, have been confirmed in other patient groups.<sup>38</sup> In a study of nearly 2000 adult patients who refused blood transfusion,<sup>42</sup> decreasing preoperative Hb concentrations were associated with increased risk for 30-day mortality, especially for patients with Hb of 6 g/dL or lower. The risk of mortality was particularly high in patients with preexisting cardiovascular disease who had a preoperative Hb of 10 g/dL or less.

Like noncardiac surgery, there is also abundant literature on preoperative anemia and outcomes in cardiac surgery. In a cohort of 7738 adults undergoing cardiac surgery between 2003 and 2009, preoperative anemia was an independent predictor of mortality (OR 1.44, 95% CI, 1.02–2.03), postoperative renal dysfunction (OR 1.73, 95% CI, 1.43–2.1), and prolonged hospital length of stay (OR 1.3, 95% CI, 1.15–1.47 for >7-day length of stay).<sup>43</sup> Similarly, in a multicenter study of nearly 5000 adults undergoing coronary bypass

surgery, preoperative anemia was associated with both renal and cerebrovascular adverse events.<sup>34</sup> More recently, in a study of more than 4000 adults undergoing elective cardiac surgery, progressively lower Hb concentrations were associated with adverse postoperative outcomes such as acute kidney injury and prolonged hospitalizations.<sup>35</sup> Importantly, the relationships between Hb concentrations and outcomes were not uniform across sexes, with differential thresholds identified for increasing perioperative risk in men (14 g/dL) and women (13 g/dL). This result suggests that reliance on WHO definitions of anemia may not be ideally suited for identifying Hb concentrations associated with the greatest risk of adverse perioperative outcomes.

While most published literature on preoperative anemia and surgical outcomes is focused on adults, there is also substantial evidence that preoperative anemia is similarly detrimental in children. In a study of more than 50,000 children between ages 1 and 18 years in the ACS NSQIP database, approximately one quarter were anemic at the time of surgery.<sup>44</sup> Preoperative anemia was associated with increased in-hospital mortality after multivariable adjustment, with an odds ratio of 1.75 (95% CI, 1.15–2.65) compared to propensity-matched nonanemic patients. Like in adults, preoperative anemia is also an independent risk factor for perioperative RBC transfusion and prolonged hospitalization in children.<sup>45</sup>

Beyond preoperative anemia, postoperative anemia is also extremely common, occurring in up to 90% of patients.<sup>46</sup> Anemia after surgery is most often related to inadequately optimized preoperative red cell mass, perioperative blood loss, prolonged inflammatory states, diagnostic phlebotomy, and impaired nutritional status. In noncardiac patients that received intraoperative red cell transfusion, severe anemia immediately postoperatively (i.e., initial postoperative Hb <7.5 g/dL) was associated with increased odds for acute kidney injury, cerebral ischemia, and mortality.<sup>47</sup> Similarly, several studies have assessed the relationships between postoperative Hb concentrations and clinical outcomes in patients that declined RBC transfusion.<sup>48,49</sup> In those studies, mortality rates were low with nadir postoperative Hb concentrations between 7.1 and 8.0 g/dL (range 0–1.5%), though composite morbidity/mortality rates were higher, ranging between 9% and 23%. Most importantly, every 1 g/dL decrease in postoperative Hb was associated with progressive increases in morbidity and mortality, with outcomes markedly inferior in those with nadir Hb concentrations less than 5 g/dL.

### **Preoperative anemia optimization**

Given the detrimental impact of anemia on perioperative outcomes, there is increasing recognition of the importance of evaluating and treating anemia prior to surgery. Formal preoperative anemia programs or clinics have been created to fill this niche,<sup>50</sup> though these are far from ubiquitous in modern medical practice despite numerous benefits. Preoperative anemia programs are tasked with identifying anemia in advance of surgery, determining the underlying cause(s) of anemia which may include further diagnostic testing (e.g., endoscopy, referral to a hematologist), prescribing appropriate targeted treatments, and ensuring optimal hemoglobin recovery prior to surgery.<sup>36</sup> Diagnostic evaluation usually entails thorough medical history and medical record review to identify likely sources of anemia (e.g., blood loss, nutritional deficiencies, hemolysis, chemotherapy, thalassemias, sickle cell anemia, and end-stage renal disease) followed by laboratory testing to assess iron status (i.e., transferrin saturation, ferritin,

and reticulocyte hemoglobin content) and noniron nutritional status (i.e., B12 and folate levels). Additionally, labs are typically assessed to exclude anemia secondary to hemolysis or severe renal dysfunction.<sup>36</sup>

Iron deficiency is the leading cause of preoperative anemia and can be treated with either oral or intravenous (IV) iron replacement. The advantages of IV iron are related to quicker resolution of iron deficits, faster hemoglobin recovery, and the avoidance of gastrointestinal side effects that are commonly encountered with oral therapy. As such, IV iron may be considered first-line therapy for preoperative iron deficiency anemia, particularly in those with surgery occurring within 4–6 weeks of preoperative evaluation and in those with severe anemia. Fortunately, current IV iron formulations have an excellent safety profile,<sup>36</sup> though costs associated with IV therapy are much greater than over-the-counter oral iron supplements. Anemia of inflammation is another common cause of preoperative anemia, which is typically a diagnosis of exclusion in patients without other obvious etiologies of anemia and with evidence of chronic illness or inflammatory conditions. These patients typically show an abrupt hemoglobin response with erythropoietic stimulation after ensuring optimization of iron stores.

Numerous observational studies and several randomized clinical trials have evaluated the efficacy and safety of preoperative anemia programs on hemoglobin concentrations, perioperative red cell transfusion utilization, and patient outcomes. For those with iron deficiency anemia, clinical trials have shown that targeted therapy with IV iron or the combination of IV iron and low-dose erythropoietin results in successful augmentation of hemoglobin concentrations and reductions in perioperative red cell transfusions.<sup>51,52</sup> While anemia treatments should optimally be tailored to the underlying cause(s) of anemia, even in those with anemia not exclusively related to iron deficiency, IV iron improves hemoglobin recovery through six months postoperatively after major abdominal surgery and reduces readmission rates for the management of postoperative complications.<sup>53</sup> Additionally, the utilization of a “kitchen-sink” approach to anemia management including a combination of IV iron, erythropoietin, vitamin B12, and folic acid administered only one day prior to cardiac surgery resulted in improved hemoglobin recovery and reduced perioperative transfusion utilization. In addition to finding similar improvements in hemoglobin concentrations and transfusion reductions with preoperative anemia programs, observational data support substantial reductions in hospital costs and reductions in hospital lengths of stay following the implementation of formal preoperative efforts to address anemia.<sup>54</sup> Given these findings, preoperative anemia programs appear to be a clear win for both patients and healthcare systems.<sup>55</sup> Further, it is important to provide the same vigilance for anemia throughout the perioperative spectrum of care.<sup>36</sup>

It is logical to ask whether preoperative transfusion of RBCs to increase red cell mass in anemic patients positively impacts perioperative clinical outcomes. While formal studies of RBC transfusion for anemia correction before surgery are lacking, the overwhelming evidence from observational data and transfusion trials in other clinical settings, as detailed in the following section, suggest that RBCs are unlikely beneficial and may be harmful in this context. Briefly, allogeneic RBCs are associated with substantial risk, do not address the underlying causes of anemia, and do not reliably improve tissue oxygenation except in those with the most critical hemoglobin deficits (i.e., hemoglobin <5–6 g/dL).<sup>56</sup> Preoperative

anemia optimization efforts should, therefore, be focused on addressing underlying causes of anemia whenever possible with nontransfusion-based modalities.

## Impact of RBC transfusions on surgical patient outcomes

Red blood cell transfusions are widely utilized in the perioperative setting. Earlier studies around the year 2000 showed that 40–70% of all RBC transfusions are given in association with surgical procedures.<sup>57–59</sup> A decade later, the 2011 AABB National Blood Collection and Utilization Survey (NBCUS) documented that surgical departments used 19.8% of all RBCs distributed.<sup>60</sup> This decrease in surgical blood use over a decade, consistent with other recent evaluations demonstrating overall reductions in RBC utilization,<sup>61</sup> is likely due to the emerging emphasis on patient blood management initiatives over the same time period, at least in part.<sup>62</sup>

The rationale underpinning RBC transfusion is to enhance oxygen delivery ( $\text{DO}_2$ ) in patients with a decreased red cell mass, as inferred by the presence of a low Hb. This is particularly relevant in patients in whom impaired compensatory mechanisms, such as coexisting cardiovascular disease, mean their expected tolerance for such a state is diminished.<sup>63</sup> In the surgical arena, decisions to transfuse may be guided by amount of ongoing blood loss, evidence of evolving coagulopathy, hemodynamic signs of anemia, and recent hemoglobin values. Ultimately, the decision-making process necessitates weighing the risk of transfusion against the risk of anemia. As described above, historical studies have shown an association between anemia and morbidity and mortality in surgical patients. However, until recently, it has been difficult and impractical to determine specific thresholds at which RBC administration to correct anemia results in improved outcomes. In the absence of strong data to support superior surgical outcomes with more liberal transfusion practices, and with increased appreciation for the cost, resource utilization,<sup>64</sup> and potential risks associated with transfusion,<sup>65–67</sup> restrictive transfusion practices have been advocated by patient blood management programs. For example, in 2019, Will *et al.*<sup>47</sup> proposed transfusing to a target postoperative hemoglobin value between 7.5–11.5 g/dL to achieve superior outcomes, as determined by hospital free days.

The influence of RBC transfusions on outcomes in surgical patients has been assessed in multiple studies. A recent study evaluated postoperative morbidity, in-hospital mortality, and long-term survival of Jehovah’s Witness patients who underwent cardiac surgery compared to a similarly matched group of cardiac surgery patients who received RBC transfusions.<sup>68</sup> Between 1 January 1983 and 1 January 2011, 322 Jehovah’s Witness cardiac surgery patients were compared with 87,453 non-Jehovah’s Witness patients who also underwent cardiac surgery. Transfusions were given to 48,986 of the non-Jehovah’s Witness patients. When compared to matched patients who received transfusions, Jehovah’s Witnesses had better outcomes for the following:

- Myocardial infarction, 0.31% versus 2.8% ( $p = 0.01$ ).
- Additional operation for bleeding, 3.7% versus 7.1% ( $p = 0.03$ ).
- Prolonged ventilation, 6% versus 16% ( $p < 0.001$ ).
- Intensive care unit length of stay (15th, 50th, and 85th percentiles), 24, 25, and 72 hours versus 24, 48, and 162 hours ( $p < 0.001$ ).
- Hospital length of stay (15th, 50th, and 85th percentiles), 5, 7, and 11 hours versus 6, 8, and 16 days ( $p < 0.001$ ).

The 95% one-year survival in Jehovah's Witness patients was significantly higher than the 89% one-year survival of transfused patients ( $p = 0.007$ ). In contrast, 20-year survival was similar (34% vs. 32%;  $p = 0.90$ ). The results of this study suggest that nontransfused cardiac surgery patients are not at increased risk for morbidity or mortality compared to similar patients who receive perioperative transfusions.

Carson *et al.*<sup>69</sup> studied the impact of RBC transfusions on 30-day and 90-day postoperative mortality in a retrospective cohort study of 8787 consecutive patients, 60 years of age or older, who underwent surgery for the repair of hip fractures at 20 hospitals between 1983 and 1993. The "trigger" Hb for transfused patients was defined as lowest value prior to the first transfusion, and this was compared to the lowest Hb in nontransfused patients. The overall mortality rates were 4.6% ( $n = 402$ ) at 30 days and 9.0% ( $n = 788$ ) at 90 days. Postoperative transfusions were given to 3699 (42%) patients. Transfusions were given to 55.6% of patients with an Hb between 8.0 and 10 g/dL and to 90.5% of patients with an Hb less than 8.0 g/dL. After adjustment for trigger Hb, cardiovascular disease, and other risk factors for death, the odds ratios for 30-day (0.96; 95% CI, 0.74–1.26) and 90-day mortality (1.08; 95% CI, 0.90–1.29) showed no influence of postoperative RBC transfusions on mortality. There was also no difference in 30-day postoperative mortality when patients who received preoperative RBC transfusions were compared to those who did not (adjusted OR, 1.23; 95% CI, 0.81–1.89). It was concluded that perioperative RBC transfusions at an Hb transfusion trigger of 8.0 g/dL or higher in this elderly patient population did not influence 30-day or 90-day mortality. Assessment of the influence of RBC transfusions on mortality at an Hb transfusion trigger of less than 8.0 g/dL in this population could not be made because 90.5% of patients received RBC transfusions.

The Transfusion Requirements in Critical Care (TRICC) trial is an important randomized clinical trial designed to assess RBC transfusion-related outcomes in critically ill patients.<sup>70</sup> In this trial, 838 critically ill, normovolemic patients with an Hb of less than 9 g/dL within 72 hours of admission to an intensive care unit were randomized to either restrictive or liberal RBC transfusion strategies. There were 408 patients in the restrictive group and 420 patients in the liberal group. RBC transfusions were administered to restrictive-group patients for an Hb less than 7 g/dL in order to maintain the Hb between 7 and 9 g/dL. RBCs were administered to liberal-group patients for an Hb less than 10 g/dL in order to maintain the Hb between 10 and 12 g/dL. Thirty-day mortality was not statistically different between the restrictive and liberal RBC transfusion groups, although the raw mortality rate was lower in the restrictive group (18.7% vs. 23.3%,  $p = 0.11$ ). The mortality rates were also lower in the restrictive transfusion group (8.7% in the restrictive group vs. 16.1% in the liberal group,  $p = 0.03$ ) when less acutely ill patients (Acute Physiology and Chronic Health Evaluation II score of  $\leq 20$ ) as well as when patients younger than 55 years of age (5.7% in the restrictive group vs. 13.0% in the liberal group,  $p = 0.02$ ) were compared. The restrictive strategy group also had lower rates of myocardial infarction (0.07% vs. 2.9%,  $p = 0.02$ ), pulmonary edema (5.3% vs. 10.7%,  $p < 0.01$ ), and mortality during hospitalization (22.3% vs. 28.1%,  $p = 0.05$ ). The restrictive and liberal groups had similar mortality rates for patients with a primary or secondary diagnosis of cardiac disease (20.5% vs. 22.9%,  $p = 0.69$ ). The average Hb and the number of RBC units transfused were significantly lower in the restrictive group. It was concluded that the restrictive RBC transfusion strategy with a transfusion trigger of 7 g/dL was at least as effective as, and possibly superior to, a trigger of 10.0 g/dL

in critically ill patients, with the possible exception of patients with acute MI and unstable angina.

In a large study designed to determine the optimal perioperative RBC transfusion threshold, the Transfusion Trigger Trial for Functional Outcomes in Cardiovascular Patients Undergoing Surgical Hip Fracture Repair (FOCUS) studied 2016 patients with known cardiovascular disease or cardiovascular risk factors who were randomized to restrictive or liberal postoperative RBC transfusion groups following surgical repair of hip fractures.<sup>7</sup> All study patients were at least 50 years of age (mean age, 82 years), and all had a postoperative Hb less than 10 g/dL. Patients in the liberal RBC transfusion group received one RBC transfusion immediately after enrollment and additional RBC transfusions to maintain the Hb higher than 10 g/dL. Patients in the restrictive RBC transfusion group received RBCs if they developed symptoms warranting RBC transfusions or when the postoperative Hb dropped below 8 g/dL (even in the absence of symptoms). Symptoms triggering RBC transfusions were chest pain believed to be cardiac in nature, orthostatic hypotension or tachycardia unresponsive to fluid challenge, or congestive heart failure. The primary study outcome was mortality or the inability to walk 10 feet across a room without assistance at the 60-day evaluation point. Also evaluated was a combined outcome score comprising death, in-hospital MI, or unstable angina. Another secondary outcome was death beyond the 60-day evaluation point for any reason. For the primary study outcome, similar rates were observed for the liberal and restrictive RBC transfusion groups (35.2% vs. 34.7%, OR 1.01, 95% CI, 0.84–1.22). Similar rates for the liberal and restrictive groups were also found for the composite end point of death, in-hospital MI, or unstable angina (4.3% vs. 5.2%, OR 0.82, 99% CI, 0.48–1.42). When evaluated individually, the rate of MI was higher (although not statistically significant) in the restrictive group (3.8% vs. 2.3%, relative risk [RR] 1.65, 95% CI, 0.99–2.75). The death rates for the liberal and restrictive groups at 60 days were similar (7.6% vs. 6.6%, OR 1.17, 99% CI, 0.75–1.83). The FOCUS trial demonstrated that, when compared to a liberal postoperative RBC transfusion strategy at a threshold of 10 g/dL, a restrictive RBC transfusion strategy (threshold of 8 g/dL or for symptoms) was not associated with worse outcomes in hip fracture repair patients with cardiovascular disease or cardiovascular risk factors. The only exception to this conclusion might be related to the risk for MI where there was a marginally increased rate in the restrictive RBC transfusion group. The results of this trial, therefore, support the concept that, for patients with a postoperative Hb of 8.0 g/dL or higher, RBC transfusion decisions can be guided by symptoms rather than a higher Hb threshold.

Two landmark studies in cardiac surgery patients provide support for the concept that an Hb of 8 g/dL is a safe RBC transfusion trigger.<sup>71,72</sup> In the first study, 428 consecutive coronary artery bypass graft (CABG) patients were assigned to one of two postoperative RBC transfusion groups.<sup>71</sup> One group was transfused with RBCs for an Hb <8 g/dL, whereas the other group was transfused for an Hb <9.0 g/dL. No between-group differences in mortality or morbidity were seen. The restrictive group, however, received fewer RBC transfusions (0.9 vs. 1.4 RBC units/patient), which resulted in a reduction of 500 RBC transfusions per 1000 CABG procedures.

In the second cardiac surgery trial, 502 consecutive patients were randomized to liberal or restrictive RBC transfusion strategies.<sup>72</sup> Patients in the liberal strategy were transfused to maintain the Hct at or above 30% throughout surgery and the postoperative period. Patients in the restrictive strategy were transfused to maintain the

Hct at or above 24% throughout surgery and postoperative period. The combination of 30-day all-cause mortality, acute renal injury requiring dialysis or hemofiltration, acute respiratory distress syndrome (ARDS), and cardiogenic shock was chosen as the composite primary outcome. No difference in the rates of the composite end points was observed (liberal group 10% vs. restrictive group 11%). Overall, regardless of restrictive or liberal RBC transfusion strategy, the number of RBC transfusions was correlated with death and other clinical complications (HR 1.2 per transfused RBC unit). These two trials support a restrictive RBC transfusion approach (i.e., Hb or Hct transfusion triggers of 8 g/dL or 24%) in cardiac surgery patients.

A recent Cochrane review evaluated strategies for guiding allogeneic RBC transfusion decisions.<sup>73</sup> Nineteen randomized trials comparing lower (restrictive) versus higher (liberal) RBC transfusion thresholds involving a total of 6264 medical and surgical patients were identified. Most of the studies assessed outcomes in patients transfused at Hb thresholds between 7 and 10 g/dL, although specific thresholds differed between trials. The analysis showed that, when compared to liberal RBC transfusion strategies, restrictive transfusion strategies resulted in the following:

- A lower rate of receiving RBC transfusions (46% vs. 84%, RR 0.61, 95% CI, 0.52–0.72).
- 1.19 fewer RBC units transfused per patient.
- Trends suggesting lower 30-day mortality (RR 0.85, 95% CI, 0.70–1.03) and overall infection rates (RR 0.81, 95% CI, 0.66–1.00) (no differences seen in pneumonia rates, however).
- No differences in functional recovery, hospital length of stay, or intensive care length of stay.
- No differences in MI risk when all trials were evaluated (RR 0.88, 95% CI, 0.38–2.04).

The two largest studies included in the Cochrane analysis, the TRICC trial and the FOCUS trial, however, showed different results pertaining to the risk of MI associated with a restrictive RBC transfusion strategy.<sup>7,70</sup> The TRICC trial documented a lower risk of MI in the restrictive group (0.7% vs. 2.9%, RR 0.25, 95% CI, 0.07–0.88), and the FOCUS trial showed a higher (although not statistically significant) risk in the restrictive group (3.8% vs. 2.3%, RR 1.65, 95% CI, 0.99–2.75). Though some ambiguity in the literature exists regarding the best way to manage patients considered to be high risk for major perioperative cardiovascular events,<sup>72,74–76</sup> conservative approaches remain advocated, with careful attention to individual signs of end organ ischemia.

Regarding RBC transfusion decisions in patients with active bleeding, in a single center trial, 921 patients with acute upper GI bleeding were randomized to a restrictive (threshold Hb 7 g/dL) or a liberal (threshold Hb 9 g/dL) RBC transfusion strategy.<sup>6</sup> Excluded from the study were patients with acute coronary syndrome, massive bleeding, a history of peripheral vascular disease, a history of stroke, or an Hb more than 12 g/dL. Emergent upper endoscopy was performed on all patients, and endoscopic therapy was provided, as necessary. The patients in the restrictive RBC transfusion group as compared to the liberal group had the following symptoms:

- Lower percent of patients receiving RBC transfusions (49% vs. 86%).
- Fewer number of RBC units transfused (mean 1.5 units vs. 3.7 units).
- Fewer complications (40% vs. 48%).
- Lower percent of patients having subsequent bleeding (10% vs. 16%, hazard ratio [HR] 0.62, 95% CI, 0.43–0.91).

- Lower mortality due to uncontrolled bleeding (0.7% vs. 3.1%).
- Lower all-cause mortality (5% vs. 9%, HR 0.55, 95% CI, 0.33–0.92).

The results of this study suggest that a restrictive RBC transfusion strategy might be safe and associated with improved outcomes in actively bleeding, hemodynamically stable patients when there are no additional comorbidities that impart risk such as unstable coronary artery disease and when surgical intervention is promptly available. Similar findings in the TRIGGER study support this approach.<sup>77</sup>

## The utility of preoperative hemostatic testing

Preoperative hemostatic assessment is important to ensure that patients at-risk for abnormal procedure-related bleeding are identified, evaluated, and managed appropriately to minimize this risk. The components of this assessment include a clinical bleeding history, a physical examination, and often laboratory tests of hemostasis. Ideally, the preoperative hemostatic assessment utilizes an individual patient's clinical history and physical examination findings in the context of the bleeding risk associated with a planned procedure to guide whether preoperative laboratory screening is necessary, and if so, the extent of such testing. In many cases, however, the clinical history and physical examination do not reveal evidence of an increased perioperative bleeding risk. The performance of preoperative hemostatic testing in the absence of evidence from the history and physical examination assumes that the results have predictive value regarding the risk of perioperative bleeding and allow for measures to be taken to prevent or respond to bleeding should it arises. The question to be considered is whether this is a valid assumption, and therefore, a valuable approach when it comes to preoperative patient management. A 1983 review of preoperative hemostatic evaluation considered the question of whether clinical history alone is sufficient to determine perioperative bleeding risk.<sup>78</sup> The author presented four reasons in support of the performance of laboratory tests in addition to the clinical history:

- Testing “may protect against the doctor who fails to take an adequate history” (i.e., inadequate information is obtained to accurately assess perioperative bleeding risk).
- “Some patients give an unreliable history” (i.e., some patients do not know they bled excessively in association with previous surgery or trauma).
- Some patients have hemostatic abnormalities but have not been exposed to hemostatic challenges serious enough to reveal their presence (e.g., factor XI deficiency).
- Some patients who have “withstood surgery without abnormal bleeding may later acquire a hemostatic defect” (e.g., thrombocytopenia that developed after prior surgical interventions that has remained asymptomatic and has not been subjected to significant hemostatic challenges).

Although these arguments in favor of performing preoperative hemostatic screening tests seem to reflect common sense, the critical question that remains is: do the results of screening tests of hemostasis in the absence of clinical information suggestive of an increased bleeding risk provide relevant information regarding the likelihood of perioperative bleeding?

## Performance characteristic of the common coagulation screening tests

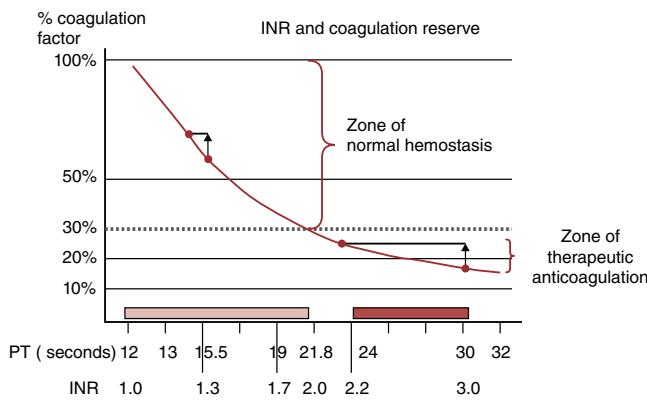
The international normalized ratio (INR) determined from the prothrombin time (PT) and the activated partial thromboplastin time (aPTT) are frequently used to guide hemostatic interventions,

including in the perioperative setting. Indeed, several societal guidelines specify INR values that may warrant treatment.<sup>22,79,80</sup> However, the interpretation of coagulation test results is fraught with challenges. Coagulation test result interpretation is hampered by a lack of clarity regarding the relationship of INR or aPTT values with in vivo coagulation factor concentrations and clinical hemostasis.<sup>20,23</sup> This uncertainty regarding the clinical significance of INR and aPTT results as gauges of true bleeding risk almost certainly contributes to the well-documented inappropriate use of plasma products.<sup>81,82</sup> To this point, it has been demonstrated in a variety of clinical settings that the INR and aPTT are poorly predictive of clinically significant hemostatic defects or periprocedural bleeding.<sup>83</sup> Furthermore, the administration of plasma often fails to produce an effective and sustained correction in coagulation factor content. Indeed, for mild-to-moderate prolongations of the INR (1.1–1.85), plasma administration frequently fails to “normalize” the coagulation test result.<sup>15,17</sup> The poor correlation between coagulation screening tests, in vivo coagulation factor levels, and clinical hemostatic dysfunction is perhaps best explained by the nonlinear relationship between the INR and serum coagulation factor levels<sup>84</sup> (Figure 39.1).

As noted in the Figure 39.1 legend, the curve is a generalized view that will vary for individual patients, which further affects the utility of the INR in evaluating the bleeding risk of a given individual patient. In settings such as liver disease or vitamin K depletion, when multiple mild coagulation factor deficiencies exist, the INR and the aPTT have been shown to overestimate the extent of coagulation defects.<sup>85</sup> This phenomenon was demonstrated by Burns *et al.*,<sup>86</sup> who observed that an in vitro plasma mixture containing a single coagulation factor at 75% activity and all the remaining factors at 100% activity produced normal INR and aPTT results. In contrast, an in vitro plasma mixture containing two coagulation factors at 75% activity and all the remaining factors at 100% activity resulted in prolonged INR and aPTT values.

### Clinical utility of preoperative hemostatic testing

Early publications proposed that preoperative assessment with the PT and the aPTT was a worthwhile effort to identify patients at-risk for bleeding.<sup>87,88</sup> Subsequent studies, however, have not provided



**Figure 39.1** The nonlinear relationship between the concentration of coagulation factors and coagulation test results. The concentration of factors (y-axis) is shown as a function of the international normalized ratio (INR) or prothrombin time (PT, in seconds). In general, a concentration of 30% is adequate for biologic hemostasis. Note that when starting at an elevated INR, a small increase in the concentration of factors will have a large impact on the measured INR. In contrast, an equivalent rise in the concentration of factors will have a negligible effect on mildly elevated INR values. The curve is a generalized view and will vary for individual patients.

strong evidence in support of preoperative hemostatic testing as a significant benefit to patient care.

A study of 480 patients with no evidence of abnormal bleeding and 139 patients with evidence of an increased risk of bleeding (by history and physical examination) evaluated the performance of the PT and aPTT done prior to surgery.<sup>89</sup> Abnormal results were obtained in 13 (2.7%) patients with no evidence of a bleeding risk and in 25 (18.0%) patients with evidence of an increased risk. One of the thirteen patients without evidence of a bleeding risk had to return to the operating room for arterial bleeding. The authors stated that the prolonged PT or aPTT was of “no apparent clinical importance” in the remaining 12 patients, and the value of preoperative coagulation testing is obscured by a high incidence of apparently false positive results. Furthermore, the authors stated that 2.28% of the healthy population would be expected to have a prolonged PT or aPTT if the reference range extended to two standard deviations above the mean normal value. Therefore, the 2.7% incidence of abnormal PT and aPTT results obtained in patients with no prior evidence of bleeding in this study could be explained by random variation in healthy subjects.

The usefulness of a protocol for the selective ordering of 12 preoperative tests was evaluated in 3866 consecutive surgery patients during a one-year period.<sup>90</sup> Test selection was based upon patient clinical status and the type of procedure planned. Based upon the protocol, the aPTT, PT, platelet count, and bleeding time were not ordered for 75%, 76%, 92%, and 99% of the patients. The value of the omitted tests was assessed relative to the occurrence of intraoperative and postoperative events. Of the nonordered tests, 0.4% were considered “potentially useful” and 0.2% “truly useful” according to the opinion of the anesthetists. Only in one case it was determined that a nonordered test (platelet count) would have been “truly useful” for patient care. This protocol for selective performance of preoperative testing was believed to have minimal adverse patient consequences, while significantly reducing test utilization.

Two prospective studies that evaluated whether preoperative hemostatic testing was justified in major surgery patients without an abnormal bleeding history were described in a single publication.<sup>91</sup> In the first study, a preoperative platelet count, bleeding time, PT, and aPTT were performed in 111 patients without an abnormal bleeding history. Mild thrombocytopenia was detected in one patient (platelet count:  $130 \times 10^9/L$ ), and eight additional patients had a prolonged aPTT. None of these patients experienced abnormal perioperative bleeding. The second study identified 49 of 1872 patients with higher than anticipated perioperative transfusion requirements over a four-month period. None of these patients had demonstrable hemostatic abnormalities, and the transfusions were given to address technical problems. The authors concluded that, in the absence of history and physical findings suggestive of a bleeding problem, preoperative testing for hemostatic testing appears unnecessary. Many similar clinical studies have reached consistent conclusions that routine preoperative hemostatic testing in the absence of clinical information suggestive of an increased bleeding risk is not useful.<sup>92-99</sup>

A systemic review of routine preoperative hemostatic testing disclosed incidences of abnormal results for the bleeding time, the PT, and the aPTT of 3.8%, 4.8%, and 15.6%, respectively.<sup>100</sup> The abnormal test results rarely led to changes in patient management. The authors concluded that the evidence did not support a policy of routine preoperative hemostatic testing for all patients. Another systemic review reported on the predictive value of the patient bleeding history and unselected coagulation testing for abnormal

perioperative bleeding.<sup>101</sup> The PT and aPTT were found to be poor predictors of bleeding. It was recommended that a bleeding history be performed in all preoperative patients, and if that history is negative, then coagulation testing is not required. If the bleeding history is positive or if clinical conditions that clearly impart an increased risk of perioperative bleeding are present (e.g., liver disease), then a comprehensive hemostasis assessment should be performed prior to surgery.

One preoperative laboratory screen that may be of use is a platelet count. A recent observational cohort study of adult elective surgery patients assessed the clinical significance of preoperative thrombocytopenia.<sup>26</sup> Preoperative thrombocytopenia was associated with significantly increased odds for intraoperative transfusions in anemic (odds ratio 2.60;  $p < 0.001$ ) and nonanemic patients (odds ratio 3.39;  $p < 0.001$ ). Preoperative thrombocytopenia, therefore, seems to correlate with intraoperative transfusion requirements.

In addition to routinely available tests such as the PT, aPTT, and platelet count, other assays have been evaluated for predicting the risks of perioperative bleeding. In the absence of a history suggestive of a bleeding tendency, the bleeding time has not been shown to be useful as a preoperative screening test (normal and abnormal results have poor predictive value) and it has not been recommended to serve in this capacity.<sup>102,103</sup> Although fairly rapid tests have been developed to measure native and drug-induced platelet dysfunction (e.g., PFA-100, Plateletworks<sup>®</sup>), because of questionable abilities to predict bleeding,<sup>104</sup> these assays are likewise not recommended as screening tests for perioperative bleeding risk.

Consistent with the above literature, the American Society of Anesthesiologists Task Force on Preanesthesia Evaluation has concluded that routine preoperative tests (tests intended to detect a potentially significant abnormality in an asymptomatic patient) do not provide an important contribution to the perioperative assessment and management of the patient.<sup>105</sup> Preoperative laboratory testing, including coagulation testing, should be ordered only as necessary based upon information obtained from the medical record review and the patient history and physical examination in the context of the types of procedure and anesthesia planned for an individual patient.

In conclusion, based upon the evidence currently available, with the possible exception of knowing a patient's preoperative platelet count, it seems reasonable to conclude that preoperative screening with hemostatic tests should be restricted to patients with a family history of an inherited bleeding disorder (in conjunction with a hemostasis consult with perioperative recommendations for management), a history of abnormal bleeding or a family history of abnormal bleeding (such patients should undergo a comprehensive preoperative hemostatic evaluation), and those with underlying conditions associated with an increased risk of bleeding such as chronic liver disease.

### **Point-of-care viscoelastic assays instead of preoperative hemostatic testing**

With growing concern regarding the ability of PT/INR, aPTT, and platelet function tests to predict bleeding events, in addition to their frequently unacceptable turnaround times for results, recent focus has shifted toward the use of modern viscoelastic assays such as thromboelastography (TEG) and rotational thromboelastometry (ROTEM).<sup>106–109</sup> These tests appear able to provide a more accurate and timely point-of-care assessment of thrombus formation in whole blood.<sup>110</sup> Although used most extensively in cardiac surgery

and liver transplantation, early data suggest that these tools may better guide individual transfusion decisions and reduce overall blood product exposure.<sup>111,112</sup> Indeed in 2011, Görlinger *et al.*<sup>113</sup> found that the use of ROTEM in cardiac surgical patients resulted in increased utilization of specific factor concentrates, while simultaneously producing an overall reduction in blood product transfusions, without any associated increase in morbidity or mortality. The use of test systems, such as point-of-care viscoelastic assays, to monitor and manage hemostatic status in surgical patients, deserves further investigation as a potentially useful adjunct or alternative to preoperative hemostatic testing.

### **Intraoperative use of the activated clotting time (ACT)**

The activated clotting time (ACT) is the preferred test for intraoperative monitoring of anticoagulation because it has a rapid turnaround time and a reportable range for patients receiving high-dose heparin during cardiopulmonary bypass. Although sensitive to factors in the intrinsic coagulation cascade and unfractionated heparin, the ACT can also be affected by antithrombin and fibrinogen levels, low platelet or red cell counts, quantitative platelet abnormalities, hypothermia, lupus anticoagulants, and antiglycoprotein IIb/IIIa medications.<sup>114–116</sup>

Several ACT devices are currently approved in the United States and each is unique regarding sample type, activator used, and clot detection mechanism.<sup>114</sup> Several studies have demonstrated that the ACT values obtained with these various devices are not interchangeable.<sup>114,117–119</sup> In one study, the Hemochron ACT and the i-STAT ACT were directly compared, and the mean difference between the two methods was  $24 \pm 101$  seconds.<sup>120</sup> This study suggested, for anticoagulation monitoring during cardiopulmonary bypass, a Hemochron ACT of  $>480$  was correlated with an i-STAT ACT of  $>400$  seconds. Similarly, Maslow *et al.* compared the i-STAT ACT to the Hemochron ACT and found that the i-STAT consistently had lower values when the ACT was  $>180$  seconds, and this difference grew as heparin levels increased.<sup>121</sup> In contrast, Lewandrowski *et al.* compared the iSTAT ACT to the Medtronic ACT Plus and found a correlation coefficient of 0.94.<sup>122</sup> Additionally, Carroll *et al.* recently demonstrated a complete lack of correlation of the Hemochron ACT with Anti-Xa levels after heparinization for cardiopulmonary bypass.<sup>123</sup>

With such wide variation in response to high-dose anticoagulation, it seems prudent for each practice to develop a well-validated testing strategy and anticoagulation goals for each perioperative need. The most recent guidelines from the Society of Thoracic Surgeons, The Society of Cardiovascular Anesthesiologists, and The American Society of ExtraCorporeal Technology on anticoagulation for cardiopulmonary bypass suggest a target ACT of  $>480$  seconds, while specifically highlighting the need to incorporate instrument bias and technology into this target.<sup>124</sup>

### **Management of perioperative anticoagulation and antiplatelet therapies**

Management of anticoagulation in the perioperative period can be challenging. Clinicians must weigh the risk of thromboembolic complications versus procedural bleeding and determine the best overall strategy for optimizing outcomes (e.g., passive anticoagulant interruption, versus active reversal with vitamin K or coagulation factor replacement, versus bridging with heparin therapy and/or IVC filter placement). Currently, there is little strong evidence in

the literature from randomized clinical trials to provide definitive recommendations, and many of the existing guidelines are based upon expert opinion. In clinical practice, thromboembolic and bleeding risks depend upon both patient and procedural characteristics. Therefore, determining the optimal management strategies will depend upon specific individual risk factors and procedural details. For patients with mechanical heart valves, the risk of thromboembolism is highest in the first three months after valve surgery, particularly when considering the mitral valve.<sup>125</sup> In this instance, simple measures such as avoiding elective surgery during this time frame can help to prevent unnecessary interruptions in anticoagulation that would place the patient at greatest risk of thromboembolic complications.

In 2012, the American College of Chest Physicians (ACCP) recommended careful planning of elective procedures with cessation of warfarin seven days before surgery thus allowing INR to gradually normalize. This approach avoids the need for vitamin K administration that may prolong achievement of an effective antithrombotic state with warfarin.<sup>126</sup> This guideline also stressed the importance of regular INR checks during this time to allow for initiation of bridging anticoagulation (either with heparin or low-molecular-weight heparin) until the time of surgery. An INR check is specifically endorsed on the day prior to surgery to allow for low-dose oral vitamin K supplementation if clinically indicated.

For patients with atrial fibrillation (AF), the risk of thromboembolism may be best characterized using existing risk stratification scores such as the CHADS2 or CHADS2-VASc.<sup>127</sup> However, it must be recognized that these scores have not been specifically validated for predicting risk of thromboembolism in the postoperative period. Of note, the Randomized Evaluation of Long-Term Anticoagulant Therapy (RE-LY) trial recently evaluated thromboembolic risk in patients with nonvalvular AF.<sup>128</sup> This investigation found a rate of thromboembolic complications (including stroke, pulmonary embolus, or cardiovascular death) of 1.2%, with the greatest risk seen in patients undergoing urgent surgery.

Finally, for patients whose indication for anticoagulation is thromboembolic disease, risk of subsequent thromboembolism with anticoagulant interruption is believed dependent on the time from the initial thromboembolic event. Where feasible, deferring surgery and accompanying anticoagulation interruption may be of benefit.<sup>129,130</sup> If emergency surgery is required, bridging with heparin is often endorsed.<sup>131</sup> However, further randomized trials are warranted in this area to determine the optimal perioperative management of anticoagulation in this setting.

For patients taking direct oral anticoagulants (DOACs) including factor Xa inhibitors such as rivaroxaban and apixaban, or direct thrombin inhibitors such as dabigatran, evidence guiding perioperative interruption is more limited. Based on their elimination half-lives (7–11 hours for factor Xa inhibitors and 12–14 hours for thrombin inhibitors in patients with normal renal function),<sup>132</sup> these agents are typically stopped 2–3 days ahead of elective surgery to minimize surgical bleeding.<sup>133,134</sup> This interval may be extended in particularly high-risk operations or shortened in particularly low-risk procedures.

Importantly, coagulation studies are not routinely indicated in DOAC-treated patients. For example, while the aPTT has been advocated to assess coagulation status in patients treated with direct thrombin inhibitors,<sup>135</sup> this method lacks sufficient evidence to make strong recommendations for its use. Indeed, for the most part, the INR and aPTT have not been validated for the assessment of coagulation status in patients treated with any DOAC.<sup>136</sup>

Nonetheless, for emergent operations or in patients presenting with a bleeding episode, assessment of coagulation status may be desired. Specific factor Xa activity assays, if available, may help to guide management of factor Xa inhibitors.<sup>137</sup> If factor Xa assays are not available, the PT may show prolongation in patients taking factor Xa inhibitors; however, significant interassay variability exists so PT and/or INR measurements are not strongly advocated in this setting.<sup>136</sup> If available, ROTEM can be used to assess coagulation status as factor Xa inhibitors will produce a dose-dependent prolongation of R and K time with no change in maximal amplitude.<sup>137</sup> At present, dabigatran anticoagulation may be best measured using the thrombin time or dilute thrombin time.<sup>138</sup> Overall, while prolonged coagulation times may point to residual anticoagulation effect in a patient treated with DOACs, normal coagulation test results cannot be considered evidence of normal coagulation; therefore, treatment should not be delayed awaiting the results coagulation testing.

If rapid correction of anticoagulation with rivaroxaban is required, prothrombin complex concentrates (PCCs) may be effective.<sup>139,140</sup> Furthermore, a specific reversal agent for factor Xa inhibitors (andexanet alfa) has recently been approved for the management of life-threatening bleeding.<sup>141</sup> Similarly, idarucizumab is a monoclonal antibody that can be used for emergency reversal of dabigatran.<sup>142</sup> Each of these reversal agents, however, carries the risk of thrombosis.<sup>143</sup> Alternatively, in the setting of acute bleeding, dabigatran may be successfully filtered by hemodialysis,<sup>144</sup> offering a unique method for reversing anticoagulation. Additionally, the use of oral activated charcoal may be considered for recently ingested oral anticoagulants, while antifibrinolytics, such as tranexamic acid, may also be effective. Ongoing randomized clinical trials are needed to determine the best way to reverse DOACs.<sup>145</sup> As with warfarin, decision-making regarding interruption of anticoagulation should weigh the risk of thrombosis versus the risk of bleeding for each individual patient and procedure. Due to the rapid onset of action of these agents relative to warfarin, bridging with heparin is often not required when reinitiating DOAC therapy postoperatively.

With an aging population, the use of antiplatelet agents, such as aspirin and clopidogrel, is widespread to manage the risk of major cardiac and vascular thromboembolic events. As with anticoagulants, similar risk to benefit assessments must be made ahead of elective surgery.<sup>146</sup> Both aspirin's and clopidogrel's antiplatelet properties last for the lifespan of the platelet due to their irreversible inhibition of cyclooxygenase 1/2 and the platelet adenosine diphosphate (ADP) P2Y12 receptor, respectively.<sup>147</sup> Due to concern for increased perioperative bleeding,<sup>148–150</sup> antiplatelet agents have historically been withheld for 7–10 days preoperatively. Most recently, this notion was supported by findings from the Perioperative Ischemic Evaluation (POISE-2) randomized controlled trial in which aspirin administration was not associated in any significant decrease in risk of death or nonfatal myocardial infarctions; however, major perioperative bleeding was increased.<sup>151</sup> Of note, patients with recent coronary stents and those taking thienopyridines or Ticagrelor were excluded from this study, raising concern about the generalizability of these findings. Moreover, prior observational studies have suggested that early perioperative aspirin use may reduce both death and ischemic complications in patients undergoing cardiac surgery.<sup>152,153</sup> Similarly, Biondi-Zoccali *et al.*<sup>146</sup> noted a threefold increase in thrombotic complications in moderate- to high-risk patients in whom aspirin was withheld perioperatively. This controversy has led to a re-exploration of the risks and benefits of antiplatelet therapies in the perioperative setting. Indeed

the 2012 ACP Guidelines suggest continuing aspirin around non-cardiac surgery in those deemed to be at moderate to high risk of cardiovascular events (with aspirin cessation for 7–10 days in those at low risk).<sup>126</sup> Of note, these guidelines were published ahead of the POISE-2 trial findings becoming publicly available.

Considering the ongoing equipoise, individual assessments of risk-to-benefit ratios are required for each patient. In general, currently accepted evidence is in favor of withholding antiplatelet agents when prescribed only for primary prevention (low thrombotic risk) and/or when bleeding risk may be life threatening, for example with intracranial or ophthalmic surgery. Conversely, patients with bare metal stent placement within six weeks, or drug eluting stent within six months, should not routinely discontinue antiplatelet therapy for elective surgery. For those patients requiring urgent surgery with an intermediate or high risk of bleeding, options include discontinuation of clopidogrel with continuation of aspirin or discontinuation of both agents with bridging therapy around the time of surgery.

### **Conservative versus liberal transfusion practices**

Historically, blood products have been administered to treat patients with both life-threatening hemorrhage and those with mild derangements in laboratory parameters. For the latter, the assumption has been that the normalization of these laboratory abnormalities would improve patient outcomes. Indeed, in the middle of the last century, it was recommended that RBCs be transfused to maintain an Hb concentration >10 g/dL based upon the assumption that increasing the patient's Hb would improve tissue oxygenation.<sup>154</sup> This became a longstanding, accepted standard of practice for surgical patients for many years. Similarly, even now, plasma is often administered to correct modest abnormalities in the INR in the absence of active bleeding.<sup>17,21,155–158</sup> Importantly, the perioperative setting remains a leading environment for the administration of blood component therapies. This makes the perioperative environment a prime setting to improve blood product utilization moving forward. Additionally, recent evidence suggests that massive transfusion protocol activations are more common in the perioperative setting than in any other area of care delivery.<sup>159</sup>

A substantial body of literature is now available evaluating RBC transfusion practices in various clinical situations. As outlined above, multiple RBC transfusion studies have demonstrated the safety and effectiveness of more restrictive transfusion practices.<sup>6,7,70,160–163</sup> A more restrictive approach has been shown safe in the setting of liver resection<sup>160</sup> as well as in patients with sepsis,<sup>161</sup> gastrointestinal bleeding,<sup>6</sup> and traumatic brain injury.<sup>162</sup> A growing body of literature also suggests safety in the setting of cardiac surgery<sup>163</sup> and acute coronary syndrome.<sup>164</sup> Additionally, Yu *et al.*<sup>165</sup> identified a positive dose-response relationship between the number of blood products transfused and risk of adverse outcomes in patients undergoing cardiac surgery. In aggregate, the prevailing conclusion of available clinical trial literature suggests that liberal RBC transfusion practices do not offer a morbidity or mortality advantage in stable, nonhemorrhaging patients. To the contrary, most observational research studies have suggested that restrictive transfusion practices may be associated with a higher probability of survival and fewer complications such as infection, acute respiratory distress syndrome, cardiogenic shock, acute kidney injury, and multiorgan dysfunction.<sup>166–168</sup>

Despite the growing evidence supporting restrictive RBC transfusion strategies, widespread implementation of conservative transfusion practices in the operating room environment has not been without challenges. Although many societal guidelines and patient blood management programs now advocate restrictive transfusion strategies,<sup>169–174</sup> transfusions continue to be frequently administered outside of published guidelines. In an effort to improve the intraoperative transfusion practice, the American Society of Anesthesiologists' (ASA) practice guidelines recommend (1) periodic visual assessment of the surgical field and communication with the surgical team, (2) continuous monitoring for evidence of inadequate perfusion and oxygenation of vital organs, (3) use of standard methods for quantitative measurement of blood loss, including checking suction canisters, surgical sponges, and surgical drains, and (4) implementation of a blood management protocol with the determination of whether hemoglobin concentrations between 6 and 10 g/dL justify or require red blood cell transfusion based on potential or actual ongoing bleeding (rate and magnitude), intravascular volume status, signs of organ ischemia, and adequacy of cardiopulmonary reserve.<sup>171</sup> Furthermore, these guidelines endorse the use of blood recovery techniques and permissive hypotension to decrease blood loss, when appropriate. Outside of massive transfusion protocols, it is also recommended that RBC transfusions occur one unit at a time, with subsequent evaluation of the clinical response before determining the need for additional RBC transfusions.<sup>171</sup>

### **Blood avoidance techniques**

Considering the potential for transfusion-related adverse events, the limited supply of blood components, and the occasional religious objection to blood transfusion, the avoidance of transfusion, in some situations, may represent a more safe, cost-effective, and morally acceptable option. Although blood transfusion is not always avoidable, several interventions may be employed to facilitate the avoidance of blood component therapies. Prior to elective surgery, phlebotomy should be limited to only tests essential for clinical decision making.<sup>175</sup> The rationale for limiting phlebotomy is based on a growing body of data outlining the prevalence and severity of iatrogenic hospital-acquired anemia (HAA) and its effect on patient outcomes.

A large observational study by Koch *et al.*<sup>176</sup> describes an incidence of HAA of 74%. Although felt to be multifactorial, phlebotomy has been highly correlated with HAA. Data suggest a decrease in Hb of 0.08 g/dL for every 1 mL of blood drawn<sup>177</sup> or an 18% increased risk of developing moderate-to-severe HAA with every 50 mL of blood drawn.<sup>178</sup> Equally, if not more important, HAA has been associated with increased hospital length of stay, hospitalization costs, and in-hospital mortality.<sup>176</sup> Minimizing phlebotomy volumes has been previously demonstrated to reduce transfusion requirements in critically ill patients and should be a standard measure employed as part of blood avoidance.<sup>179</sup> Postoperatively, iatrogenic anemia can likewise be minimized through the judicious use of phlebotomy.

In certain populations, particularly patients with preexisting anemia, and in those who refuse transfusion, erythropoietin and iron supplementation should be considered, especially if preoperative autologous blood donation will take place.<sup>180</sup> In one study, preoperative IV iron therapy prior to major abdominal surgery was associated with fewer blood transfusions, a shorter hospital stay, increased iron stores, and a higher mean hemoglobin four weeks

after surgery.<sup>52</sup> If safe to do so, cessation or avoidance of blood thinning drugs—including nonsteroidal anti-inflammatory drugs, anti-platelet agents, and anticoagulants—is also prudent.<sup>181</sup>

Intraoperatively, there should be meticulous attention to hemostasis and rapid control of hemorrhage.<sup>176</sup> Permissive hypotension may be considered as a method to minimize precipitous blood loss.<sup>182,183</sup> To this end, a recent randomized trial evaluating resuscitation of trauma patients demonstrated that patients resuscitated to a mean arterial pressure (MAP) of 50 mmHg required significantly fewer blood products and reduced crystalloid resuscitation volumes compared with those resuscitated to an MAP goal of 65 mmHg.<sup>182</sup> These patients also had a lower early postoperative mortality and experienced coagulopathy less frequently and with a lesser severity. A subsequent meta-analysis of 1158 patients from five RCTs in adult trauma demonstrated a small survival benefit (OR 0.7, 95% CI, 0.53–0.92), fewer transfused blood products, and lower estimated blood loss in permissive hypotension; however, the studies were rated as low to moderate quality and were in general under-powered.<sup>184</sup> More robust evidence is needed before recommending this as a viable practice in critically ill patients.

Other measures to minimize intraoperative blood loss include the avoidance of hypothermia,<sup>185,186</sup> and when possible, cell salvage mechanisms should be utilized to facilitate rapid autologous transfusion. Although it has been well established that coagulation worsens with hypothermia, there is ongoing enthusiasm and controversy regarding targeted temperature management to improve neurologic outcomes after cardiac arrest.<sup>187</sup> Although this remains controversial, transfusion experts should be aware of its continued use as it may conflict with the goal of maintaining normothermia to promote hemostasis.

Modern cell salvage technologies are unique in their ability to provide rapid, large volumes of blood to patients without the need for preoperative donation or hemodilution that may be limited by patient tolerance and hemodynamic reserve. These methods have been most extensively used in hepatic,<sup>188,189</sup> cardiac,<sup>190–192</sup> obstetrics,<sup>193,194</sup> and orthopedic<sup>195</sup> surgeries and have been shown to be cost-effective and safe. In recent decades, the use of cell salvage in patients with active malignancy and bacteremia has been questioned due to the concern of hematogenous spread.<sup>196,197</sup> Despite a recent meta-analysis of 4354 patients from nine RCTs in a variety of cancers showing no differences in five-year survival, five-year disease-free survival, or five-year recurrence rates,<sup>198</sup> there is no consensus on cell salvage in surgical oncology and its use should likely be considered a relative contraindication.

Although not broadly utilized, acute normovolemic hemodilution (ANH) remains a viable option for many types of surgery by minimizing the loss of red cell mass through dilution and sequestering coagulation factors and platelets until after surgical hemostasis is achieved.<sup>199–201</sup> A recent meta-analysis in cardiac surgery including 2439 patients from 29 RCTs demonstrated that the use of ANH was associated with fewer red cell transfusions and less post-operative blood loss;<sup>202</sup> however, a detailed assessment of the effects of ANH vs. controls on coagulation parameters after cardiac surgery was modest at best.<sup>203</sup> Regardless, ANH may be considered as a component of a comprehensive patient blood management protocol.

Although aprotinin is no longer approved for use by the US Food and Drug Administration, lysine analogs such as  $\epsilon$ -aminocaproic acid and tranexamic acid are advocated by some, including the Society of Thoracic Surgeons and Society of Cardiovascular

Anesthesiologists (STS/SCA), to minimize total blood loss.<sup>204</sup> Efficacy for these agents is most robust in the settings of cardiac surgery, orthopedic surgery, trauma resuscitation, and in postpartum hemorrhage, particularly when used in the first few hours of resuscitation.<sup>205,206</sup> In addition, topical hemostatic agents may be utilized (see below).

Over the past several decades, there has been a growing emphasis placed on blood conservation and transfusion avoidance techniques due to the association with improved patient outcomes and lower complication rates.<sup>207</sup> This emphasis has recently been amplified due to recent crisis-related blood shortages due to disasters and the COVID-19 pandemic. Consequently, multiple international societies have recommended PBM campaigns as a component of best practice to address these situations.<sup>208</sup>

## Topical hemostatic therapies

Topical hemostatic therapies offer a variety of options to manage localized bleeding that could prevent the need for transfusion. While suture ligation of a damaged vessel remains the single most effective method for hemostasis, this is not always technically feasible. In this situation, the use of electrocautery, sclerosing agents, argon laser beam coagulation, and direct packing and compression are common practices.<sup>209</sup> Topical hemostatic therapies provide additional options to achieve local hemostatic control of bleeding sites during surgery. There are two general categories of topical hemostatic therapies: physical agents, which provide an environment where hemostasis can occur, and biologically active agents, which actively enhance hemostatic mechanisms at bleeding sites. They are used to treat bleeding in many locations including the following:<sup>210</sup>

- Areas close to nerves
- Vital structure regions that should not sustain cautery-induced injury
- Suture holes in vessels or grafts
- Cut surfaces of bone
- Pleural or peritoneal surfaces
- Cut surfaces of solid organs<sup>211–213</sup>
- The nose<sup>214,215</sup>
- Dental extractions<sup>216</sup>

## Physical agents

Physical agents consist of materials that foster hemostasis through activation of platelets and the extrinsic coagulation pathway while also serving as the structural foundation for thrombus deposition. As the thrombus grows at the site of physical agent placement, pressure is exerted locally, which can tamponade bleeding vessels. There are a number of physical hemostatic agents that can be used topically including the following:

- Bone wax
- Ostene
- Gelatin preparations
- Microfibrillar collagen
- Microporous polysaccharide spheres
- Oxidized regenerated cellulose
- Hydrophobic light-activated adhesive (investigational)

Bone wax and Ostene can be used to control bleeding from cut surfaces of medullary bone, which can occur during neurosurgery and orthopedic procedures and after median sternotomy. Bone wax stops bleeding by physically occluding blood vessels within bone.

Bone wax has been implicated as a risk factor for surgical site infections and in the generation of granulomas, resulting in interference with bone healing.<sup>217,218</sup> Ostene also occludes blood vessels within bone; however, it does not remain in the wound for long term and persist as a foreign body. Ostene has been reported to be associated with better bone healing in comparison to bone wax.<sup>219,220</sup>

Gelatin (e.g., Gelfoam and Surgifoam) preparations provide a meshwork that entraps platelets and coagulation factors, thus providing an environment that fosters clot formation.<sup>221,222</sup> Gelatin sponge preparations can absorb a significant volume of blood or fluid (40–45 times its weight) and they can expand in size by up to 200%. This creates a tamponade effect that also helps to achieve hemostasis. Pressure is maintained (often for several minutes) on the gelatin sponge after placement to secure hemostasis. The sponges can be left in place as they completely resorb in four to six weeks. However, gelatin use has been associated with granulomas, fibrosis, increased incidence of infection, and clot disruption from sponge removal.<sup>221,223</sup>

Microfibrillar collagen (e.g., Avitene) serves as a scaffold for the activation of platelets and subsequent clot formation. Microfibrillar collagen can be applied to sites of bleeding as a powder or as a foam sheet, and it will fully resorb within three months.<sup>210</sup> Indications for use of microfibrillar collagen include neurosurgery, urology, and vascular surgery. Microfibrillar collagen should not be allowed to enter cell salvage systems because the filters do not effectively block their passage; therefore, the fibers can be reinfused into the patient along with the salvaged product and pose a risk for embolization and DIC.

Microporous polysaccharide spheres (MPS) (e.g., Arista) absorb water, which serves to regionally concentrate platelets and coagulation factors, promoting clot formation. MPS are available as a powder. The powder is generously dispersed over bleeding sites following pressure with a dry surgical sponge, which is used to achieve as dry a surface as possible. After distribution of the spheres, pressure is again applied with a surgical sponge to achieve hemostasis. MPS rapidly absorb within 48 hours and they do not promote infections or cause foreign body responses.<sup>224</sup> The spheres are approved for use in neurosurgery, and they have also been used in cosmetic surgery. MPS can be used in patients undergoing cell salvage procedures that utilize cell washing and 40-μm infusion filters.

Oxidized regenerated cellulose (ORC) is sterile, dry mesh (e.g., Surgicel) that can be directly applied to sites of bleeding. ORC is typically used to control bleeding at the cut surfaces of solid organs, at vascular anastomoses, and at pelvic and retroperitoneal surfaces after lymphadenectomy. It is pliable and can be delivered to bleeding sites laparoscopically.<sup>225,226</sup> Full absorption of a single sheet of ORC is expected to occur in approximately 14 days.<sup>223</sup> Adhesions and infections, however, have occurred in association with residual ORC. Unabsorbed ORC was identified as a risk factor in a study of 360 patients with postoperative pelvic abscesses.<sup>227</sup> In some of these patients, nonresorbed ORC was identified during laparotomy more than 1 year after application.

Hydrophobic light-activated adhesive (HLAA) is a topical hemostatic agent that is being investigated.<sup>228</sup> It is a prepolymer that is mixed with a substance that can be photoactivated. HLAA is applied to bleeding sites as a liquid and, upon exposure to ultraviolet light, the material develops crosslinks and converts into a tissue glue that resists dissolution in the presence of blood or other fluid environments.

## **Biologically active agents**

Biologically active topical hemostatic agents enhance hemostasis at the site of application. Agents of this type include the following:<sup>210</sup>

- Topical thrombin
- Fibrin sealant
- Tranexamic acid

Topical thrombin is available as a lyophilized powder that requires reconstitution prior to use.

In diffuse bleeding, such as when large portions of pleural and peritoneal surfaces are oozing, topical thrombin can be delivered via a sprayer. When bleeding is more localized, it can be delivered to the site with a needle and syringe. Topical thrombin in combination with gelatin preparations (sponge or granules) takes advantage of the combination of the biological hemostasis enhancement properties of thrombin with the physical configuration of the gelatin, which serves as a scaffold for clot formation.<sup>229,230</sup> This combination has been effective in achieving hemostatic control of suture holes in vascular grafting procedures. Hemostatic control may be more rapid when thrombin is used in combination with gelatin granules (e.g., FloSeal, Surgiflo) as opposed to thrombin-soaked gelatin foam.

Currently, bovine, human, and recombinant thrombins are available. The use of bovine-derived thrombin has been associated, in a minority of patients, with the development of antibodies directed against bovine coagulation proteins in the preparation.<sup>231</sup> These antibodies include factor V antibodies and thrombin antibodies. In some patients, these antibodies can crossreact with human coagulation proteins, resulting in the development of a coagulation inhibitor. In some of these patients, the inhibitor can be clinically significant and represents a serious adverse effect of bovine thrombin. Because human-derived and recombinant preparations of topical thrombin are now widely available, the necessity to use of bovine thrombin for hemostatic control has significantly decreased.<sup>232</sup>

Fibrin sealant preparations typically consist of two separate solutions, one containing fibrinogen and factor XIII and the other thrombin and calcium.<sup>233</sup> A fibrin clot forms when these two solutions are applied simultaneously. The fibrin sealants are applied as a liquid, spray, or dressing, and they facilitate the control of bleeding from cut tissue surfaces and vascular anastomoses.<sup>234–236</sup>

Tranexamic acid is an antifibrinolytic agent that competitively blocks the binding of lysine to lysine binding sites on plasminogen molecules, resulting in the inhibition of the conversion of plasminogen to plasmin.<sup>237</sup> Topical use of tranexamic acid to achieve local hemostatic control of bleeding surfaces has been used in settings such as breast, cardiac, head and neck, and orthopedic surgery.<sup>238–242</sup> Evidence regarding the safety and efficacy of topical tranexamic acid is still rather preliminary, and more data will be required to better define its role in the management of surgical bleeding.

## **Topical hemostatic selection**

The selection of a particular topical hemostatic is affected by the site and extent of bleeding, the local availability of the various agents, the usage experience and preferences of practitioners, and cost.<sup>243</sup> In general, compared to nonbiologically active physical topical agents, biologically active agents such as thrombin or fibrin sealant tend to be more effective for more rapid bleeding and when a coagulopathy and/or defibrinated fluids are present.

## Perioperative transfusion guidelines

### Red blood cells: indications, threshold, and dose

Evidence-based indications for RBC transfusion in nonbleeding, hemodynamically stable patients have been revised and subsequently reaffirmed many times in recent decades.<sup>173,174,244</sup> Unfortunately, few of these investigations provide meaningful guidance for surgical populations, particularly when clinically significant active bleeding is encountered.<sup>170,171,204</sup> In this specific clinical circumstance, the unwavering take home message is that appropriate RBC transfusion practices require more than an arbitrary hemoglobin-based transfusion trigger. Rather, the decision to transfuse RBCs in this context should be based upon patient-specific symptoms (e.g., hemodynamic instability or evidence of tissue hypoxia) and details relating to the surgical course (e.g., expected ongoing blood loss).<sup>170,171,204</sup> To this point, the most compelling indication for intraoperative RBC transfusion remains acute hemorrhage with associated hemodynamic instability or evidence of inadequate oxygen delivery. In contrast, generalized mucosal oozing, gradual blood loss over a prolonged period of time, and asymptomatic anemia related to chronic disease or hemodilution are perhaps more frequent clinical scenarios that prompt difficult decisions regarding the appropriateness of intraoperative RBC administration. In 2006, the ASA's taskforce on perioperative blood transfusion was among the first to encourage more conservative transfusion practices for surgical populations.<sup>22</sup> The authors appropriately noted that the literature was insufficient to define a transfusion trigger in surgical patients with substantial blood loss. They concluded that the decision to transfuse should be based upon regular assessment of the surgical field, communication with the surgical team, estimated blood loss, Hb concentration, and an assessment of organ perfusion. Assessment of organ perfusion includes heart rate, blood pressure, urine output, evidence of ischemia, and, where appropriate, mixed venous oxygen saturations and echocardiography. All members of the panel agreed that RBCs should be administered when the Hb is <6 g/dL and that transfusion is rarely necessary when the Hb >10 g/dL.<sup>22</sup> These guidelines were updated in 2015, reinforcing the use of a restrictive red blood cell transfusion strategy for most clinically stable surgical patients.<sup>171</sup>

Beyond the setting of acute hemorrhage, available guidelines generally support conservative transfusion practices, considering individual patients' need for RBC transfusion only when the Hb falls below 7 g/dL.<sup>173,174,244</sup> This recommendation was also endorsed by the Society of Critical Care Medicine and the Eastern Association of Surgery for Trauma's 2009 joint clinical practice guidelines.<sup>169</sup> Specifically, this guideline intended to address indications for RBC transfusion in the critically ill, those presenting with trauma, requiring mechanical ventilation or with known stable coronary disease. Although there remains a degree of equipoise regarding optimal transfusion thresholds in patients with coronary artery disease,<sup>75,245</sup> recent clinical trials suggest safety with more restrictive approaches to RBC transfusion in these populations as well.<sup>163,164</sup> Although recommendations similar to these were re-emphasized in the AABB 2016 clinical practice guidelines, a more modest Hb threshold of 8 g/dL was suggested for patients with stable coronary disease and for those undergoing cardiac or orthopedic procedures.<sup>173</sup> This slightly higher threshold of 8 g/dL was predominantly chosen based upon findings of the FOCUS trial in which liberal (Hb < 10 g/dL) transfusion strategies were compared to a more restrictive approach (Hb < 8 g/dL or symptomatic) in surgical patients with known stable cardiovascular disease.<sup>246</sup> As the restrictive threshold chosen for this trial was 8 g/dL rather than 7 g/dL,

AABB has endorsed this slightly higher threshold acknowledging that 7 g/dL may also be safe but has not been formally studied in these specific patient populations.

The primary goal of perioperative RBC transfusion is to maintain tissue oxygenation. Although improved oxygen carrying capacity is certainly achieved with RBC transfusion, the association between RBC transfusion and improved tissue oxygenation remains much more controversial.<sup>247–250</sup> Multiple studies have attempted to address this very question and while oxygen delivery consistently improves following RBC transfusion, very few studies have been able to demonstrate an increase in tissue oxygen consumption.<sup>169,249</sup> Although the underlying mechanisms remain under investigation, it is hypothesized that this observation may be secondary to a diminished capillary diameter in critically ill or injured patients, or as a function of the many biochemical changes that occur in stored RBCs.<sup>250</sup>

In the absence of acute blood loss, the desired end points following perioperative RBC transfusion are less well defined. Available literature suggests that an individualized assessment for evidence of ischemia or hemodynamic stability with signs or symptoms suggestive of inadequate end-organ perfusion and oxygenation should guide decisions on RBC administration.<sup>170,171</sup> Where none of the above criteria are satisfied, further transfusion is unlikely to result in meaningful clinical benefit. However, respecting existing guidelines recommending RBC transfusion when the Hb falls below 7 g/dL, it would seem reasonable to transfuse patients to a target above this threshold.

In summary, modern perioperative RBC transfusion practice has seen a move toward more restrictive transfusion strategies. This substantial re-evaluation of our RBC transfusion practice has been largely driven by evolving evidence showing an association between liberal transfusion practices and adverse patient outcomes, including infection, acute respiratory distress syndrome, multiorgan dysfunction, and mortality. Although the association between liberal RBC transfusion practices and these adverse patient outcomes remains a matter of much debate, largely due to the observational nature of supporting literature, the absence of evidence suggesting the potential for harm with restrictive transfusion therapies would appear to support recommendations to avoid RBC transfusion in the majority of nonbleeding, hemodynamically stable patients when the Hb is >7 g/dL.

### Plasma: indications, threshold, and dose

It has been estimated that approximately one-third of plasma products are transfused for nonevidence-based indications.<sup>17–19</sup> In the United States, the most frequently cited rationale for deviation from published guidelines is to correct abnormal preprocedural coagulation screening tests in nonbleeding patients.<sup>84,158,251</sup> The rationale for transfusing plasma in this context is based on three basic assumptions: (1) an elevated INR reliably predicts bleeding complications, (2) administration of plasma will normalize the INR, and (3) administration of plasma will prevent bleeding complications. Importantly, all three assumptions rest on minimal evidentiary support, and when considering the growing body of evidence suggesting a poor correlation between coagulation screening test abnormalities and periprocedural bleeding complications alongside the increasingly appreciated risks of transfusion, such practice is strongly discouraged.<sup>20,84,252–256</sup>

It is important to note that, in the absence of massive transfusion or hemorrhagic shock, anticoagulant therapy, or consumptive disorders, such as disseminated intravascular coagulation,

coagulation factor depletion as an underlying cause of clinically significant bleeding is rare. Indeed, it is understood that adequate coagulation persists with approximately one-third of normal clotting factor concentrations and fibrinogen levels  $\geq 75$  mg/dL.<sup>155,257</sup> Although these abnormalities will result in derangements of laboratory coagulation parameters, thrombin generation is generally preserved, and clinical coagulopathy does not typically correlate with testing results. Accordingly, with improved understanding of in vivo coagulation, as well as the risks of transfusion therapies,<sup>66,67</sup> there have been notable changes in the clinical indications for perioperative plasma transfusion. Indeed, the historic use of plasma as a volume expander is now obsolete. At present, the strongest indication for plasma transfusion is for the treatment of active hemorrhage that is believed related to multiple coagulation factor deficiencies.<sup>155,171,258,259</sup> A substantial body of evidence supports the use of liberal plasma transfusion strategies (e.g., 1:1 RBC–plasma) in the setting of massive hemorrhage associated with military trauma in attempt to avoid trauma-induced coagulopathy.<sup>260–262</sup> The rationale for this more aggressive plasma resuscitation practice is that it may prevent subsequent persistent and unrelenting transfusion requirements resulting from delayed and/or inadequately dosed plasma transfusion. The absence of accurate and timely point-of-care laboratory tests reflecting the current coagulation status during active resuscitation, particularly in the setting of military trauma, has further supported this simplified approach to coagulation factor management in the setting of massive transfusion.<sup>263</sup>

Although such liberal approaches to plasma transfusion have been investigated in civilian trauma populations,<sup>264</sup> equipoise remains. Numerous studies have suggested that fixed-ratio plasma resuscitation strategies may reduce early transfusion requirements and potentially improve survival in civilian populations.<sup>264–266</sup> However, additional literature raises concern that such liberal approaches to plasma resuscitation may not translate well to civilian populations in whom there is often far greater prevalence of comorbid disease. The role of ratio-based resuscitation strategies is particularly unclear in circumstances not related to major trauma (e.g., obstetric, surgical, gastrointestinal bleeding).<sup>159,255,267,268</sup> Indeed, such populations may be at risk for adverse outcomes with more liberal plasma transfusion strategies.<sup>269–271</sup> Additionally, the issue of survival bias in the observational studies investigating hemostatic resuscitation strategies has also raised concern as to the true impact of these liberal transfusion practices on patient important outcomes.<sup>272–276</sup> Of note, the modern viscoelastic assays, TEG and ROTEM (discussed in the “The utility of preoperative hemostatic testing” section), may enable physicians to make point-of-care decisions regarding a patient’s ability to form thrombus and thereby offer a more individualized and evidence-based approach to plasma transfusion in a variety of civilian trauma or massive hemorrhage settings.<sup>277–280</sup> Indeed, a better understanding of the translatability of fixed-ratio plasma resuscitation strategies to civilian populations and a robust comparison of fixed-ratio plasma resuscitation strategies to strategies incorporating viscoelastic testing are highly anticipated areas of future research.

Beyond massive bleeding, indications for plasma transfusion are notably sparse. Specific examples of evidence-based indications for plasma transfusion include the following:

- Replacement of inherited single coagulation factor deficiencies for which no virus-safe fractionated product exists.<sup>258</sup>
  - Replacement of specific protein deficiencies.<sup>281</sup>
  - Replacement of multiple coagulation factor deficiencies with associated severe bleeding, massive transfusion, and/or disseminated intravascular coagulation.<sup>278,282</sup>
  - Replacement of fluid during plasma exchange in patients with TPP.<sup>283</sup>
  - Urgent reversal of warfarin anticoagulation when severe bleeding is present and PCCs are not available.<sup>284</sup>
- Notably, the frequently cited indication of  $\geq 1.5$ -fold prolongation of the PT and/or aPTT is particularly controversial based on the knowledge that elevated PT/INR or aPTT results do not necessarily correlate well with clinical coagulopathy.<sup>84</sup> Haas *et al.*<sup>83</sup> conducted a comprehensive review of the literature pertaining to the value of the PT/INR and aPTT in perioperative coagulopathy. Their study found that 6 of the 11 published guidelines for the management of perioperative bleeding continued to endorse the  $\geq 1.5$ -fold prolongation threshold; however, referenced evidence supporting this recommendation was generally from small, poor-quality studies and expert panel recommendations. Use of plasma for correction of abnormal coagulation tests prior to specific invasive procedures and in patients with liver disease is discussed elsewhere in this chapter.
- Specific contraindications to plasma transfusion include the presence of an isolated coagulation factor deficit when specific factor concentrates are available, reversal of oral anticoagulation therapy in the absence of bleeding, and treatment of hypovolemia. For patients who are anticoagulated with vitamin K antagonist therapies (e.g., warfarin), discontinuation of the oral anticoagulant therapy and supplementation with vitamin K are the primary therapeutic modalities when reversal is needed, and there is no evidence for active bleeding or requirements for emergent high-risk surgery. In the presence of clinically significant active bleeding, PCCs should be considered ahead of plasma transfusion due to their potential for more rapid reversal of the oral anticoagulant effect.<sup>278,285–287</sup> In addition, these agents are felt to carry a lower risk of transfusion-associated pulmonary and infectious complications. Finally, there is not a role for plasma transfusion as a strategy to mitigate bleeding associated with DOACs.
- In the absence of massive bleeding, plasma is typically dosed at 10–15 mL/kg predicted body weight with an expected increase in clotting factor levels of 25–30%.<sup>288</sup> This equates to approximately 1 L of plasma in a typical 70 kg patient. Importantly, however, studies have suggested that this dosing regimen may often result in an inadequate repletion of clotting factors.<sup>268,289</sup> Indeed, evidence suggests that upward of 30 mL/kg of plasma may be needed to reliably achieve desired coagulation factor levels, thereby ensuring adequate hemostasis.<sup>290</sup> However, a primary concern with this plasma dosing regimen is the potential for volume overload, particularly in those with baseline predisposing factors (e.g., congestive heart failure and advanced renal disease).
- As previously discussed, the evidence supporting plasma transfusion outside of massive hemorrhage is limited. Importantly, much of these data do not relate to patients with liver disease, casting yet more uncertainty over interpretation of PT/INR values with regard to bleeding risk. An in-depth evaluation of in vivo coagulation status in patients with liver disease by Lisman *et al.*<sup>291</sup> provided a comprehensive contemporary summary of “rebalanced hemostasis.” In this review, they summarize the evidence for the simultaneously enhanced procoagulant state (due to elevated von Willebrand factor and factor VIII, along with decreased ADAMTS-13, proteins C and S, antithrombin, and plasminogen) as well as an anticoagulant state (due to thrombocytopenia and platelet dysfunction; depletion of

factors II, V, VII, IX, X, XI and XIII; vitamin K deficiency; dysfibrinogenemia; along with elevated tissue plasminogen activator, nitric oxide, and prostacyclins).<sup>291</sup> In light of the complexity of hemostatic derangements in this patient population, traditional coagulation tests must be interpreted with particular caution and should not be the sole indication for plasma transfusion.<sup>292</sup>

Although a number of observational studies have attempted to examine the efficacy of plasma transfusion, no randomized trials have evaluated liberal versus conservative plasma transfusion strategies as has been performed with RBCs. This will be an important future step in refining current transfusion practice.

### Platelets: indications, threshold, and dose

As with other blood component therapies, platelet transfusion is generally considered appropriate in the presence of thrombocytopenia or abnormal platelet function with clinically significant active bleeding.<sup>293–297</sup> Recommendations for non-neuraxial surgery advocate single unit platelet transfusions with a target platelet count of  $\geq 50 \times 10^9/L$ .<sup>294,296,297</sup> In patients undergoing invasive neurologic or ophthalmologic procedures, a slightly higher threshold of  $100 \times 10^9/L$  has been recommended.<sup>296–298</sup> As with plasma transfusion, early military data suggest a potential role for early transfusion of platelets in order to avoid persistent bleeding following massive transfusion. Specifically, when the application of a 1:1:1 ratio of RBCs, plasma, and platelets was applied in military populations, improved survival was noted.<sup>260,262</sup> The pragmatic, randomized optimal platelets and plasma ratios (PROPPR) trial found that patients who received higher ratios of platelets and plasma to red blood cells were more likely to achieve hemostasis (86.1% vs. 78.1%,  $p = 0.006$ ) and experience fewer deaths due to exsanguination by 24 hours (9.2% vs. 14.6%).<sup>264</sup> These data may support a more liberal approach to platelet transfusion in the setting of trauma-related massive hemorrhage. However, the data supporting this strategy are not robust and clinical uncertainty persists. This again is an area in critical need of additional scientific study.

Current evidence suggests that the risk of bleeding due to thrombocytopenia is minimal when the platelet count is  $\geq 50 \times 10^9/L$ .<sup>296,297,299</sup> Importantly, however, this assumes that platelet function is normal. Frequently, there are clinical scenarios that may result in platelet dysfunction despite normal platelet counts (e.g., cardiopulmonary bypass, antiplatelet therapies, and congenital disorders). In these specific circumstances, the role of platelet transfusion is often unclear.<sup>294,296,297</sup> Indeed, recent recommendations from the AABB have highlighted these uncertainties.<sup>294</sup> Increasingly, thromboelastography, platelet aggregometry, and platelet mapping assays have been advocated to help inform clinical decisions in these specific settings.<sup>300</sup> As an example, a maximal amplitude of  $<54$  mm on TEG has been noted to represent abnormal platelet count and/or function with impaired whole blood clot formation.<sup>301</sup> Platelet aggregometry with an ADP-induced aggregation  $<31$  units has been shown to be independently associated with bleeding events and transfusion in cardiac surgery.<sup>302</sup> Similarly, platelet mapping provides an estimate of the degree of platelet inhibition due to aspirin or ADP-receptor antagonists and has been shown to correlate well with light transmission aggregometry.<sup>303</sup> Nonetheless, no well-validated guidelines currently exist with regard to platelet transfusion thresholds using these more advanced laboratory testing strategies and significant knowledge gaps remain, precluding broad endorsement of these testing strategies at this time.<sup>304,305</sup>

Aside from active bleeding, evidence-based guidelines suggest the administration of platelet components for platelet counts

$<10 \times 10^9/L$  to prevent spontaneous hemorrhage ( $<20 \times 10^9/L$  when fever, sepsis, heparin therapy, DIC, or other conditions leading to increased platelet consumption coexist).<sup>296,297</sup> These indications are mostly supported by a number of studies evaluating transfusion strategies in patients with leukemia and bone marrow failure and do not specifically focus on the needs of nonbleeding surgical populations.<sup>306</sup> Therefore, the optimal platelet count triggering platelet transfusion in the nonbleeding postoperative patient remains poorly defined and specific recommendations are largely lacking.

Following platelet transfusion, the expected increments in the platelet count for a typical 70 kg adult are  $5–10 \times 10^9/L$  per whole-blood-derived unit transfused and  $30–60 \times 10^9/L$  per apheresis platelet transfused.<sup>307</sup> These expected increments should be used to guide dosing with the aforementioned transfusion goals in mind. Platelet refractoriness can complicate the transfusion support of patients who require long-term platelet transfusion therapy.<sup>307</sup> Questions related to dosing and efficacy of platelet transfusion have been further examined by the PROPPR and platelet dose (PLADO) trials.<sup>264,308</sup> In the absence of massive hemorrhage, guidelines generally recommend the administration of up to a single apheresis unit or equivalent as greater doses are not more effective, and lower doses may be equally effective.<sup>294,297</sup>

### The role of plasma transfusion in special circumstances

#### Central venous catheter (CVC) insertion

Serious complications of CVC placement are rare and include pneumothorax, air embolus, and hemothorax. In fact, significant bleeding following subclavian vein catheterization occurs in patients with intact hemostasis and results from inadvertent injury of the subclavian artery, right atrium, and superior vena cava.<sup>309</sup> Among patients with advanced liver disease, the insertion of a central venous catheter for transvenous hepatic biopsy provides an excellent model to demonstrate the failure of abnormal coagulation tests to predict bleeding complications at the time of central line placement. Goldfarb and Lebrec<sup>310</sup> reported results of 1000 consecutive CVC placements in patients with liver disease, all of whom had abnormalities of laboratory coagulation tests. Only one patient experienced a significant hematoma despite 74 of 1000 also having inadvertent carotid artery puncture. Similarly, Foster *et al.*<sup>311</sup> performed central line insertions on 259 patients with advanced liver disease awaiting transplantation, all of whom had serious coagulation derangements. Importantly, no preprocedure products were given, and no important bleeding complications were observed.

Comparable results have been replicated in multiple subsequent studies. Petersen<sup>312</sup> compared bleeding complications in anticoagulated (aPTT of 1.5 times control) versus nonanticoagulated patients undergoing CVC placements. Of the 22 hematomas observed, 13 were found in anticoagulated patients and 9 were in nonanticoagulated patients. None of the patients with hematomas required treatment beyond topical care. Of note, among 22 episodes of inadvertent puncture of the carotid artery, the incidence of subcutaneous hematoma formation was the same in both the anticoagulated patients (4/12) and the nonanticoagulated patients (3/10). Another recent randomized clinical trial compared pretreating coagulopathic patients with FFP versus no transfusion prior to an invasive procedure (CVC placement, percutaneous tracheostomy, chest tube, or abscess drainage).<sup>313</sup> Although the overall numbers were

small, there was no difference in bleeding complications between the groups with the vast majority having no bleeding events at all.

Fisher and Mutimer<sup>314</sup> reported results of 658 subclavian and internal jugular cannulations in patients with advanced liver disease (median INR 2.4–2.7 and median platelet count 81–83 × 10<sup>9</sup>/L) in which no preprocedural plasma or platelets were given. INR values >5.0 and platelet counts <50 × 10<sup>9</sup>/L were associated with a higher incidence of superficial hematomas or site oozing; however, the strongest predictor (OR 8.0) of a procedure-related hematoma was a failed guide wire attempt. This paper underscored the fact that technical mishaps remain the dominant cause of hematoma formation and that bleeding complications other than skin hematoma or oozing are rare regardless of the coagulation test results.

A recent summary of available data was published by van de Weerd et al.<sup>315</sup> and included one RCT and 21 observational studies. Of 3150 patients with severe coagulopathy that remained uncorrected, the bleeding incidence ranged from 0% to 32%, and the risk of bleeding could not be predicted by the severity of coagulopathy on lab testing. Additionally, no study demonstrated a beneficial effect from prophylactic platelet or FFP transfusion.<sup>315</sup>

### Liver biopsy

Closed liver biopsy has generally been considered one of the higher risk bedside invasive procedures due to the fact that patients with liver disease often have multiple hemostatic test abnormalities and biopsies are performed without the aid of direct pressure on the wound sites. For these reasons, there is a strong desire to identify patients at increased risk for bleeding. In some instances, abnormalities in hemostasis screening tests sway providers to preferentially perform open surgical or laparoscopic biopsy over closed biopsies or a biopsy followed by Gelfoam plugging.<sup>316</sup>

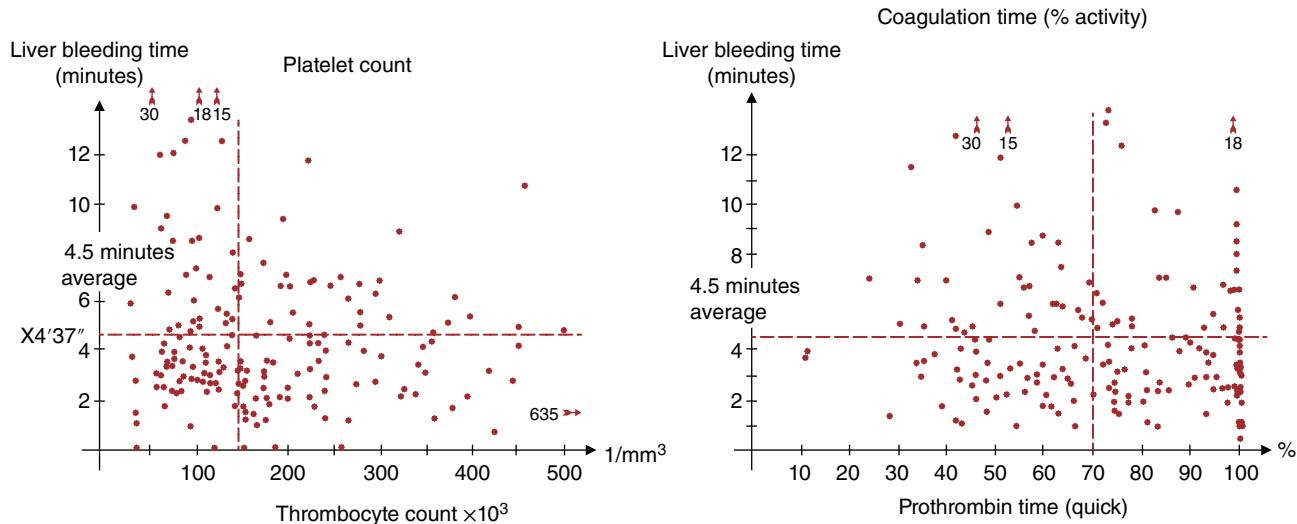
One of the earliest studies evaluating hemostasis in patients with liver disease found that among 200 patients with nonmalignant liver disorders undergoing laparoscopic liver biopsy, most patients had at least one abnormal coagulation test, but none were treated with blood components preprocedurely.<sup>317</sup> These

investigators found no correlation between the length of time the liver bled after puncture and the preprocedure laboratory values (Figure 39.2).

Similar findings have been duplicated by others, including Terjung et al.<sup>318</sup> In this study, patients undergoing percutaneous liver biopsies with at least one abnormality in preprocedural coagulation tests were evaluated for bleeding outcomes. The authors found no correlation between bleeding risk and mild-to-moderate abnormalities in the preprocedure INR, aPTT, platelet count, or bleeding time.

Given the strong (albeit unfounded) reliance on the INR as a key measure of hemostasis in liver disease, and the dependence of the INR test on factor VII, it is not surprising that investigators might explore the role of recombinant factor VIIa given before liver biopsy. Jeffers et al.<sup>319</sup> studied 71 patients who received recombinant factor VIIa in doses ranging from 5 to 120 µg/kg 10 minutes before laparoscopic liver biopsy. Although PT correction was fairly uniform, especially with the 20, 80, and 120 µg/kg doses, hemostasis at 10 minutes failed in 26% of patients, and an 80 µg/kg “rescue” dose of recombinant factor VIIa was administered. No toxicity was observed. This study unfortunately lacked a control group, and no follow-up testing for hematoma formation was performed. Although the ability of recombinant factor VIIa to improve the INR in patients with liver disease readily occurs, the fundamental question is whether preprocedure treatment with it is superior to treatment with FFP or to no treatment at all. This would be best answered in a prospective, randomized controlled clinical trial.

Similarly, in a recent report of 1321 transjugular liver biopsies in 932 patients, only 8 patients (0.6%) had an intraperitoneal hemorrhage, all of which were nonfatal.<sup>320</sup> When comparing the major and minor complication rates at three days and at one month, there was no association with the severity of coagulopathy (platelet count or PT/INR). Overall, these results continue to suggest that liver biopsy remains a relatively safe procedure with minimal bleeding complications that cannot be adequately predicted with preoperative coagulation studies or prevented with coagulation factor therapy.



**Figure 39.2** Lack of correlation between bleeding from the site of liver biopsy and preprocedure coagulation test or platelet count. The duration of bleeding from the site of puncture of the liver (y-axis) is plotted as a function of the preprocedure platelet count (left-hand panel) or the preprocedure coagulation test (expressed as % activity, right-hand panel). There is no correlation between preprocedure laboratory test results and the duration of bleeding. Source: Ewe (1981).<sup>317</sup> Reproduced with permission of Springer.

## Thoracentesis and paracentesis

Given the lack of evidence in favor of preprocedure transfusions for CVC placement or for closed liver biopsy, it is not surprising that published evidence also fails to support the use of preprocedure prophylaxis for less invasive procedures such as thoracentesis or paracentesis. McVay and Toy<sup>321</sup> reported a retrospective review of outcomes in 608 consecutive procedures. Patients were not transfused before the procedure, and no patient had serious bleeding. The proportion of procedures that were accompanied by a decline in Hb was comparable in the group with normal INR/aPTT/platelet values versus abnormal INR/aPTT/platelet values.

Similarly, a retrospective analysis of ultrasound-guided thoracenteses was performed comparing those with preprocedural correction of abnormal coagulation labs versus no correction.<sup>322</sup> Of 1009 procedures, there were only four hemorrhagic complications (0.4%), all of which occurred in those that received preprocedural platelet or FFP transfusion, suggesting that attempting to correct abnormal labs in this population is unlikely to yield any benefit.

Bleeding following paracentesis was also examined by Webster *et al.*<sup>323</sup> who reported a retrospective analysis of 179 outpatients. Four patients developed intraabdominal or abdominal wall bleeding that required a transfusion of RBCs. The INR values of these four were indistinguishable from those of the 175 patients who did not bleed.

In another retrospective study of ultrasound guided paracentesis, of 3116 patients (most with abnormal coagulation labs due to cirrhosis), 6 patients (0.19%) suffered a postprocedure hemorrhage and one of these patients required radiologic embolization.<sup>324</sup> No preprocedure testing results could be identified that predicted a bleeding complication, including abnormal PT/INR or platelet count, further suggesting the futility in transfusing to correct isolated abnormal lab values in the absence of bleeding.

## Procedures on the upper airway, bronchoscopy, and transbronchial lung biopsy

Special care to provide good hemostasis is prudent when procedures are being performed on the oropharynx, trachea, or bronchi, given that excessive bleeding can be rapidly fatal. Tonsillectomy was an early subject for studies assessing preoperative hemostatic screening. As with other surgical procedures, the predictive value of an isolated abnormal coagulation screening test for bleeding at tonsillectomy or adenoidectomy is very poor. The majority of patients who bleed during these procedures have normal presurgical tests.<sup>325</sup>

Tracheostomy is commonly performed in very ill patients in intensive care, many of whom may have abnormal laboratory tests for coagulation. Auzinger *et al.*<sup>326</sup> studied patients with severe liver disease undergoing percutaneous tracheotomy. In this study, all but two patients had derangements of laboratory coagulation tests or platelet count. Only one patient had excessive bleeding (defined as >150 mL), which was external (extratracheal). Similar findings have been confirmed in multiple small observational studies<sup>327,328</sup> and in critically ill patients on VV-ECMO.<sup>329</sup> In aggregate, these publications appear to refute the notion that mild-to-moderate prolongation of the INR or aPTT or mild-to-moderate thrombocytopenia represent absolute contraindications to safe tracheotomy.

In 1994, Kozak and Brath<sup>330</sup> reviewed 274 patients undergoing fiberoptic bronchoscopy and biopsy procedures. Abnormal coagulation screening tests were found in 10% ( $n = 28$ ) of patients. Overall, 35 patients bled, 32 of whom had normal preprocedure laboratory values. Three patients had severe bleeding, and each of them had normal preprocedure test results.

A larger retrospective review of bronchoscopies by Diette *et al.*<sup>331</sup> found that bleeding did not correlate with coagulation parameters or platelet count, and that performing a transbronchial biopsy did not independently increase the risk of bleeding. Such findings have now been replicated several times in both human and animal models.<sup>332,333</sup>

## Epidural anesthesia, diagnostic lumbar puncture, and neurosurgical procedures

Because bleeding in the closed space of the subarachnoid or epidural region can produce paraparesis or paraplegia following lumbar puncture (LP), avoiding preventable bleeding is more important for lumbar punctures and epidural anesthesia than for most invasive procedures. The chance of peridural hematoma appears to be increased among patients with full-dose anticoagulation who undergo placement or removal of an epidural catheter for spinal anesthesia. Ruff and Dougherty<sup>334</sup> reported five cases of paraplegia among 342 patients who received a diagnostic LP followed by administration of full-dose heparin. In addition, the introduction of low-molecular-weight heparin treatment in the United States was accompanied by an observed increase in reported spinal hematomas among patients with epidural catheters, estimated to occur at a rate of 1 in 10,000 patients.<sup>335</sup> In patients with low-dose anticoagulation undergoing vascular surgery, Rao and El-Etri<sup>336</sup> did not find evidence of epidural/peridural hematomas following placement of either an epidural catheter or a subarachnoid catheter. In a similar manner, low-dose heparin was not associated with spinal hematoma in over 5000 patients undergoing spinal or epidural anesthesia.<sup>337</sup>

The true incidence of epidural hematoma formation with epidural placement is not precisely known, especially in the setting of coagulopathy. The risk of hematoma appears greatest both at the time of epidural placement as well as when removed. A recent retrospective review of 11,600 epidurals identified 629 patients with associated abnormal coagulation studies when placed (278 patients) or removed (351 patients).<sup>338</sup> Of this coagulopathic cohort, only two epidural hematomas occurred for a combined incidence of 1 in 315 (95% CI, 1 in 87–2597).

Despite some institutions commonly transfusing FFP or platelets to patients with coagulopathy prior to neuraxial procedures, the practice remains controversial. Two recent attempted Cochrane reviews aimed to assess the efficacy of FFP<sup>339</sup> or platelet<sup>340</sup> transfusions before lumbar punctures or epidural placement; however, they were unable to find any completed or ongoing trials to include. They estimated that each intervention (platelets and FFP) would need 47,000 patients to detect a 1/1000 difference in epidural hematomas.

Interestingly, in 2001, Schramm *et al.*<sup>341</sup> reviewed outcomes among 1211 patients undergoing craniotomy, spinal surgery, or other neurosurgical procedures at the Royal Melbourne Hospital in Australia. The authors found that 17% of preoperative laboratory tests were abnormal, but neither prolongation of the INR nor depression of the platelet count predicted excessive surgical bleeding. Of note, procedure-related bleeding was associated with patients who demonstrated a prolonged aPTT and these prolonged aPTTs were predictable by history in most of these patients.

## Angiography

The increasing use of interventional radiology and cardiology has created a new cohort of patients at risk for arterial bleeding following angiography. Not surprisingly, there is little evidence that pre-procedure blood components are indicated for angiographic

procedures. Darcy *et al.*<sup>342</sup> prospectively studied 1000 consecutive patients undergoing femoral arterial puncture for diagnostic or therapeutic vascular procedures. Analysis was restricted to patients with INR values <1.5. The incidence of hematoma formation was the same in patients without any abnormality in preprocedural INR (9.7%) as those with abnormal results (8.1%). Neither the INR nor the aPTT was predictive of hematoma formation; however, hematoma formation was more common among patients with platelet counts <100 × 10<sup>9</sup>/L, with the use of catheters larger than 5 Fr, and among patients with a documented history of bleeding disorders.

### The role of platelet transfusion in special circumstances

The efficacy of prophylactic platelet transfusion remains a point of contention with somewhat limited experimental evidence to support its use in a wide variety of clinical scenarios. A platelet count of <10 × 10<sup>9</sup>/L is widely accepted as the threshold to transfuse platelets in order to prevent spontaneous hemorrhage. However, recent data demonstrates that prophylactic platelet transfusion at higher thresholds does not necessarily reduce bleeding complications or support improved outcomes in the critically ill,<sup>343</sup> surgical patients,<sup>344</sup> and those undergoing invasive interventional radiology procedures.<sup>345</sup> Indeed, the most recent Cochrane review concluded there was insufficient evidence to recommend prophylactic perioperative platelet transfusions.<sup>346</sup> In 2015, the AABB provided their recommendations for prophylactic platelet use in various clinical circumstances.<sup>294</sup> More recently, in 2020, the European Society of Intensive Care Medicine (ESICM) provided their recommendations for prophylactic transfusion in the critically ill.<sup>347</sup> Various clinical applications of these recommendations are summarized below.

#### Central venous catheter placement

Equipoise regarding the appropriate threshold with which to prophylactically transfuse platelets in patients undergoing invasive procedures was highlighted in 1997, when Ray and Shenoy<sup>348</sup> found no evidence of increased bleeding, infection, or thrombosis in a prospective study of 105 patients undergoing prophylactic platelet transfusion for central line placement. Further small studies and one randomized controlled trial subsequently noted increased bleeding and/or mortality in those receiving preprocedural platelet transfusions.<sup>349,350</sup> More recently, in 2017, Warner *et al.*<sup>345</sup> conducted a propensity matched analysis of adults undergoing vascular interventional radiology procedures, including central line placement, to assess the relationship between platelet transfusions and periprocedural RBC transfusion requirements. They concluded that prophylactic platelet transfusion did not result in a lower periprocedural RBC requirement in patients with a platelet count of at least 50 × 10<sup>9</sup>/L, suggesting that this intervention may indeed lack efficacy for this population. The AABB clinical practice guideline supports the use of prophylactic platelet transfusion in patients requiring elective central venous catheter placement with a platelet count of <20 × 10<sup>9</sup>/L.<sup>294</sup> In the same vein, the ESICM recommend against prophylactic platelet transfusion for platelet counts greater than 50 × 10<sup>9</sup>/L, but makes no recommendation regarding prophylactic platelets for counts between 10 and 50 × 10<sup>9</sup>/L.<sup>347</sup>

#### Liver biopsy

Sparse focused literature exists to definitively guide prophylactic platelet transfusion for liver biopsy. While various sources recommend threshold ranging from 50 to 100 × 10<sup>9</sup>/L, data from as far

back as 1981 have shown no correlation between degree of bleeding after liver biopsy and any laboratory measure of hemostasis.<sup>317</sup> However, conflicting data exist to suggest increased bleeding events at platelet counts below 60 × 10<sup>9</sup>/L.<sup>351</sup> Further, much of these data make no account for platelet function. Overall the risk of bleeding with either transjugular or percutaneous liver biopsy is believed to be <1%.<sup>351</sup> In 2003, a retrospective review of 51 patients with thrombocytopenia related to hematological malignancy undergoing transjugular liver biopsy found no hemorrhagic complications despite a mean platelet count of 38 × 10<sup>9</sup>/L.<sup>352</sup> In patients with liver disease, interpretation of bleeding risk for any given platelet count is further complicated by complex coexisting disturbances in both pro- and anticoagulant systems. Warner *et al.*<sup>345</sup> recently demonstrated that peri-procedural bleeding complications did not correlate with preprocedural platelet count in patients undergoing liver biopsy. In light of these data, the American Association for the Study of Liver Disease makes no specific threshold recommendation, but instead advocates for an individualized peri-procedural risk assessment including identification and correction of modifiable risk factors such as antithrombotic drug use, or concurrent acute kidney injury or systemic infection.<sup>353</sup>

Two TPO receptor agonists have been approved for the treatment of thrombocytopenia in patients with chronic liver disease. While each agent was superior to placebo at achieving a target platelet count of 50 × 10<sup>9</sup>/L, there were no statistically significant differences in bleeding complications; therefore, routine use is not recommended at this time.<sup>354,355</sup> Furthermore, because each of these agents require 2–8 days to be effective, they are unlikely to be useful for emergent liver biopsies.

#### Thoracentesis and paracentesis

Published evidence to guide the use of platelet transfusions in the setting of thoracentesis and paracentesis is scant. Though few focused studies exist, such patients have been included in both historical and contemporary evaluations of periprocedural bleeding risk, without clear evidence of benefit for prophylactic platelet transfusion.<sup>321,345</sup>

#### Procedures on the upper airway, bronchoscopy, and transbronchial lung biopsy

While careful attention to hemostasis in the airway is critical, available data do not support the use of prophylactic platelet transfusion below a threshold of 50 × 10<sup>9</sup>/L. The only available RCT to evaluate such practice was terminated early due to a low number of bleeding events and physician's unwillingness to transfuse prophylactic platelets to correct mild thrombocytopenia (threshold under investigation 40 × 10<sup>9</sup>/L).<sup>350</sup> Contemporary retrospective analyses have supported similar conclusions, with a lack of evidence that prophylactic platelets reduce periprocedural RBC requirements or mortality.<sup>345</sup> In 2016, Nandagopal *et al.*<sup>356</sup> found that overall there was a very low incidence of bleeding complications in patients undergoing bronchoscopy (with or without lavage), none of which were considered to be clinically significant, even in patients with platelet counts below 30 × 10<sup>9</sup>/L. They concluded that such procedures could be safely performed with severe thrombocytopenia. The AABB makes no formal recommendations regarding this clinical scenario, while the ESICM maintains that, in the absence of clear benefit, prophylactic platelet transfusions are not recommended for bronchoscopy patients with a platelet count of >50 × 10<sup>9</sup>/L.<sup>347</sup>

## Epidural anesthesia, diagnostic lumbar puncture, and neurosurgical procedures

As with other clinical scenarios already described, limited data exist to support the use of platelet transfusions for bedside procedures such as epidural anesthesia and lumbar puncture (LP). Several studies provide evidence that thrombocytopenia is not a major contraindication to diagnostic LP. Howard *et al.*<sup>357,358</sup> reported an extensive experience with >5000 LP procedures performed in 958 children with leukemia. Despite the fact that platelets in these children may have been dysfunctional due to leukemia or antibiotics in addition to the varying degrees of thrombocytopenia observed ( $10\text{--}100 \times 10^9/\text{L}$ ), no patient developed spinal hematoma or a clinical bleeding complication. The authors noted that the likelihood of finding >500 red cells/ $\mu\text{L}$  in the spinal fluid was slightly higher in patients with platelet counts  $<100 \times 10^9/\text{L}$  compared to those with counts  $>100 \times 10^9/\text{L}$ . However, among patients with counts  $<100 \times 10^9/\text{L}$ , the likelihood of an LP with >500 red cells/ $\mu\text{L}$  did not increase as the platelet count declined to levels as low as  $10 \times 10^9/\text{L}$ ; thus, the authors concluded that a platelet count of  $10 \times 10^9/\text{L}$  was adequate to perform a routine LP for administration of intrathecal chemotherapy. Similar findings have subsequently been confirmed in adult populations,<sup>359,360</sup> although the authors recommended prophylactic transfusions be given if the platelet counts were  $<20 \times 10^9/\text{L}$ .<sup>359</sup> These studies provide evidence that LPs that are critically important for patient care should not be delayed or withheld because of thrombocytopenia.

In 2016, Warner *et al.*<sup>344</sup> described the impact of preoperative platelet transfusions on perioperative RBC transfusion needs in almost 14,000 thrombocytopenic patients (threshold  $<100 \times 10^9/\text{L}$ ) undergoing noncardiac surgery, including neurological and spinal surgery. They noted that preoperative prophylactic platelet transfusion did not appear to attenuate perioperative RBC requirements; however, it was associated with an increased ICU admission rate and prolonged hospital stay, suggesting that a more conservative approach may be warranted. Though evidence is weak, for patients undergoing neurosurgical procedures a platelet count  $<100 \times 10^9/\text{L}$  is believed to be associated with an increased risk of hemorrhagic complications.<sup>361</sup> While this is the threshold often cited for the management of these patients, acuity and magnitude of the drop in platelet count likely impacts individual risk and should be considered when deciding to transfuse platelets.

Current AABB guidelines support the use of prophylactic platelet transfusions in patients requiring elective lumbar puncture with platelet counts  $<50 \times 10^9/\text{L}$ . They do note that evidence is weak, and some sources advocate for a lower thresholds of  $10\text{--}20 \times 10^9/\text{L}$  in patients with hematological malignancies and immune thrombocytopenia.<sup>359,362</sup> The AABB makes no specific comment on epidural catheter placement; however, a threshold of  $80 \times 10^9/\text{L}$  is often cited in the absence of stronger data to support lower thresholds.<sup>363</sup> Similarly, no specific recommendations are provided for neurological surgery or in the setting of intracranial hemorrhage due to scant and conflicting data.

## Cardiac surgery

Strong definitive evidence to support an approach to platelet transfusion in the cardiac surgical patient remains sparse, and further investigations are warranted. In a recent meta-analysis of available literature, it was concluded that platelet transfusion did not appear to be independently associated with added perioperative risk—including operative mortality, stroke, myocardial infarction, reoperation for bleeding, infection, or dialysis requirements.<sup>364</sup> The AABB dissuades the routine administration of preoperative prophylactic platelet transfusion in thrombocytopenic patients scheduled to undergo cardiac surgery with bypass. They do, however, advocate for platelet transfusion in the presence of clinical signs of bleeding and/or platelet dysfunction.<sup>294</sup>

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## CHAPTER 40

# Transfusion therapy in the care of trauma and burn patients

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### The evolution of the approach to blood product resuscitation for severely injured patients

The oldest reports of successful blood transfusions for trauma come from the American Civil War. However, it was more than 50 years later, at the end of World War I, that recognizably modern blood banking was developed.<sup>1</sup> During the Korean War (1950–1953), the US Army's Surgical Research Team studied blood loss, the evolution of shock, and the effects of fluid resuscitation with the tracer deuterium heavy water.<sup>2</sup> Further, they reported on the safety of massive transfusion with stored universal donor blood and on the use of plastic bags for fluid and blood administration. A decade later in the Vietnam War, these concepts and products were integrated into systems for early resuscitation, rapid transportation, massive transfusion, and early surgical intervention which reduced the death rate from wounds in field hospitals to 3%.<sup>3,4</sup> Altogether, the US armed forces transfused 600,000 units of whole blood or red cells in Vietnam.<sup>5</sup> They further demonstrated the relative safety of group O universal donor red cells, with over 100,000 units transfused, given that all seven fatal hemolytic transfusion reactions in Vietnam occurred in patients receiving crossmatched but mistransfused blood. The use of large volumes of crystalloid for resuscitation was also common in the Vietnam War and likely contributed to the first described cases of acute respiratory distress syndrome (ARDS).<sup>6</sup>

The Coconut Grove nightclub fire in Boston on 28 November 1942 was a critical event in the history of burn care. It led to the first burn resuscitation formula, the first systematic evaluation of the use of blood plasma in burn resuscitation, and the first burn unit in the United States.<sup>7,8</sup> While the first two concepts remain controversial, a strong case can be made that the ready availability of blood and blood components has improved survival among elderly burned patients and patients with associated medical problems who otherwise would not have tolerated a low hemoglobin level.<sup>9</sup> Interoperative transfusion also allowed wider more complete debridement procedures, rapidly reducing the inflammatory burden of irrevocably damaged tissue, thereby decreasing the number of surgical procedures and the length of hospital care needed by a burned patient.<sup>10</sup>

The development of trauma centers and burn units in the late 1970s coincided with the development of blood bags and blood component therapy and with a general increasing demand for blood components in hospitals to support more advanced surgery and The War on Cancer.<sup>11</sup> Despite an expanding blood supply system, blood products were frequently in short supply, especially universal donor group O Rh-negative red cells (6% of donors) and group AB plasma (4% of donors). Managing a limited inventory of AB plasma meant keeping it frozen in the blood bank until the patient had a blood type and an indication in the form of a prothrombin time (PT) or activated partial thromboplastin time (aPTT) greater than 1.5 times normal.<sup>12</sup> In addition, studies of hemorrhagic shock conducted in animals during this time period supported the increased use of crystalloid during resuscitation.<sup>13</sup> These studies demonstrated that poor tissue perfusion secondary to hypovolemic hypotension became life threatening after approximately 30% blood loss, but perfusion could be restored by volume replacement with crystalloid fluids.<sup>14</sup> With continued blood loss and volume replacement, red cell mass became critically low after 60% of total blood volume loss. Red cell transfusion corrected this problem. Decreasing colloid osmotic activity led to blood flow-limiting edema after 120% blood volume removal that could be corrected with albumin. With concomitant volume and red cell and albumin replacement, hemorrhagic complications occurred spontaneously after 180% volume was lost that were related primarily to plasma coagulation factor deficiency. Platelets became limiting only after 220% volume removal and replacement of the other blood components. These isovolemic models of controlled hemorrhage and blood component replacement suggested that volume and oxygen transport were the critical early issues in the treatment of hemorrhagic shock and that coagulopathy was largely dilutional and a late complication. This logic was codified in the ATLS resuscitation algorithm in which injured patients were given crystalloid fluids to maintain volume and red cells to maintain oxygen transport only if more than 2 L of crystalloid fluid was required to maintain blood pressure.<sup>15</sup> Plasma was to be given based on laboratory measures of a prolonged PT or aPTT greater than 1.5 times normal, platelets when the platelet count was less than 50,000/ $\mu$ L, and cryoprecipitate

when the fibrinogen was less than 100 mg/dL. This approach worked well for moderately injured patients and led to reduced overall mortality, especially from renal failure.

However, in severely injured patients with uncontrolled hemorrhage, the use of large volumes of crystalloid fluid led to massive tissue edema and severe coagulopathy. Cold crystalloid resuscitation caused hemodilution and hypothermia which further exacerbated coagulopathy, a phenomenon that came to be known as the “bloody vicious cycle.” This led to the approach of damage-control surgery and complications related to abdominal compartment syndrome.<sup>16</sup> Nevertheless, for patients who needed 10 units of RBCs, mortality was typically 40%, and for those who needed 20 units, it was 50%.<sup>17</sup>

In the past two decades, three areas of clinical investigation led to the use of more plasma and less crystalloid in the resuscitation of severely injured patients. The first involved reducing the amount of crystalloid fluid given for volume expansion because there were situations in which it was not necessary and certain patients appeared to do better without the extra volume. The second line involved experiences, almost entirely military, in which dramatically better hemostasis was achieved with the use of fresh whole blood. The third involved the purposeful administration of plasma early in resuscitation to prevent coagulopathy because once coagulopathy had developed it proved difficult to treat with conventional blood components.

Bickell and colleagues used an aortic tear in swine to demonstrate that crystalloid resuscitation could be harmful.<sup>18</sup> In this model, a loop of stainless steel suture was placed in the aorta and brought out through the skin. A week later, with the animal awake, the suture was torn out to create a 0.45 cm aortic tear. Pigs sustaining this injury typically lay down, dropped their blood pressure to 30 Torr systolic for an hour while the injury bleeding clotted firmly, and then the blood pressure slowly returned to normal with 85% of the animals surviving. Attempting to resuscitate the swine with crystalloid raised their blood pressure causing further bleeding and coagulopathy resulting in 100% fatality. This observation led to a randomized controlled trial for patients sustaining penetrating truncal trauma in Houston, TX.<sup>19</sup> Hypotensive patients were randomized to standard crystalloid resuscitation vs. no crystalloid resuscitation beginning in the field and extending to the operating room. Mortality was higher in the resuscitated group. A second smaller randomized study conducted by Dutton and colleagues also showed no benefit to early resuscitation in the short time between admission and exploratory surgery in hypotensive injured patients in a level 1 trauma center.<sup>20</sup>

US military experience with the use of fresh whole blood to treat battlefield casualties in situations where platelets were not available also led to a widening appreciation of the ability of whole blood to rapidly achieve hemostasis in badly injured soldiers. In the first Iraq war, in Somalia, Bosnia, Kosovo, and then in Afghanistan and the second Iraq war, situations repeatedly occurred where casualties with uncontrolled coagulopathic bleeding after resuscitation with red cells and crystalloid were able to achieve hemostasis after the administration of fresh whole blood.<sup>21</sup>

The recognition that crystalloid fluids and red cells did not prevent coagulopathy led to many attempts at more balanced resuscitation. Hiipala wrote about the importance of early administration of plasma in massively bleeding trauma patients in the 1990s, but groups like the British Committee for Standards in Haematology continued to advocate for limiting plasma use and strict transfusion triggers.<sup>22,23</sup> However, with the descriptions of the acute coagulopathy of trauma in 2003, and reports of devastating injuries from improvised explosive devices in the second Iraq war, the need to prevent and reverse coagulopathy early in resuscitation came to the fore.<sup>24</sup> The US military published its first theater guideline recommending early initiation of

balanced hemostatic resuscitation in 2004 and published a national recommendation in 2007 stating red cells, plasma, and platelets be given in 1:1:1 ratios to the most seriously injured.<sup>25</sup> Since that time, a series of consecutively more rigorous studies of the resuscitation of massive hemorrhage were conducted in the civilian community including the Trauma Outcomes Group study (2009), the Prospective Observational Multicenter Massive Transfusion Trial (PROMMTT, 2012), and the Pragmatic Randomized Optimal Plasma and Platelet Ratios trial (PROPPR, 2015). These data constitute the best evidence for resuscitation of massive hemorrhage using balanced ratios of conventional blood products. The American College of Surgeons has followed with educational projects such as the Educational Initiative on Critical Bleeding in Trauma and the Trauma Quality Improvement Project Best Practice Guidelines for Massive Transfusion published in 2012.<sup>26,27</sup> In Northern Europe, a similar process has been led by Johansson through his work at the University of Copenhagen and in the Scandinavian Conferences on Critical Bleeding.<sup>28</sup> The use of whole blood is also returning in the civilian community as described below.

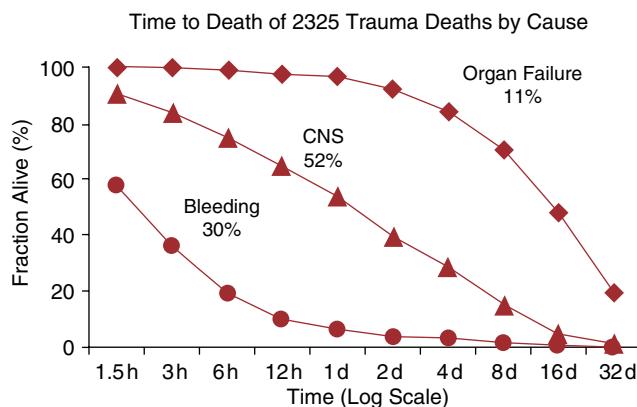
## The epidemiology of physical injury

Injury is common and over the last decade mortality has increased.<sup>29,30</sup> In the United States, approximately 1 individual in 10 receives a significant physical injury requiring medical attention every year and 1 in 100 is hospitalized because of such an injury annually. Approximately 1 in 1000 individuals receives a blood transfusion for injury each year, and just over 180,000 people die of physical injury annually. Costs are greater than \$400 billion each year. About 95,000 of these deaths are the result of unintentional injury, with the remainder divided between suicides using physical methods and deaths from injuries intentionally inflicted by others. Injury is the third leading cause of death overall, and because it occurs so frequently in children and young adults, it is the leading cause of loss of years of productive life through the age of 75 years. As noted, to deal with these injuries, the United States and Canada have established more than a thousand trauma centers and more than 100 burn centers.

Motor-vehicle-related injuries, interpersonal violence, and falls are the most common causes of fatal injuries. Work and recreational accidents also contribute to the toll. All of the common causes of injury are subjects of ongoing efforts at primary prevention through engineering and social controls, but faster cars, higher caliber handguns, and a rising elderly population limit the decline in the overall incidence of both injuries and deaths.

Half of all injury-related deaths in civilians occur outside the hospital, either because of rapid death from massive injury or because they occur in remote or unobserved locations so that movement to advanced care is too late. In the Vietnam War, 85% of battlefield deaths occurred in the field with 40% occurring essentially instantaneously, 65% being dead within 5 minutes, 80% within 30 minutes, and 90% within 2 hours of injury.<sup>31</sup> For civilian casualties that arrive at the hospital alive and subsequently die a hemorrhagic death, a similar compression of early deaths occurs with 50% of such patients dead in two hours and 80% dead in six hours (Figure 40.1). Later deaths are caused largely by central nervous system injury or multiple organ failure.<sup>32</sup> Autopsy series of those who die in the field suggest that 15–20% have a potentially correctable hemorrhagic cause of death.

Blood use in the injured is strongly related to injury severity. In an examination of blood usage rates in a trauma center, only 8% of all admissions received RBCs, and only 3% of all admissions received 10 or more units.<sup>33</sup> Increased anatomic injury severity was strongly associated with increased RBC use and mortality. Nevertheless, there does not appear to be an upper limit to the number of RBC units



**Figure 40.1** Time to death of 2325 trauma patients directly admitted to Maryland Shock-Trauma who survived at least 15 minutes and died in care. Median time to death for patients with uncontrolled hemorrhage as their major cause of death was two hours. Only 4% of all admissions died during their primary hospital admission. Source: Data from Dutton et al. (2010).

that can be successfully transfused to a surviving patient; survivorship after massive transfusion was 60%, after transfusion of 20 U of RBC it was about 45%, and after 50 units of RBC it was in the 15–20% range.<sup>34</sup> Thus, while massive transfusion is associated with a high mortality, it is not a marker of therapeutic futility.

Due to the large distances and limited resources in rural areas in the United States and Canada, access to blood products can be limited. Rural hospitals have a limited supply and can only support the storage of frozen plasma creating inherent delays in administration. This delay creates a need for alternative blood products for austere environments such as freeze-dried plasma and frozen platelets.<sup>35</sup> Further investigations are needed for widespread adoption of these strategies.

### Clinical approach to the trauma patient

Traditionally, there are four phases of trauma care: prehospital, emergency department, operative care, and postoperative intensive care. From the perspective of resuscitation, these phases have become less distinct as hemostatic resuscitation has been pushed further forward into the prehospital phase of care. The military has been pushing blood products into the forward surgical environment, and in the civilian community many aeromedical services and some ground services have begun to carry either red cells and liquid plasma or whole blood. Early studies have demonstrated improved patient outcomes and minimal blood product wastage from these programs.<sup>36</sup>

Half of all civilian trauma deaths occur in the prehospital environment. Rapid movement of patients from the local site where injury occurred to a trauma center provides the greatest benefit because most rapidly fatal injuries are truncal and require surgical or radiographically guided hemorrhage control. Nevertheless, opportunities exist to limit extremity bleeding in the prehospital environment through the use of tourniquets, a measure which has objectively improved prehospital mortality in potentially survivable trauma. This mortality reduction was first described in the military, where Eastridge *et al.* reported a nearly eightfold reduction in extremity hemorrhage deaths after implementation of routine tourniquet use.<sup>37</sup> Teixeira *et al.* noted a similar reduction with tourniquet use in civilians, where prehospital tourniquet application was independently associated with a sixfold mortality reduction in patients with peripheral vascular injuries.<sup>38</sup> Neck, scalp, and truncal soft tissue bleeding can also be reduced by the application of direct pressure

and hemostatic bandages such as QuikClot Combat Gauze, a kaolin impregnated dressing used to stop bleeding with pressure recommended in the Tactical Combat Casualty Care Guidelines.

Keeping patients warm and limiting blood dilution with crystalloids are also important goals. The administration of crystalloid fluids in the field to prevent acute renal failure does not appear to be necessary with modern casualty evacuation times in urban and suburban areas, and raising blood pressure is associated with the loss of more of the patient's blood through vascular defects particularly in penetrating trauma. In situations where red cells and plasma are available in the field or in transport, they can be given, but only about 15% of patients undergoing helicopter transport to trauma centers appear to be candidates for transfusion even when blood products are available.<sup>39</sup> The recent PAMPer study confirmed the benefit of such a practice. This phase 3 cluster-randomized controlled trial enrolled hypotensive tachycardic trauma patients to receive either two units of FFP or standard resuscitation. Primary outcome was 30-day mortality. The plasma group had a significantly lower 30-day mortality (23% vs. 33%,  $p = 0.03$ ) with lower median prothrombin time as well.<sup>40</sup> While the COMBAT study also examined prehospital plasma use in the same trauma patient population, there were some notable differences in comparison with PAMPer. While both groups had similar median BP, COMBAT patients had a higher incidence of penetrating injury (50% vs. 20%) as well as higher ISS (27 vs. 22). In contrast, patients enrolled in COMBAT experienced shorter transport times (median transport time 19 minutes compared to 42 minutes in PAMPer) which could have contributed to the lower mortality in the COMBAT study when compared to PAMPer (12.5% vs. 30%). Taking the information from both studies into account, the benefit of pre-hospital blood product transfusions may only exist with long transport times and in the sickest trauma patients.

In the emergency room, there is an emphasis on rapid and systematic evaluation. The primary survey of airway (A), breathing (B), circulation (C), neurologic disability (D), and full exposure (E) (ABCDEs) is followed by a more systematic evaluation and the mobilization of teams to deal with specifically identified problems. Hypotension and penetrating injury suggest major internal bleeding and urgent need to move to the operating room (OR) as do significant tachycardia and the presence of fluid in body cavities assessed in a focused ultrasonographic survey for trauma (FAST). A combination of these two signs as the ABC score has close to 80% power to predict massive transfusion and served as a major inclusion criterion in the PROPPR study.<sup>41</sup> For patients who do not require immediate operative intervention, further studies leading to a complete secondary survey of the patient are the next goal. Hemorrhage must always remain a concern. There are multiple body cavities where it is possible to lose enough blood to cause shock from hypovolemia: pleural spaces and the mediastinum in the chest, peritoneal and retroperitoneal spaces in the abdomen, pelvis and thighs, as well as subcutaneous spaces. Whole body imaging is important for patients stable enough to tolerate it, and computerized tomographic (CT) scanning identifies additional sites of injury such as intracranial bleeding, pericardial hemorrhage, major vascular disruption, major organ fracture or laceration, and bowel, biliary, and urinary tract disruption that need prompt attention. Moreover, musculoskeletal injuries are frequent and need at least initial stabilization to make patients movable for nursing care. Simply placing a pelvic binder to reduce the volume of the pelvic ring can often limit venous bleeding and prevent further arterial injury in the early phases of patient assessment.

In the OR, time remains of the essence. Historically, it was observed that 40 minutes of open abdominal exposure in a hemorrhaging

trauma patient resulted in the loss of a degree centigrade in core temperature. Thus, attention on the rapid administration of blood products with active warming is important. A damage-control approach to surgery seeks to minimize OR time by focusing on the goals of hemorrhage control and control of contamination from intestinal, biliary, and urinary tract injury with delayed reconstruction. The need for damage-control surgery rates has declined as iatrogenic resuscitation injury is avoided.<sup>42</sup> This can allow for the identification of correctable sources of bleeding that can be dealt with by vascular repair or ligation, repair or removal of injured organs, and closure.

Increasingly, close collaboration between surgeons and interventional radiologists (IR) has led to procedures that are partially performed by one specialty and partially by the other. In order to manage multiple sites of bleeding, some patients require an initial procedure in the OR followed by embolization in the IR suite. Modern hybrid ORs now allow these procedures to be done simultaneously.<sup>43</sup> Transfusion support for such highly complex and high blood loss situations is similar to other massive transfusion situations and involves ongoing balanced resuscitation shaped to the clinical situation and the available laboratory data. At the end of such procedures, patients are moved to intensive care units (ICUs) for continued resuscitation. Bleeding is often slowed but not stopped, and provision for continuing blood product support and planning for potential return of frank hemorrhage if packing fails or is reinitiated with unpacking is prudent. In the recovery room or ICU, bleeding is usually slow enough that its replacement can be guided by laboratory measures. It is still important to remember that the limitations of resuscitation imposed by multiple different blood components diluting each other make it prudent to avoid components such as platelets in additive solution that do not maximize plasma dose.

### Trauma-associated coagulopathy

The physiology of massive injury leads to both rapid degradation of the body's already limited coagulation capacity as well as the release of inflammatory mediators further enhancing the coagulopathy. Dilution, acidosis, hypothermia, consumption, and fibrinolysis all contribute to an inability to take full advantage of the limited blood clotting resources that the body has available.<sup>44</sup> In combination, these factors result in many severely and most profoundly injured patients presenting with coagulopathy when they arrive at medical care.

Loss of coagulation factors and platelets start with the acute blood loss of injury which can be in excess of 40% in patients arriving in deep shock. Attempts to raise blood pressure with a sanguinous fluid to sustain tissue perfusion will lead to further blood loss through uncontrolled defects in vascular integrity. Dilution of platelet numbers and coagulation factor concentrations occurs naturally as remaining intravascular blood is watered down by physiologic vascular refill. Normally, blood pressure pushes water into the tissues which returns through the lymphatic circulation. As blood pressure falls in hemodynamic shock, water moves back into the vascular space down a concentration gradient to dilute the colloid osmotic activity of plasma proteins.

Acidosis, which develops rapidly with tissue hypoperfusion, has profound effects on plasma coagulation. Normally, activated plasma coagulation factors assemble into complexes on negatively charged phospholipid rafts on the surfaces of exposed subendothelial cells, platelets, and endothelium in ways that increase the clotting activity of the enzymes in these complexes by 10,000- to a million-fold. However, the increased concentration of protons present when pH drops destabilizes these coagulation factor complexes and reduces their activities. The reduction is 50% at pH 7.2, 70% at pH 7.0, and 80% at pH 6.8.<sup>45</sup> All these levels are commonly seen in patients suffering severe hemorrhagic shock.

Hypothermia also affects the plasma coagulation enzymes, reducing their activities by about 10% per °C, but actually has a much greater effect on platelet activation. Normally, platelets activate when they adhere to exposed subendothelium through bridging von Willebrand factor. The von Willebrand factor binds to the platelet glycoprotein receptor Ib with one end and to collagen on the other end, creating traction and torsion of the receptor leading to platelet activation. However, this receptor torsion–platelet activation coupling is lost with mild cooling and is essentially gone at 30 °C.<sup>46</sup> In the past, the combination of a low core temperature and severe injury with uncontrolled bleeding was viewed as fatal, but now the combination of extracorporeal blood warming, avoidance of blood dilution, and an array of hemorrhage control strategies allow many trauma patients who present with severe hypothermia to survive.

Consumption of platelets and coagulation factors can occur both within wounds, and diffusely with the embolization of tissue factor-bearing tissue fragments, phospholipids, and diffusion of thrombin. In high energy blunt or penetrating trauma, the extent of endothelial disruption may extend to billions of endothelial microtears, each associated with the exposure of tissue factor-bearing cells and subendothelial basement membrane collagen. Under these circumstances, factors VII, VIII, and V as well as platelets can be depleted. Factor VII is present normally in only nanomolar amounts, so the exposure of a few hundred grams of mesothelial cells presents enough tissue factor to bind all of the factor VII available. Factors VIII and V are classically consumable factors, activated by thrombin and inactivated by protein C. The blood can be exposed to multiple cycles of activation and inactivation in its course through injured tissue with resultant consumptive loss of much procoagulant activity. Finally, platelet activity can be consumed with either reduction in platelet numbers or depletion of platelet granule contents, membrane, and energy. The net result is that severely injured patients typically present with normal numbers of platelets which then decline over the first hour in the hospital, and their remaining platelets appear to have reduced activity, a phenomenon called platelet fatigue.<sup>47</sup>

Fibrinolysis, in the context of trauma-associated coagulopathy, is the early and inappropriate breakdown of fibrin clot resulting in the loss of hemostasis and the substrate for further coagulation and vascular healing. Pathologic fibrinolysis following trauma is typically caused by plasmin or neutrophil elastase. Plasmin is activated by tissue plasminogen activator released in response to low blood flow, and its breakdown is delayed by early destruction of plasminogen activator inhibitor by protein C. Neutrophil elastase is released in large amounts in injured tissue, and levels of its signature fibrin fragments increase significantly following severe injury.

An endotheliopathy of trauma is also being increasingly recognized as another manifestation of trauma-associated coagulopathy. Shock and hypoperfusion lead to shedding of the endothelial glycocalyx along with increased circulating syndecan-1 and danger-associated pattern molecules (DAMPs). The injured endothelium leads to vascular hyperpermeability, while the increased levels of syndecan-1 and DAMPs are associated with shock, inflammation, and are independent predictors of mortality. In animals, vascular hyperpermeability and increased levels of syndecan-1 and DAMPs are attenuated when resuscitation is performed using plasma but not crystalloids.<sup>48</sup>

In severely injured patients, all of these activities limiting stable clot formation can be going on at once. In fact, a review of the risks for uncontrolled coagulopathy following injury by Cosgrove and colleagues found that the risks were additive.<sup>49</sup> Patients with severe injury but without shock, acidosis, or hypothermia were rarely coagulopathic. With profound injury about 10% were coagulopathic at presentation, and with shock requiring diluting resuscitation it increased to 40%. With profound injury and hypothermia,

coagulopathy was present in 50% and with acidosis in 60% of all patients. However, when three or more of these factors were present simultaneously, the incidence of coagulopathy increased to 85–98%. Thus, the most severely injured patients will develop the most profound coagulopathies.

What was not widely recognized until a decade ago is that about one in four severely and profoundly injured patients arrive at the hospital with coagulopathy already established. In 2003, Brohi and his colleagues described a thousand patients arriving at the Royal London Hospital by helicopter from motor vehicle collisions on the London ring road. These patients had received only 400 mL of crystalloid fluids on average before arrival, and yet a quarter showed prothrombin time (PT) prolongation beyond 1.5 times normal.<sup>50</sup> Moreover, the quarter with the prolonged PT had a fourfold excess in-hospital mortality. Brohi called the finding the acute coagulopathy of trauma. A month later, MacLeod and her colleagues from the Ryder Trauma Center in Miami reported a similar finding in 20,000 patients seen over a five-year period.<sup>51</sup> In their series, 28% of all seriously injured patients had a prolonged PT that was associated with a 35% excess in-hospital mortality, and 8% had a prolonged partial thromboplastin time (PTT) that was associated with a 426% excess in-hospital mortality. In 2007, Hess and his colleagues described the prevalence of abnormal coagulation measures in 35,000 direct admissions to the Cowley Shock-Trauma Center in Baltimore over a seven-year period. They showed greater prevalence of PT, PTT, fibrinogen, and platelet count abnormalities with increasing injury severity. Furthermore, the magnitude of these laboratory abnormalities was paralleled by increased in-hospital mortality.<sup>52</sup> At the highest levels of injury severity observed, the prevalence of abnormal admission coagulation tests was 45%, and in-hospital mortality was 80%. In a search for clinical and mechanistic drivers of the acute trauma coagulopathy, Cohen and his colleagues from the Prospective Observational Multicenter Massive Transfusion Trial (PROMMTT) found that 42% of 1198 severely injured patients had an INR  $\geq 1.3$  and that they generally had depressed concentrations of factors I, II, V, and VIII, and evidence of increased concentrations of activated protein C.<sup>53</sup> Decreased concentrations of platelets on admission were much less frequent. As noted, most patients with severe injury present with normal platelet counts. However, when platelet levels on admission are low, a dose-dependent increase for in-hospital mortality is observed.

It should be noted that there has been an argument in the literature about whether the acute coagulopathy of trauma is a separate pathophysiologic entity or is just the early and hypocoagulable form of disseminated intravascular coagulation (DIC).<sup>54</sup> On the one hand, the International Society of Thrombosis and Hemostasis supported the latter view and used trauma as their prototypical example. On the other hand, it is confusing and imprecise to call a condition DIC that is not disseminated, intravascular, or associated with increased coagulation. Rather, others believe that acute coagulopathy of trauma is a concise and descriptive term for a process initiated by multiple local injuries, with hemorrhagic manifestations, and extravascular or at least extra-endothelial mechanisms. This argument is purely semantic.

### **Damage-control resuscitation**

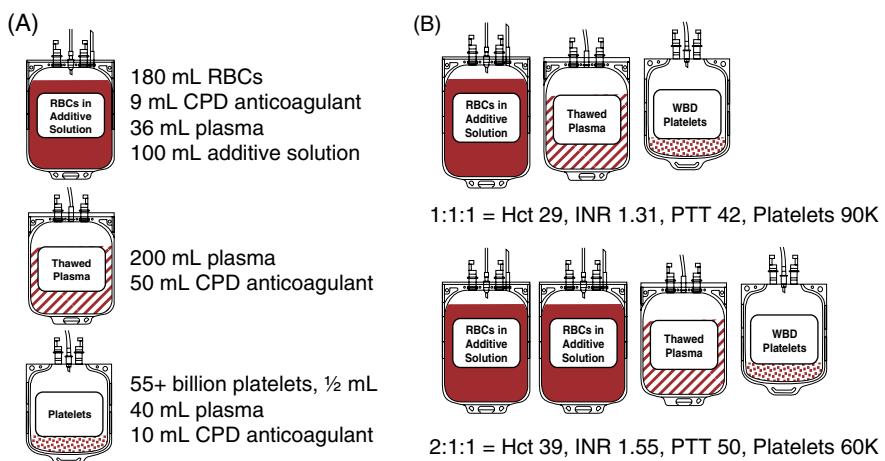
Several large autopsy series suggest that the majority of potentially savable lives that are currently lost to injury are in the group of seriously injured patients with uncontrolled hemorrhage who die in the early hours after presentation.<sup>55,56</sup> The autopsy series suggest that such patients may represent 8–19% of all injury deaths, and the deaths represent a combination of missed diagnostic and therapeutic opportunities that in turn result in delayed hemorrhage control.

As these deaths clearly overlap with the excess mortality associated with the acute coagulopathy of trauma, designing resuscitation systems to limit coagulopathy is a priority.

In an instructive case, a young soldier seen in the combat support hospital in Baghdad with massive injuries from an improvised explosive device received 18 units of RBCs during approximately 50 minutes of hospital care, but died before type-specific plasma was thawed. In reviewing the case among the hospital staff in Baghdad, the surgeons pointed to the need to get plasma into the patient sooner while the supporting transfusion service pointed to the limited supplies of universal donor group AB fresh-frozen plasma (FFP) and the potentially massive waste of plasma products associated with the administrative requirement to use FFP within six hours of thawing. The transfusion service had adopted the protocol of first obtaining the patient's ABO type and only then thawing type-specific plasma to provide the best matched product, limit frozen plasma wastage, and balance usage by ABO type. However, the end result was dead patients. An outside consultant suggested that the problem could be partially ameliorated by the simple expedient of relabeling thawed FFP as "thawed plasma" that could be kept for five days under refrigerated conditions. Furthermore, keeping four units of AB plasma thawed at all times would provide a buffer of plasma for immediate use, and the patients might benefit as well from prompt initial treatment of the acute coagulopathy of trauma. After confirming the definitions of thawed plasma and assuring a continuing supply of AB FFP, this plan was adopted. It was further agreed that units of red cells and plasma would be given alternately to the profoundly injured to keep the combined hematocrit of the administered products close to 30% and provide an easy protocol for administration in the chaotic situation of a massive injury resuscitation (Figure 40.2).

The clinical results were dramatic. All involved in the immediate care of the wounded casualties viewed the new resuscitation strategy as an improvement. More patients appeared to survive initial resuscitation, their injured tissues were easier to treat in the operating room with less spontaneous bleeding and edema, and the required duration of ventilator support was shorter because patients received less blood and fluids altogether. Other surgical groups in theater, both United States and allied in Iraq and Afghanistan, adopted the procedures and by the end of 2004 the use of 1:1 unit ratios of red cells and plasma for resuscitation had become the standard theater guideline. A retrospective review of cases performed at the Baghdad hospital showed a strong correlation between the plasma:red cell transfusion ratio and survival.<sup>57</sup>

Because this 1:1:1 (red cells:plasma:platelets) blood component therapy (BCT) resuscitation strategy was at variance with both conventional surgical dogma and with evolving transfusion medicine doctrine, as exemplified by the drive to reduce plasma usage generally, the authors gathered panels of experts to review the findings. These panels were asked to develop data and position papers on critical issues surrounding early massive transfusion for trauma. The meeting took place at the US Army Institute of Surgical Research in San Antonio, 25–27 May 2005, and the results were published as a supplement to the *Journal of Trauma* in September of 2006.<sup>58</sup> Several major findings were presented in these publications. First, when comparing data from the German Trauma Registry and the US Miami and Baltimore Trauma Registries, significant correlations were observed between common clinical and laboratory data (injury severity, number of units of RBCs transfused, and admission coagulation measures) and outcomes (time to death and in-hospital mortality).<sup>59</sup> Second, in contacting 80 academic trauma centers, formal massive transfusion protocols were rare.<sup>60</sup> Third, a number of high volume trauma centers including those in Sydney,



**Figure 40.2** (A) Whole blood-derived blood components processed by the platelet-rich plasma method have the average contents shown above. (B) Giving RBC, plasma, and platelets in a 1:1:1 unit ratio results in a hematocrit (Hct) of 29%, a plasma concentration of 65%, and a platelet concentration of about 90 K/mcL. An additional unit of RBC dilutes the plasma to 52% and the platelet count to 60 K/mcL while raising the Hct unnecessarily. Source: Data from Arman and Hess, 2003 (*Transfus Med Rev* 2003 Jul;17[3]:223–31) and Kornblith et al., 2014 (*J Trauma Acute Care Surg* 2014 Dec; 77[6]:818–27).

Baltimore, and Helsinki had switched their resuscitation blood orders from emergency un-crossmatched transfusions to type compatible blood in a 1:1:1 BCT ratio as soon as a blood type was available. Fourth, concerns about the safety of red cell and plasma transfusion were widespread, especially among groups writing standards, but the size of the associated risks and the effects of risk reduction strategies such as leukoreduction were not known. Fifth, for the group of severely injured patients with ongoing massive uncontrolled hemorrhage, resuscitation with a 1:1:1 ratio was recommended as long as resuscitation was running ahead of available laboratory data. Finally, it was widely acknowledged that there was a general lack of useful data to allow the identification of injured patients at risk for massive transfusion; determine what the best clinical and laboratory tests were to guide subsequent transfusion; and evaluate the safety and efficacy trade-offs involved in blood-product-based resuscitation. Furthermore, while early balanced resuscitation appeared to reduce morbidity and mortality, no groups performed such resuscitation in an ideal way, and the trauma community was poorly positioned to gather the data that would be needed to justify the costs and risks of such a commitment of resources. The meeting was a watershed event.

In the five years following the conference, a number of retrospective studies of massive bleeding were published that described differences in outcome associated with differences in resuscitation on large, essentially consecutive series of patients. Borgman and his colleagues published the results of the Baghdad series showing a 50% reduction in mortality with high-plasma as compared to low-plasma resuscitation of combat injuries. Johansson and colleagues reported a 33% drop in massive transfusion mortality at the University of Copenhagen Hospital after instituting hemorrhage control resuscitation.<sup>61</sup> Cotton and colleagues reported a 40% improvement in 30-day survival after damage-control laparotomy when damage-control resuscitation was used.<sup>62</sup> Holcomb and the Trauma Outcomes Group gathered 466 cases of massive transfusion from the records of 16 trauma centers and showed a strong relationship between the plasma to red cell transfusion ratio and outcome.<sup>63</sup> Also noted was a strong relationship of platelet count on admission, and platelet transfusions, to outcome. However, all of these important studies were subject to the criticism that it was impossible to

separate out the effects of receiving blood products from the effects of surviving long enough to receive the products, known as “survival bias.”<sup>64</sup> In a review of 10 published series, Stansbury and her epidemiologic colleagues pointed out a number of reasons for believing that the reported effect of a high plasma to RBC ratio was real, most importantly the declining overall mortality. However, the investigators ultimately concluded that randomized and prospective data would be necessary to overcome the biases introduced by patient selection and time to treatment differences.<sup>65</sup>

Obstacles to conducting randomized trials in trauma patients are multiple. They include problems with study design, informed consent, study team building, and support. The trials of recombinant human blood clotting factor VIIa (rFVIIa) conducted by its manufacturer, and of hypertonic saline by the Resuscitations Outcomes Consortium (ROC), provided experience and object lessons. In the rFVIIa hemorrhage trials, informed consent turned out to be a difficult problem as half of all hemorrhagic deaths occurred in the first two hours after arrival in the trauma center, and the mean time to obtaining consent from next of kin or other legally authorized representative was three hours. This meant that a \$40 million trial with a planned mortality of 40% could have only 11% mortality and a statistically negative outcome in the patients who could be consented.<sup>66</sup> Fortunately, the hypertonic saline trials provided experience with conducting large publicly funded resuscitation research under the exception from informed consent (EFIC) regulations of the US government. While these studies showed no benefit for this low-volume resuscitation technique, the investigations were successfully completed and provided a model for the conduct of transfusion resuscitation trials.<sup>67</sup>

The first large prospective trial of hemostatic trauma resuscitation, PROMMTT, was observational only.<sup>68</sup> Designed to gather data in preparation for a randomized trial, in 15 months it screened 12,560 patients with highest level trauma team activations at 10 level 1 trauma centers. PROMMTT enrolled 1245 patients with apparent uncontrolled hemorrhage who survived at least 30 minutes and received at least one unit of red cells within six hours of admission. A subset of 905 of these patients who went on to receive at least three blood products in the next six hours became the analysis cohort. In the first six hours of observation, 95 patients died

including 77 of uncontrolled hemorrhage, whereas in the period between six hours after admission and 30 days only 18 of 125 further deaths were associated with uncontrolled bleeding. A Cox proportional hazards analysis showed that deaths in the first six hours were strongly associated with the ratio of plasma to red cell units transfused; the hazard ratio for death was four times greater in the patients who received less plasma (plasma:red cell ratios of less than 0.5). However, even these high-volume level 1 trauma centers had trouble delivering plasma in a timely manner, 65% of patients received no plasma in the first hour, 40% received no plasma in the first two hours, and 20% received none in the first three hours. Infusion times for platelets were even slower. Median time to hemorrhagic death was 2.6 hours. Ultimately, while 25% of patients in the evaluation cohort died, only one-third died of hemorrhage, and most of them during the first six hours when active resuscitation was occurring. The study showed that it was possible to gather accurate time of transfusion data on large numbers of trauma patients in a short time period. However, the study unfortunately also showed that the process was labor-intensive and required screening large numbers of patients to enroll the few patients likely to benefit. Furthermore, and even in the best centers, the resulting data would still be subject to risks of survival bias because of long delays in delivering plasma and platelets to the bedside. Nonetheless, considering these caveats, timely administration of balanced resuscitation appeared to provide a large therapeutic benefit, reducing hemorrhage-related mortality by as much as 75%.

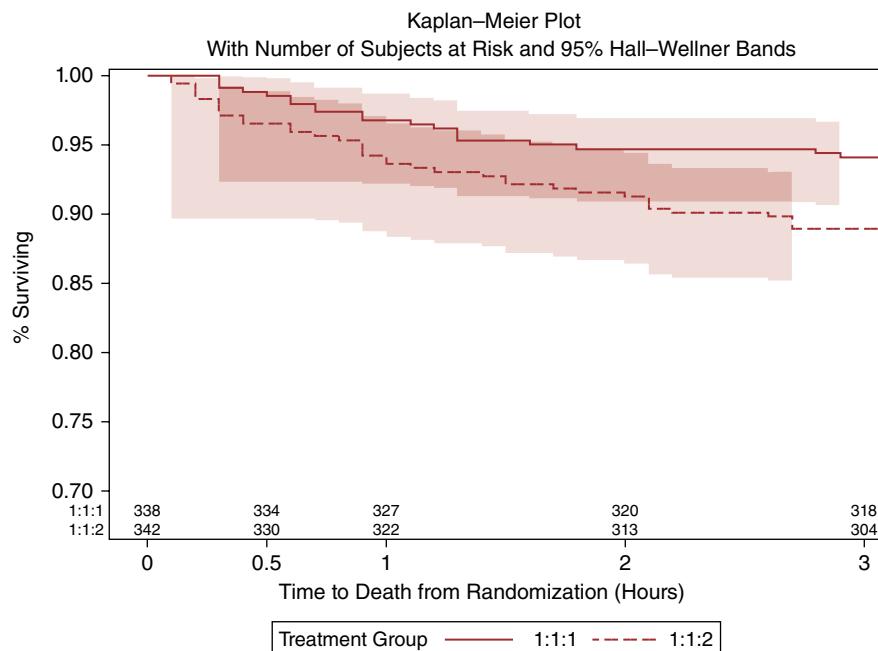
The PROMMTT study suggested that a prospective randomized trial of 1:1:1 BCT transfusion in severely injured and massively bleeding trauma patients was possible. However, such a trial would require access to more than 10,000 trauma patients and the ability to screen patients and enroll those at high risk for active ongoing hemorrhage while simultaneously excluding those with severe head injury unlikely to benefit from resuscitation. It would need to submit them to randomization quickly with EFIC, and to treat them

promptly with blood products available within minutes after arrival so that there were no delays between the time of randomization and treatment that might create survival bias.

The PROPPR study was conducted between August 2012 and January 2014.<sup>69</sup> It screened 14,000 patients and enrolled 680 who had an Assessment of Blood Consumption (ABC) score of 2 or more and no evidence of unsurvivable head injury. The 12 participating trauma centers had to prove that their transfusion service could make six units of universal donor RBCs and plasma available within 10 minutes of notification and have six more units available within 20 minutes of patient arrival. The patients were randomized to receive plasma:platelets:red cells in either 1:1:1 or 1:1:2 unit ratios and followed to 24-hour and 30-day mortality end points. The trial was completed with better than 97% protocol compliance and 100% follow-up for the primary end point.

The results are complicated only in the sense that the US Food and Drug Administration (FDA) required that the primary end points be 24-hour and 30-day survival as a condition of granting the permission to conduct the study under EFIC regulations. The results showed no significant differences between these two ratios at these primary end points. However, over the course of resuscitation from 10 minutes to 3 hours, a significantly greater proportion of patients treated with the 1:1:1 ratio survived, and the absolute difference in mortality was significant at the end of resuscitation and persisted from 3 hours to the end of the study (Figure 40.3). In the PROPPR study, the number of hemorrhagic deaths occurring during resuscitation was reduced by almost 50%, and the relative reduction in total deaths over 30 days was 18%. Overall mortality in the treated arm was 18%, much lower than the 40–70% mortality in similar patients in series reported a decade ago.

What the PROPPR study did not answer was whether the benefit of balanced hemostatic resuscitation was the result of the extra plasma, the platelets, or both. It addressed only the difference between resuscitation with a 1:1:1 versus a 1:1:2 BCT ratio. Planned



**Figure 40.3** Mortality in the resuscitation phase of the PROPPR trial comparing resuscitation with a 1:1:1 unit ratio of RBC-plasma-platelets with a 2:1:1 ratio as a Kaplan-Meier plot with 95% Hall-Wellner confidence intervals. Data from Holcomb et al. (2015).

blood sampling at admission and two hours after admission missed most of the intermediate differences that led to excess early deaths in the 1:1:2 arm. Regardless of these caveats, rapid delivery of balanced hemostatic resuscitation has become the new standard of care. It has been adopted by the American College of Surgeons in their Trauma Quality Improvement Guidelines for Massive Transfusion Protocols required for trauma center accreditation,<sup>70</sup> and it is required for Patient Blood Management program accreditation through the AABB.<sup>71</sup>

## Clinical approach to the mass casualty trauma situation

Mass casualty trauma situations are uncommon, and those requiring large amounts of blood are rare. In the United States, between the years 1975 and 2000 there were only such four events that required more than 100 units of RBCs to treat all of the resulting casualties in their first 24 hours of care.<sup>72</sup> Furthermore, these events are used by media to encourage the population to donate blood, resulting in a temporary increase in blood collections and a subsequent outdating of large quantities of that blood. For example, 600,000 extra units of blood were collected after 11 September 2001, most of which had to be destroyed. The exception to this is mass shootings, with the deadliest being the Las Vegas shooting on 1 October 2017. This mass casualty event resulted in 58 deaths and injured more than 600 people. The shooting utilized more than 500 units of blood in the first 24 hours, while 800 units of blood were donated by the public immediately after the shooting, resulting in only 17% waste.<sup>73</sup>

The rarity of massive blood use in multicasualty events is to be contrasted with the frequency of massive blood use in single individuals with poly-trauma or transplant; these cases more commonly create situations where one patient uses more than 100 units of RBCs in 24 hours. A critical difference between these scenarios is the need to have well-practiced protocols to maintain the unique identity of each patient in a multicasualty setting. The use of universal donor products in disaster situations where casualties may be unidentified or more importantly misidentified is an important precaution. It should be remembered that more than 100,000 universal donor whole blood and red cell units were given in the Vietnam War and that all seven fatal acute hemolytic reactions there were associated with the misidentification of recipients of crossmatched red cells.

## Initial resuscitation of the burn patient

The resuscitation of patients with severe burns is different than that of patients with penetrating and/or blunt force trauma. For trauma patients in hemorrhagic shock with ongoing acute blood loss, the priorities are hemorrhage control and resuscitation with blood products. Burn patients without mechanical trauma, however, develop hypovolemia due to systemic capillary leak. Often these patients will have a higher initial hemoglobin concentration due to the redistribution of plasma water into the interstitium and intracellular space. The priority in burn patients is to replace this volume loss and prevent the patient from progressing to severe hypovolemic shock.<sup>74</sup> Therefore, the initial resuscitation utilizes crystalloid and colloid solutions, and blood product transfusion is less common. However, later during the treatment course, cumulative blood loss increases due to burn excision and grafting, anemia of critical illness, and laboratory testing. It is during this latter period

in the hospital course that attention should be given to increased blood product utilization and the type and quantity of blood transfusions given to patients with severe burns.

In the initial period (24–48 hours after injury), resuscitation is conducted according to standard burn resuscitation formulas; however, no single formula provides an exact volume required. Resuscitation formulas are a starting point, from which physicians will need to titrate fluid volumes to the patient's response. Burns of less than 20% total body surface area (TBSA) typically do not require fluid resuscitation, except in children and older adults, for whom a 15% TBSA cutoff is generally used. Utilizing the American Burn Association Consensus Formula, fluids for the initial 24 hours resuscitation are calculated at 2–4 mL/kg of fluid per percentage of TBSA. The first half of the calculated volume is given over the first eight hours from injury, not from the beginning of resuscitation; the rest is given over the next 16 hours.<sup>75,76</sup> Lactated Ringers' solution is preferred given the large volumes administered and increased electrolyte derangements with other isotonic fluids. Children should also receive maintenance fluids containing dextrose in addition to the resuscitative fluid. The presence of inhalation injury can increase the volume of fluid required to achieve adequate resuscitation. Colloids may minimize the total volume of fluid administered during resuscitation, though it is debated whether or not this improves mortality or outcomes like respiratory complications. Albumin 5% is the colloid of choice started at a rate of 0.3–0.5 cc/kg/%TBSA and titrated alongside the crystalloid rate based on resuscitation end points. Colloids should be considered for patients with extensive burns or for those requiring fluid volumes in excess of Consensus Formula prediction.<sup>77–79</sup> Fluids are titrated to the patient's specific hemodynamics with a goal urine output of 0.5–1 mL/kg/hour.<sup>76</sup> It bears repeating that the consensus formula is a starting point, fluid must be titrated to urine output and patient physiology.

Release of vasoactive mediators from injured tissue results in a diffuse capillary leak starting soon after injury. In larger burns, this occurs in both the burned and unburned tissues. Immediately after injury, the loss of microvascular integrity results in extravasation of crystalloid and colloid solutions. However, after 6–12 hours, this will begin to subside in nonburned tissue, and this is the time frame in which many centers will initiate albumin resuscitation.<sup>76</sup>

It is important to note that there is renewed interest in the use of plasma in the resuscitation of burn patients. While plasma was used historically as a volume expander in burns, it is not widely used in modern burn centers. However, animal models have demonstrated that plasma may have benefits beyond its oncotic effects. In particular, plasma may play a role in preserving the endothelial glycocalyx (EGC). In a rat burn model, resuscitation with plasma was associated with preservation of the pulmonary ECG.<sup>80</sup> Currently, there is a multicenter randomized trial to investigate the use of plasma in acute burn resuscitation.

## Transfusion therapy in the care of the severely burned

Patients with less than 10% total body surface area (BSA) burns rarely receive transfusions.<sup>81</sup> Increasing burn size, age, and presence of anticoagulants significantly increases the use of blood transfusions, but deciding when to transfuse can be difficult.<sup>82</sup> The hypermetabolic response to severe burns significantly alters normal physiologic parameters that are commonly relied upon to determine transfusion indications. When considering transfusions in

burn patients, several factors are evaluated including the acuity of the anemia, blood loss mitigation techniques, and the potential physiologic benefits as compared to transfusion risks.

During the initial resuscitative period, burned patients will have an acute blood loss anemia that is not associated with large volume blood loss, but rather from destruction of erythrocytes within the cutaneous circulation and by hemorrhage into the burn wound. This initial anemia is typically not significant enough to require transfusion as evident by data showing an average of  $5.3 \pm 0.3$  days from admission to the first transfusion.<sup>81</sup> The greatest blood loss, and subsequently the use of large volume transfusions, occurs during burn wound excision and grafting. Further blood loss can occur from postoperative dressing changes on areas of open wounds. The average transfusion requirement for a 40% burn, for example, is at least 11 units of packed red blood cells.<sup>83</sup>

Efforts made to mitigate the use of transfusions during burn excision include the use of hemostatic techniques such as topical thrombin, epinephrine-soaked gauze, tourniquets, and fibrin sealants.<sup>84,85</sup> Intraoperative blood loss is an important factor limiting the extent of excision and grafting performed. Initial steps to control hemorrhage use electrocautery devices or pressure. Thrombin spray and laparotomy pads saturated with thrombin and epinephrine can be applied directly to the wound bed to limit intraoperative blood loss. The use of tourniquets for excision and grafting of extremities can further decrease blood loss during extremity excision.<sup>86</sup> As in the care of trauma patients, the importance of normothermia in preventing coagulopathy is recognized, and patients are actively warmed, or more importantly not allowed to get cold at the time of surgery.

Data from a multicenter study evaluating the effects of blood transfusion in severe burns revealed that 70% of blood transfusions for burn victims occur outside of the operating room.<sup>87</sup> This anemia is multifactorial in origin and due in part to mild ongoing blood loss from dressing changes and phlebotomy, impaired red blood cell production from erythropoietin insensitivity, malnutrition and metabolic demands. As with operative excisions, some efforts can be undertaken to mitigate this anemia as well. Minimizing blood draws and using pediatric blood collection tubes can decrease iatrogenic anemia. Maintaining adequate nutrition is important for all patients, and some studies have identified up to 13% of critically ill patients are deficient in nutrients necessary for erythropoiesis.<sup>88</sup> Administration of exogenous erythropoietin has been extensively studied in multiple critically ill populations but has yet to show any efficacy in patients with burns.

Transfusing patients with thermal injuries must be tempered with the known risks and benefits of blood and blood products. Increasing hemoglobin concentrations is the only method to increase oxygen carrying capacity, but the known transfusion associated morbidity documented in multiple specialties is also seen in burn patients. The safety of a restrictive transfusion strategy, to maintain a hemoglobin level of 7–8 mg/dL, was demonstrated in the Transfusion Requirements in Critical Care (TRICC) trial.<sup>89</sup> However, patients with a 3 g/dL drop in hemoglobin or ongoing bleeding were excluded, thus the applicability of this trial to the burn population is limited. Nonetheless, burn patients remain at risk for complications of transfusion. In a large, multicenter retrospective analysis of 666 burn patients, the number of infections per patient increased with each unit of blood transfused. This translated to a 13% increase in developing an infection per unit of blood transfused.<sup>81</sup> The Transfusion Requirement in Burn Care Evaluation (TRIBE) study was a multicenter, controlled trial that randomized

345 burn patients across 18 centers to a restrictive or more liberal transfusion strategy.<sup>90</sup> The restrictive group received a median 8 (IQR 3–24) units of red cells compared with a median of 16 (IQR 7–40) in the liberal group ( $p < 0.0001$ ). There were no differences in any outcome, including mortality or infection. No intraoperative adverse outcomes were related to protocol adherence. This study provides level 1 evidence for pursuing a more restrictive transfusion strategy for red cells in burn patients.

The appropriate use of plasma during intraoperative blood loss from burn wound excision is not well defined. As described above, increasing the volume of plasma in relation to packed red blood cells has shown definitive benefit in trauma patients with hemorrhagic shock. In one study comparing higher FFP usage in burn patients (1:1 vs a 4:1 [RBC:FFP]), there was a decrease in overall blood product usage and a trend toward shorter duration of hospital stay, infectious complications, and organ dysfunction in the 1:1 group.<sup>87</sup> However, in military patients with severe thermal injuries, the use of plasma was associated with the development of acute respiratory distress.<sup>91</sup> There is a recent trend to use viscoelastic analysis in guiding transfusion resuscitation, and early use of this test during burn wound excision shows promise in reducing the amount of blood products transfused, but further investigation is warranted.<sup>92</sup>

## Adjuncts in transfusion therapy for trauma and burn patients

### Tranexamic acid

Tranexamic acid (TXA) and epsilon-aminocaproic acid are lysine analogs used as antifibrinolytics.<sup>93</sup> Newly formed fibrin polymer has exposed lysine residues that serve as binding sites for plasmin and facilitate the plasmin-dependent breakdown of fibrin polymer. During normal coagulation, a strong thrombin burst activates the thrombin-activated fibrinolysis inhibitor (TAFI) that removes these lysine residues to prevent fibrin breakdown.<sup>94</sup> However, after trauma and especially with the acute coagulopathy of trauma, the intensity of thrombin activation is reduced and as a result TAFI is not activated. Furthermore, activation of plasmin by tissue plasminogen activator secreted in response to hypotension and inactivation of plasminogen activator inhibitor by activated protein C led to high plasmin activity. The combination of high plasmin activity and widely available plasmin binding sites on fibrin leads to rapid degradation of fibrin polymer. Using TXA to inhibit fibrinolysis stabilizes newly formed clot and reduces blood loss.

TXA has been demonstrated to reduce blood loss in several non-trauma settings such as knee and hip joint replacement, craniosynostosis surgery, and after tooth removal in patients with hemophilia. Knee and hip joint replacement trials have shown reduced blood drainage after surgical site irrigation with a suspension of the drug.<sup>95</sup> In infants undergoing craniosynostosis surgery, a randomized trial demonstrated an almost 50% reduction in blood loss and red cell replacement requirements with IV TXA.<sup>96</sup> In hemophilia patients undergoing dental extraction, oral wash and swallow with a suspension of the drug reduced the duration of bleeding.<sup>97</sup>

For trauma patients, the international CRASH-2 trial and the MATTERs study are the basis for recommendations for wide use of the drug. The CRASH-2 trial was a large simple randomized trial of TXA use or nothing in 20,211 trauma patients in 274 hospital in 40 countries.<sup>98</sup> Use of the drug led to a 1.5% reduction in overall mortality with a reduction in the odds of hemorrhagic mortality by 15%. However, in patients treated more than three hours after

injury, mortality was increased. Criticisms of the CRASH-2 trial include the participant centers were mostly in third world countries, only half of the patients enrolled received a blood transfusion, and only 30% of the patients were hypotensive. These caveats have led some to question the mechanistic impact of TXA. The Military Application of Tranexamic acid in Trauma Emergency Resuscitation study (MATTERs) was a retrospective review of 896 consecutive admissions to a British Army medical facility in Afghanistan that demonstrated better survival in patients treated with TXA.<sup>99</sup> The mortality benefit observed in patients receiving TXA was almost 50% in those receiving more than 10 units of RBCs. Lastly, there exists a potential benefit for TXA in isolated Traumatic Brain Injury patients as demonstrated in the CRASH-3 trial (mortality 18.5% with TXA and 19.8% without); however, the mortality benefit did not carry over to a recently published US multicenter RCT (14% vs. 17%,  $p = 0.26$ ).<sup>100</sup>

Despite occasional reports of thrombotic complications after TXA administration, a large meta-analysis of randomized trials of the drug did not reveal excess thrombosis or mortality after its use. This suggestion of broad safety has led to recommendations that TXA be given in prehospital situations and this drug is rapidly becoming a standard of care for hemorrhagic shock trauma patients.

Interestingly, studies suggest that fibrinolysis is not a major contributor to coagulopathy in burns, and the use of TXA or other fibrinolytics should be limited to burn patients with demonstrated fibrinolysis.

### **Plasma-derived coagulation factors**

Plasma-derived coagulation factors potentially allow rapid restoration of coagulation system activity in severely injured patients. Recent studies have focused on the use of four-factor prothrombin complex (PCCs) and recombinant activated factor VIIa (rVIIa). Potential benefits of PCC include its ease of storage and reconstitution, high concentrations of extrinsic pathway factors, blood-type independence, low volumes, and virologic safety profile. Disadvantages include the 20-minute reconstitution time, the lack of other plasma proteins including factors I, V, VIII, XI, XIII (which, when deficient, confer a very high mortality), as well as the low volume of the product that does not address volume loss in a hemorrhaging trauma patient.

Data regarding four-factor PCC in trauma is limited. A small single center randomized controlled trial from Innerhofer and colleagues in Austria randomized coagulopathic trauma patients to FFP or coagulation factor concentrates. The study was halted early due to medical safety concerns as the FFP group required more massive transfusion and (30% vs. 12%) and had a higher need for rescue therapy (52% vs. 4%).<sup>101</sup> A retrospective propensity matched ACS-TQIP study by Zeeshan and colleagues demonstrated reduced blood product transfusion (6 units vs. 10 units,  $p = 0.02$ ), reduced respiratory distress syndrome (1.3% vs. 4.7%,  $p = 0.04$ ), and a lower mortality (17.5% vs. 27.7%,  $p = 0.01$ ) in patients receiving PCC.<sup>102</sup> While both studies had numerous limitations, the results are intriguing and pave the way for future multicenter randomized controlled trials to determine the role for PCCs in the management of hemorrhaging trauma patients who need volume as well as clotting factors.

rVIIa has been identified as an additional adjunct to the treatment of the bleeding trauma patient. Boffard *et al.* renewed trauma interest in the drug after publishing a randomized controlled trial demonstrating reduction in blood product usage (by 2.6 units) and need for massive transfusion, as well as trends toward reduced mortality.<sup>103</sup> A subsequent retrospective cohort study by Rizoli *et al.* out of

Toronto again found decreased blood product usage and a significant mortality benefit (OR 3.5); however, the study had few patients receiving the rVIIa (n=38) with expected wide range of potential benefit.<sup>104</sup> rVIIa received additional attention in 2009 with the FAST trial in acute cerebral hemorrhage patients with the medication being associated with decreased growth of the hematoma; however, there was no impact on survival or functional outcome.<sup>105</sup> With earlier access to blood products and restricted crystalloid use, the initial enthusiasm for using rVIIa in mature trauma systems has faded.

### **Cryoprecipitate**

The glycoprotein fibrinogen has been shown to mediate platelet and endothelial function and is thought to play a role in the stabilization of syndecan-1 and correction of endotheliopathy (in addition to the effects stated above). There is also evidence that fibrinogen is the first clotting factor to become critically low in trauma patients and is independently associated with mortality.<sup>106</sup> Cryoprecipitate is the most common method of fibrinogen supplementation. Prepared from plasma, cryoprecipitate contains fibrinogen, von Willebrand factor, factor VIII, factor XIII, and fibronectin. CRYOSTAT-1 was an unblinded RCT in 2015 that demonstrated the feasibility of early cryoprecipitate transfusion in bleeding trauma patients<sup>107</sup> CRYOSTAT-2 is an international multicenter randomized controlled trial designed to determine whether the addition of early cryoprecipitate to massive transfusion protocols improves survival in major trauma hemorrhage.

### **Freeze-dried plasma**

While high ratios of plasma:pRBCs and early plasma transfusion have been shown to reduce mortality and correct trauma associated coagulopathy, there remain significant limitations to plasma-first resuscitation including the transport, storage, availability, and administration of FFP. Due to these factors, there is a renewed interest in freeze-dried plasma as it is easier to store (room temperature), readily transportable, and reconstitutes easily (three minutes). This has led to approval in Germany, France, and South Africa, as well as FDA emergency use authorization for military use in the United States in 2018. Numerous studies have shown that clotting factors are well preserved in freeze-dried plasma, and that this product can attenuate trauma-induced coagulopathy in animals and *in vitro*. Several studies have also confirmed its safety profile and efficacy in trauma patients. A retrospective 2018 study at a level 1 trauma center by Nguyen *et al.* examined the effects of French lyophilized plasma versus FFP in patients receiving 2U RBCs.<sup>108</sup> They found several advantages of lyophilized plasma, including a shorter time to transfusion, a higher plasma:RBC ratio, faster time to 1:1 ratio, and significantly fewer cases of massive transfusion. This retrospective study's findings were corroborated in a French randomized controlled trial by Garrigue *et al.* in 2018.<sup>109</sup> This trial enrolled adult trauma patients deemed by the attending physician to need massive transfusion who were subsequently randomized to French lyophilized plasma or FFP. The lyophilized plasma group had a shorter time from randomization to transfusion of plasma (14 minutes vs. 77 minutes) leading to higher fibrinogen and factor concentrations and reduced prothrombin times. Both of these studies were carried out at centers without access to thawed plasma, so the improvements were likely related to shorter times to plasma transfusions. Lyophilized or freeze-dried plasma present reasonable alternatives for hospitals without access to thawed plasma (due to reduced need or increased waste); however, the FDA has not yet approved this product for civilian use.

## Whole blood

With the trend of reduced mortality with BCT in 1:1:1 physiologic ratios, there has been a renewed interest in whole blood (WB) transfusion. The concept is logical, to replace bleeding trauma patients blood loss with equivalent ratios of three separate blood products or whole blood. Whole blood has a 21-day shelf life at 4 °C. Several retrospective military studies have demonstrated a mortality benefit when WB was used.<sup>110,111</sup> Cotton *et al.* performed a pilot RCT in 2013 comparing WB without platelets and BCT in a civilian patient population of 107 patients and failed to find a difference in transfusion volumes or a mortality benefit.<sup>112</sup> However, when excluding TBIs in the sensitivity analysis, there was significantly reduced transfusion volumes in the WB group. This study is supplemented by a propensity matching study at two level 1 trauma centers by Hazelton *et al.* comparing 91 WB and 182 BCT hypotensive bleeding trauma patients.<sup>113</sup> This study demonstrated that WB transfusions increased hemoglobin and hematocrit at 24 hours and decreased trauma bay mortality (2.2% in WB, 8.8% in BCT,  $p = 0.039$ ); however, there was no difference in 30-day mortality. Finally, Hanna and colleagues performed a retrospective TQIP study of transfused trauma patients, comparing those that received WB and BCT to therapy with BCT alone.<sup>114</sup> While this is a database retrospective study, they did find that WB transfusions were associated with significantly lower 24-hour mortality (17% vs. 25%,  $p = 0.002$ ), reduced in-hospital mortality (29% vs. 40%,  $p < 0.001$ ), fewer major complications (29% vs. 41%,  $p < 0.001$ ), and shorter length of stay (9 vs. 15 days,  $p = 0.011$ ). Overall, the results are promising, and many major trauma centers have implemented WB resuscitation into their transfusion algorithms (as well as the prehospital algorithms). However, more conclusive randomized controlled trials are needed prior to exclusively focusing on WB resuscitation early in hypotensive trauma patients.

## Summary

As described in this chapter, transfusion support of the trauma and burn patients has undergone significant improvements over the last two decades. For example, recent modifications to trauma resuscitation and trauma care protocols have led to an almost

50% reduction in the incidence of hemorrhagic deaths in trauma centers carrying out damage-control resuscitation. These improvements in BCT, which were validated in the PROPPR study, centered on the early administration of balanced hemostatic resuscitation with plasma and platelets aimed at early correction of the acute coagulopathy of trauma. The reduction in blood transfusion in the care of burned patients has also been dramatic (three- to fivefold) as burn surgeons have learned to manage blood loss and accept lower transfusion triggers. These are examples of patient blood management evolving in surgical fields, sometimes occurring independently of transfusion-medicine-driven changes.

The challenges of extending these improvements in care to injured patients arriving in smaller centers and to soldiers and civilians in the field will center on developing ways to deliver current blood products earlier in care and the wider use of hemostatic adjuncts such as tranexamic acid, hemostatic bandages, and fibrinogen concentrates. It will also require a national commitment to collecting the amounts of universal donor blood products needed to make the system work. Achievement of this goal will additionally be facilitated by developing a new generation of blood products such as more accessible freeze-dried plasma, more effective plasma derivatives, and frozen platelets.

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## CHAPTER 41

# Transfusion support for the oncology patient

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Transfusion support for patients with cancer is a complex, multifaceted medical therapy meant to assist in the management of complications related to chemotherapy, radiation, transplantation, or widespread metastatic disease. Oncology patients frequently require chronic transfusion support and present several unique challenges in providing appropriate blood products. For these patients, greater attention must be paid to the selection, preparation, modification, and response to blood components to help ensure better outcomes. This chapter highlights the most challenging aspects of transfusion therapy for oncology patients encountered by clinicians and transfusion services on a routine basis.

## Red cell transfusion

### Indications

The goal of red blood cell (RBC) transfusion in oncology patients is the same as in other populations—to increase the oxygen-carrying capacity of whole blood for patients with anemia. Unfortunately, there is currently no evidence-based laboratory criteria upon which to determine when transfusion is appropriate for oncology patients. Studies in other critically ill patient populations have shown that hemoglobin levels of 7–10 g/dL can be tolerated without the need for allogeneic transfusion.<sup>1</sup> There is no evidence to suggest that higher hemoglobin levels provide any therapeutic benefit, and there is some evidence to suggest that “hypertransfusion” may be detrimental to patient outcomes.<sup>2</sup> Therefore, oncology patients should be transfused for symptomatic anemia or at predetermined hemoglobin or hematocrit levels defined by a hospital’s oncology service.

### Selection of ABO group for RBC transfusion

For most oncology patients, no special consideration is required for the appropriate selection of ABO group for RBC units as well as other blood products. However, for patients who have received an allogeneic hematopoietic stem cell transplant (HSCT), selecting the appropriate ABO group can be more difficult. This is particularly true in the case of ABO mismatch between the hematopoietic progenitor cell (HPC) donor and recipient. Up to half of HSCTs are

ABO mismatched as a consequence of priority given to matching for human leukocyte antigens (HLA).<sup>3,4</sup> While ABO mismatch between the donor and recipient does not seem to have any short-term drawbacks, providing appropriate transfusion support in the period after transplantation can be complex.<sup>5,6</sup> These patients generally require more transfusion support due to delayed cellular engraftment and red cell aplasia. They are also at risk for acute and delayed hemolytic reactions from ABO incompatibility while engraftment occurs.<sup>6,7</sup> Although outcome results following ABO mismatched HSCTs have been mixed, ABO-mismatched HSCT does not appear to adversely impact outcome or long-term survival in either pediatric or adult populations.<sup>5,6,8</sup>

An ABO mismatch, however, can lead to concerns during HSC infusion as well as post-transplantation. In the setting of allogeneic transplantation, there are several possibilities for ABO antibody mismatch including minor mismatch, major mismatch, as well as a combination of both a minor and a major mismatch. When the donor possesses antibodies against recipient red cells, patients are deemed to have a minor mismatch. An example of a minor mismatch is when a patient (recipient) with group A red cells receives HPCs from a group O donor. On the other hand, a major mismatch occurs when the recipient has antibodies targeted to donor red cells. This most frequently occurs when a group O patient (recipient) receives HPCs from a group A, B, or AB donor. Finally, “two-way” incompatibility can occur in the presence of both a major and a minor mismatch, for example when a group A patient (recipient) receives HPCs from a group B donor. Table 41.1 summarizes both major and minor incompatibility scenarios by blood group. The key to successful transfusion for any of these mismatches is to prevent or reduce hemolysis during HPC infusion. For patients with a minor mismatch, this can be achieved by plasma reduction of the HPCs to significantly lower the amount of offending antibody. For patients with a major mismatch, red cell depletion of the HSC product can be used to prevent acute hemolysis during infusion.<sup>9</sup> However, it is important to keep in mind that the latter process will likely decrease the content of progenitor cells in the product as well. Therapies used post-transplant to manage red cell aplasia include glucocorticosteroids, immunosuppressive agents, and rituximab, among others. In addition, recent reports have shown success with

**Table 41.1** Compatibility by ABO Group in Hematopoietic Stem Cell Transplantation

Recipient ABO Blood Group	Donor ABO Group			
	O	A	B	AB
O	Compatible	Major	Major	Major
A	Minor	Compatible	Major and minor	Major
B	Minor	Major and minor	Compatible	Major
AB	Minor	Minor	Minor	Compatible

Compatible, major incompatibility, minor incompatibility, and major and minor incompatibility ("two-way" incompatibility).

**Table 41.2** Transfusion Protocol for HSCT Patients

Recipient	Donor O			Donor A			Donor B			Donor AB		
	RBC Type	FFP/PLT Type	Second Choice PLT	RBC Type	FFP/PLT Type	Second Choice PLT	RBC Type	FFP/PLT Type	Second Choice PLT	RBC Type	FFP/PLT Type	Second Choice PLT
O	O	O	A or B	O	A	O	O	B	O	O	AB	A
A	O	A	O	A	A	O	O	AB	A	A	AB	A
B	O	B	O	O	AB	A	B	B	O	B	AB	B
AB	O	AB	A	A	AB	A	B	AB	B	AB	AB	A or B

NOTE: If a recipient is Rh negative, they will continue to receive Rh negative products.

the use of daratumumab, the human monoclonal antibody against CD38, for the treatment of refractory red cell aplasia following ABO-mismatched HSCT.<sup>10</sup>

Transfusion support in the post-transplant period requires careful consideration given that antibodies can persist for weeks to months after transplantation, which can lead to ongoing issues in the selection of proper blood components. Patients can also present as chimeras after HSCT, with two distinct blood groups seen by routine blood typing.<sup>11</sup> Fortunately, standardized strategies have been developed to provide the most compatible ABO components for these patients. For patients with major incompatibility from recipient anti-A or anti-B directed to donor red cells, recipient-type RBC units are necessary until ABO antibodies are no longer detected.<sup>12–14</sup> For patients with minor incompatibility, recipient-type plasma and platelets should be used until the recipient's red cells are no longer detected. Donor-type RBC units can be used immediately after HSCT for patients with minor incompatibility.<sup>12–14</sup> Patients with two-way incompatibility require group O RBC units and group AB plasma and platelets until the offending antibodies and cells are no longer detectable. Table 41.2 provides a transfusion protocol that can be followed for ABO-mismatched HSCT.<sup>12–14</sup>

If a recipient is transplanted with a Rh negative HPC, they will receive Rh negative products.<sup>25</sup>

### Alloimmunization to red cell antigens

Despite significant immunosuppression, patients with malignancy can still mount immune responses to foreign red cell antigens, which can complicate transfusion and increase the risk for hemolysis.<sup>15,16</sup> Rates for red cell alloimmunization in oncology patients have been most studied in patients with hematologic malignancies undergoing HSCT. These studies have shown that alloimmunization occurs in 2–8% of patients in these populations.<sup>17,18</sup> Additionally, a multicenter case-control study evaluated the association of various malignancies and treatment modalities with alloimmunization rates and found reduced rates of alloimmunization in patients with

acute leukemia and mature lymphoma as well as stem cell transplant patients compared to patients without these malignancies. On the other hand, for patients with solid tumors or other oncologic conditions, the study found similar rates of alloimmunization compared to the general transfused population.<sup>19</sup>

### Component modification: leukocyte reduction and irradiation

Prestorage leukocyte reduction techniques decrease the number of white blood cells within transfusion components, which has been shown to reduce the risk of HLA alloimmunization.<sup>20,21</sup> In addition, leukocyte reduction can lower the rate of blood-borne transmission for pathogens that are carried within white cells, such as cytomegalovirus (CMV).<sup>22</sup> According to the 2017 National Blood Collection Utilization Survey, approximately 95.8% of all red cell units in the United States were leukocyte reduced, most before storage.<sup>23</sup>

Despite the use of leukocyte-reduced units, however, many oncology patients are still at risk for transfusion-associated graft-versus-host disease (TA-GVHD) because of their immunocompromised state. In cases of TA-GVHD, donor lymphocytes generate a profound immune response against the recipient's cells.<sup>24</sup> The underlying causes and manifestations of TA-GVHD are discussed in Chapter 50. Prevention of TA-GVHD can be accomplished by either gamma or X-ray irradiation of cellular blood components.<sup>25</sup> Some in the field of transfusion medicine have argued for a policy of universal blood component irradiation as the process has few side effects and may prevent cases of TA-GVHD in patients whose high-risk status is not known.<sup>26</sup> However, the practicality and cost-effectiveness of such a policy are still under review. The main disadvantage of irradiation is the induction of a potassium leak within the red cell membrane, thereby shortening the shelf of an RBC unit to no more than 28 days from the date of irradiation or the original expiration date, whichever comes first. There are no such concerns regarding the irradiation of platelets or plasma products. The latter component does not require irradiation as it does not contain viable cells.

### Alternatives to allogeneic red cell transfusion

For oncology patients undergoing a surgical intervention, intraoperative blood recovery—a process where shed whole blood is centrifuged, washed of contaminants, and then reinfused—may be of benefit to reduce allogeneic blood usage.<sup>27</sup> Although concern has been raised about the promotion of tumor cell metastasis with the use of such blood recovery procedures in some cancer patients, several trials conducted in patients with hepatobiliary and genitourinary cancers have shown no evidence of decreased patient survival.<sup>28–31</sup> Additional techniques including acute normovolemic hemodilution (ANH) and the use of preoperatively donated autologous whole blood can also be considered. However, these techniques are often not feasible in oncology patients who are often too anemic and who can require surgery at unpredictable times. The lack of cost-effectiveness for preoperative autologous donation also limits its utility to oncology patients undergoing major surgery.<sup>32</sup>

Another alternative to allogeneic red cell transfusion utilized in the past was the administration of erythropoietin-stimulating agents (ESAs). However, given the growing awareness of the severe associated risks, ESAs are no longer recommended. Several studies have found that the use of ESAs in patients with cancer and other critical illnesses can lead to an increased risk for venous thromboembolism and stroke.<sup>33</sup> In addition, erythropoietin may accelerate tumor growth and lead to decreased survival. Finally, studies have also shown that the use of ESAs does not significantly decrease the need for allogeneic transfusion in critically ill patients.<sup>34,35</sup> These findings led to a black box warning regarding the use of ESAs in the setting of cancer-related anemia.<sup>36</sup> As such, it is recommended that before initiation of an ESA, a detailed history, physical, and diagnostic work-up be performed to identify causes of anemia aside from hematopoietic malignancy or chemotherapy.

## Platelet transfusion

### Indications

More than one-third of platelets in the United States are administered to support oncology patients.<sup>37</sup> The goal of platelet transfusion is to stop or prevent bleeding in thrombocytopenic patients. The use of platelet transfusion is a first-line therapy for acute hemorrhage in patients with thrombocytopenia or those receiving anti-platelet medication. The use of platelet transfusion for bleeding prophylaxis, however, is somewhat controversial. Initial studies that supported the use of platelets for bleeding prophylaxis arose from trials in leukemic patients who were noted to have decreased bleeding episodes after platelet transfusion.<sup>38</sup> Later studies also demonstrated that prophylactic transfusion resulted in better outcomes and lower mortality compared to the use of therapeutic transfusion in patients with hematologic malignancy.<sup>39</sup> In the early 1990s, the goal for routine bleeding prophylaxis was to keep platelet counts greater than 20,000/ $\mu\text{L}$ .<sup>40</sup> More recent trials, however, have shown that spontaneous hemorrhage is unlikely, even with platelet counts as low as 5000–10,000/ $\mu\text{L}$ . In addition, patients transfused utilizing more restrictive criteria appear to have no change in morbidity and use significantly fewer blood products, thereby reducing costs as well as adverse effects associated with transfusions including reactions and alloimmunization.<sup>41–46</sup> A 2015 Cochrane review examined prophylactic versus therapeutic platelet transfusion in patients with hematologic disorders who had received myelosuppressive chemotherapy or HSCT. The review found that the therapeutic transfusion group was associated with an increased risk of bleeding

compared to the prophylactic group.<sup>47</sup> Analysis of the multicenter platelet dose (PLADO) trial, which evaluated bleeding with different doses of platelet transfusions, found that patients younger than 18 years of age had a higher risk of bleeding compared to adults, particularly for patients receiving autologous HSCT.<sup>48</sup> This finding highlights the special considerations needed when planning transfusion support for pediatric patients.

### Selection of ABO group and Rh type for platelet transfusion

Platelets express ABO antigens, although interactions between these antigens and host antibodies do not usually mediate clinically significant transfusion reactions.<sup>49</sup> Although type-specific platelets are preferred, most blood banks, especially during periods of inventory shortages, transfuse ABO-mismatched platelets to adults without significant concern for incompatibility. The use of ABO-mismatched platelets in oncology patients, however, can result in decreased therapeutic benefit or adverse reactions. For instance, a form of platelet refractoriness is mediated by ABO antibodies, with mismatched platelets cleared from circulation minutes to hours after infusion.<sup>49</sup> Another concern is the possibility that high-titer ABO antibodies in the plasma of donor platelets units may lead to hemolytic transfusion reactions in ABO-incompatible recipients.<sup>50–52</sup> Children in particular may demonstrate clinically significant hemolysis to ABO-incompatible platelet transfusions owing to their small blood volume. Therefore, it is recommended that oncology patients with low platelet counts receive ABO-compatible platelet products when feasible. For populations undergoing HSCT, other considerations regarding major and minor mismatches are also relevant. Table 41.2 summarizes guidelines for platelet transfusion therapy in the peri-transplant period.

Even though platelets lack Rh antigens, there remains a small concern for Rh alloimmunization in RhD-negative recipients during platelet transfusion due to the potential exposure to small numbers of residual red cells in the platelet product collected from RhD-positive donors. Because of the immunogenicity of the D-antigen, even a milliliter or less of red cells can lead to alloimmunization in Rh-negative recipients. However, recently published studies have shown that the overall rate of alloimmunization is quite low.<sup>53,54</sup> There is evidence to suggest that, in part due to immunosuppression, both adult and pediatric patients with hematologic malignancies are unlikely to form anti-D responses to Rh-incompatible platelet transfusions.<sup>55,56</sup> Thus, the provision of Rh-incompatible platelet products is unlikely to cause D alloimmunization in oncology populations. In addition, this risk may be further reduced through the use of apheresis platelets as these units have fewer residual red cells.<sup>57</sup> Nonetheless, it is still advisable to prevent Rh alloimmunization in children and females of childbearing potential because of the consequences of anti-D development in these populations. Prevention of alloimmunization can be achieved with a dose of 50–300  $\mu\text{g}$  of Rh immunoglobulin (RhIg) provided within 72 hours of D antigen exposure.<sup>58</sup>

### Selection of platelet products

Historically, the majority of platelet products were derived from whole blood by centrifugation, followed by pooling into groups of four to yield a product intended to increase the platelet count by approximately 50,000/ $\mu\text{L}$ .<sup>59</sup> These platelet products are referred to as pooled random donor platelets. With advances in apheresis technology in the 1970s, an increasing number of platelet units were obtained from single donors by apheresis collection, referred to as

apheresis platelets, that provided a platelet dose equivalent to the pooled product.<sup>59,60</sup> The decision to use apheresis platelets versus pooled platelets relates to concerns about exposure to multiple donors, risk of alloimmunization, risk of adverse reactions, and platelet quality rather than increases in baseline platelet count as both products have similar viability and recovery.<sup>61</sup> The risk for many of these issues are not specific to oncology and are discussed in greater detail in Chapter 35.

One particular concern for oncology patients is a possible increase in risk for platelet alloimmunization due to the large number of transfusions administered to these patients. This was a significant concern prior to the era of leukocyte reduction, especially for pooled platelet products. However, several studies, including the Trial to Reduce Alloimmunization to Platelets (TRAP), have demonstrated that the risk of alloimmunization is identical between leukoreduced pooled platelets and apheresis units.<sup>20</sup> Once a patient has become alloimmunized, however, the use of pooled platelets may be advantageous until crossmatch-compatible or HLA-matched platelet units are available. In this scenario, an alloimmunized patient may be more likely to respond by chance to one of the four or five donors whose units are part of a platelet pool. Therefore, in alloimmunized patients, apheresis platelets generally should be used only if the unit is crossmatch-compatible or HLA-matched.<sup>59</sup>

### **Platelet refractoriness and alloimmunization**

Chronically transfused oncology patients often experience lower than expected platelet increments. This is known as platelet refractoriness. Obtaining two consecutive corrected count increments (CCIs) following platelet transfusion is recommended as the initial screen to determine if a patient is platelet refractory. An acceptable CCI value is defined as  $>7.5 \times 10^6/\mu\text{L}$ , although some use a lower threshold of  $>5 \times 10^6/\mu\text{L}$ .<sup>62</sup> Additional alloimmunization studies can be performed if the CCI is below this value. Refractoriness to platelets can be due to either immune or nonimmune causes. Platelet alloimmunization and refractoriness are further discussed in detail in Chapter 54. In oncology patients, over 70% of cases of platelet refractoriness are due to nonimmunologic factors.<sup>59</sup> There are a few clinical management options for patients with nonimmunologic platelet refractoriness. When possible, correcting the underlying cause (e.g., splenectomy for hypersplenism, removal of offending medications, and treatment of sepsis and DIC) can alleviate refractoriness. For the acutely bleeding patient or for hemorrhage prophylaxis, strategies of continuous platelet infusion (platelet drip) have shown moderate success.<sup>63,64</sup> The platelet drip, wherein half a unit of an apheresis platelet bag is infused slowly over a four-hour period, is intended to provide an ongoing source of platelets to maintain vascular integrity, while addressing the practical concern of managing an often limited blood bank platelet inventory.

Although they constitute a minority of cases, the underlying cause of refractoriness in the remaining 20–30% of oncology patients is likely due to an antibody to a platelet antigen. Platelets express class I HLA and numerous other platelet-specific antigens. Human leukocyte antigens are the most common mediator of immunologic platelet refractoriness, although studies have shown that alloantibodies can develop to any platelet antigen.<sup>59,65</sup> The treatment strategy for immune-mediated refractoriness is to provide antigen-negative components. Although often not a cause of true refractoriness, the first approach is to provide ABO-compatible units, which may lead to a more sustained increase in platelet count. The next option is to provide crossmatch-compatible platelets from

the hospital blood bank or donor center. This is typically done by a solid-phase capture method in which the patient's plasma is crossmatched with a variety of random donor platelets and selecting units that do not cause agglutination.<sup>66</sup> Crossmatch compatible platelets have been proven to be as effective as HLA-matched platelets in raising platelet counts in alloimmunized patients.<sup>59,65,66</sup> Therefore, the decision to provide HLA-matched platelets should depend upon factors such as quality of the HLA match, severity of alloimmunization, and availability of crossmatched products. If a majority of tested donors are incompatible and HLA-matched products are needed, the recipient can be HLA typed to provide antigen-matched products.<sup>59,65</sup> Polymorphisms of HLA class I antigens can complicate compatibility testing and make the process of finding fully matched donors difficult.

Unfortunately, once alloimmunization has occurred, immune modulation with corticosteroids, plasmapheresis, and intravenous immunoglobulin (IVIG) are of little benefit.<sup>67</sup> Therefore, the best strategy to reduce immune-mediated platelet refractoriness is prevention of HLA and platelet antigen exposure. Leukocyte reduction has helped reduce the incidence of alloimmune platelet refractoriness by limiting exposure to HLA.<sup>20</sup> Conservative transfusion strategies have also helped prevent exposure to HLA and platelet-specific antigens and may play a role in reducing the frequency of alloimmunization.

### **Component modification: irradiation, leukocyte reduction, and volume reduction**

As is the case with RBC units, platelets can be modified to maximize safety for oncology patients. The process of leukoreduction can achieve a four-log reduction in leukocytes. Despite this reduction in leukocytes, platelet units still contain small amounts of white cells, generally below  $5 \times 10^6$ . Therefore, patients at risk for TA-GVHD should receive irradiated platelets.<sup>24</sup> As mentioned previously, leukocyte reduction of platelet products helps reduce the rate of alloimmunization.<sup>20</sup> According to the 2017 National Blood Collection and Utilization Survey, approximately 99.2% of all whole-blood-derived platelet units were reported as leukocyte reduced.<sup>37</sup> In addition, all apheresis platelets are leukocyte reduced as part of the collection process.

Volume reduction of platelet products via centrifugation and resuspension in saline should be considered for oncology patients who have experienced severe allergic or anaphylactic reactions to platelet products.<sup>68</sup> However, centrifugation damages platelets and can result in blunted in vivo response; therefore, centrifugation should be used sparingly. Volume reduction is also efficient in removing the plasma fraction of platelet products to reduce the risk of hemolysis associated with ABO-incompatible transfusion as well as the risk of volume overload.<sup>68</sup>

Recent advances to reduce adverse effects of platelet transfusions, particularly bacterial contamination of platelets, led to the development of pathogen-reduced platelets. Pathogen reduction technology uses ultraviolet light in conjunction with photoactive agents to damage the nucleic acid of infectious agents such as bacteria and viruses. Three pathogen reduction technologies are under study, including Mirasol, which uses riboflavin (vitamin B2) as the photoactive agent, and Intercept, which uses a psoralen compound as the photoactive agent. Lastly, Theraflex uses UVC without a photoactive agent. Psoralen-UVA-treated pathogen-reduced platelets are the only one of the three protocols that are approved by the FDA for both pathogen reduction of platelet concentrates and for the prevention of TA-GVHD. The ultraviolet light manufacturing

procedure causes damage to the DNA in the donor lymphocytes, thus eliminating the risk of TA-GVHD and the need to X-ray or gamma-irradiate pathogen-reduced platelets (see Chapter 42).

### Alternatives to platelet transfusion

For severe platelet refractoriness, or for patients unwilling to undergo platelet transfusion, several medical therapies may be beneficial during bleeding episodes. A commonly prescribed drug, 1-deamino-8-D-arginine vasopressin (DDAVP or desmopressin), acts to stimulate the release of von Willebrand factor from endothelial cells, which can enhance platelet activity even at very low platelet counts.<sup>69</sup> DDAVP is also of proven benefit for platelet dysfunction and bleeding associated with uremia.<sup>70</sup> Antifibrinolytic therapies have also been employed as an adjunct to platelet transfusions for the bleeding patient. Medications such as aminocaproic acid and tranexamic acid have been successfully used to reduce hemorrhage and allogeneic transfusion requirements in the bleeding patient.<sup>71,72</sup>

Chemical and cytokine-based stimulation of the marrow to endogenously increase platelet production has also been attempted. Agents such as thrombopoietin (TPO) and megakaryocyte growth factors have been synthesized, but clinical trials with some of these drugs led to the development of neutralizing antibodies and thrombocytopenia in some recipients.<sup>73,74</sup> TPO and TPO-like growth factors are now used for some conditions such as immune thrombocytopenic purpura (ITP), and studies in oncology populations have been promising.<sup>75</sup> Several agents, such as romiplostim and eltrombopag, have been licensed by the US Food and Drug Administration (see Chapter 52). Interleukin-11 (IL11), a cytokine that drives megakaryocyte production and division, has also been approved for use in thrombocytopenic patients.<sup>73,74</sup>

Clinical studies looking at a lyophilized platelet-derived hemostatic agent, Thrombosomes, as an alternative to traditional platelets are currently ongoing. Unlike the short five-day storage life of traditional platelets, Thrombosomes can be stored for up to 24–36 months at room temperature. Studies have shown Thrombosomes are able to adhere to a collagen surface and contribute to clot formation and generate thrombin.<sup>76</sup> Further clinical trials are ongoing.

## Plasma and plasma-derived product transfusion

### Indications for plasma transfusion and product selection

The indications for transfusion of plasma and plasma-derived derivatives, such as cryoprecipitated antihemophilic factor (AHF), intravenous immune globulin (IVIG), and fibrinogen concentrate (RiaSTAP), are similar between oncology and other patient populations. For the majority of oncology patients, no additional concerns are involved with the selection of an ABO group for plasma so long as the unit is ABO compatible with the recipient. Cryoprecipitate contains significantly less plasma volume as compared to platelets and plasma. The risk of hemolysis from ABO-incompatible cryoprecipitate is very low; thus, blood banks routinely issue ABO-incompatible cryoprecipitate.<sup>77</sup> Rh compatibility between donor and recipient is not a consideration for infusion of plasma or cryoprecipitate. As mentioned in this chapter, however, for patients who have undergone allogeneic HSCT, the choice of ABO group can be complex and should be based upon consideration of major and minor mismatches. Table 41.2 summarizes guidelines for plasma

transfusion therapy in the peritransplant period. The guidelines for cryoprecipitated AHF are identical to those for plasma. Irradiation of these products is not required given that they are acellular.

### Alternatives to plasma transfusion

For some oncology patients, plasma transfusion may be contraindicated, may be ineffective, or may not provide rapid enough reversal of coagulopathic states. In these situations, there are several alternatives to plasma products, consisting of concentrated or recombinant coagulation factors. Recombinant factor VIIa (NovoSeven), a potent activator of the coagulation cascade, is one of the most commonly used agents for acute moderate to severe bleeding.<sup>78</sup> Successful use of factor VIIa has been reported to help control massive bleeding in a variety of oncology patients.<sup>79–81</sup> In addition, there is a role for use of factor VIIa as a bypass agent for those oncology patients who acquire inhibitors to circulating coagulation factors, mainly factor VIII.<sup>82</sup> However, factor VIIa failures in bleeding oncology patients have been reported for gastrointestinal bleeding and alveolar hemorrhage.<sup>83,84</sup>

Few adequate clinical trials have been conducted to determine the safety and efficacy of factor VIIa in oncology populations; therefore, it is difficult to recommend the broad application of this therapy for bleeding oncology patients. Furthermore, appropriate dosing regimens are imprecise and mostly based on data gathered in trials performed in other critically ill patient populations. Factor VIIa also carries a number of serious risks, including severe or even fatal arterial and venous thromboembolism.<sup>85</sup> Therefore, factor VIIa may be considered as an alternative to plasma infusion but should be used only after other interventions have failed.

### Intravenous immunoglobulin

IVIG is used to provide passive immunity in highly immunosuppressed individuals and has also been used as an immunomodulatory therapy for patients with conditions such as immune thrombocytopenic purpura (ITP).<sup>86,87</sup> For recipients of HPC products, IVIG has been associated with improved immune defense against pathogens such as CMV and has helped to decrease the complications of acute GVHD.<sup>88,89</sup>

IVIG infusion is usually tolerated without significant adverse events in oncology patients. The common side effects include myalgia, flushing, headache, fever, and fatigue, which are typically mild and transient.<sup>90</sup> Current IVIG products have not transmitted hepatitis B, hepatitis C, or human immunodeficiency virus, but there are rare case reports of transmission of parvovirus B19, a serious pathogen for oncology patients;<sup>91</sup> however, plasma is screened for this virus prior to being pooled.

## Granulocyte transfusion

### Indications

Although rarely used today, granulocyte infusions have been used in oncology patients with severe neutropenia to treat life-threatening, antibiotic-refractory infections such as mucormycosis. The decision to initiate granulocyte transfusion usually represents failure of other forms of therapy. Only patients with a chance at sustainable marrow recovery after resolution of the underlying infection are candidates for granulocyte products.<sup>92</sup> It has been shown that humans should produce approximately  $2-3 \times 10^{11}$  polymorphonuclear cells daily to clear significant bacterial or fungal infections. For oncology patients whose marrow cannot support

this level of production, doses of  $1 \times 10^{11}$  granulocytes per square meter of body area have been attempted to support the immunologic response.<sup>93</sup>

### **Granulocyte donation and donor preparation**

The safety and efficacy of granulocyte transfusion using G-CSF stimulation in healthy donors has been evaluated in a number of trials. Some studies suggest that granulocyte infusions may be effective for the treatment of bacterial and fungal infections.<sup>94</sup> However, it has been hypothesized that improved outcomes may be seen with higher granulocyte doses. To obtain a larger number of granulocytes, the Safety and Effectiveness of Granulocyte Transfusion in Resolving Infection in People with Neutropenia (RING) study used G-CSF and dexamethasone to stimulate donors. Although this multicenter trial found that there was no difference in success rates between antibiotic and granulocyte therapies, the study was severely limited by a low accrual rate resulting in ~47% power to detect a difference.<sup>95</sup>

Healthy donors who qualify to donate granulocytes should be ABO-compatible with the intended recipient, and products must be crossmatched before infusion as granulocyte products may contain 6–12% red cells. Typically, donors receive 5–10 µg/kg G-CSF subcutaneously approximately 12 hours before leukapheresis. Granulocyte collections can be performed daily, over 4–5 days to collect multiple doses for neutropenic patients. Granulocyte donation is generally well tolerated with mild, short-lived side effects such as bone pain, headache, and fatigue as a result of the G-CSF administration and apheresis.<sup>92,96,97</sup>

### **Alloimmunization**

Alloimmunization from granulocyte transfusion can occur.<sup>98</sup> In addition, patients previously alloimmunized to HLA may demonstrate a reduced response to granulocyte infusion.<sup>99</sup> If granulocyte transfusion is necessary for these patients, antigen matching for HLA may be required. However, this is rarely performed given the low use of granulocyte transfusions overall.

### **Granulocyte storage, component modification, and infusion**

Granulocytes are stored for up to 24 hours from the time of collection at 20–24 °C without agitation.<sup>100</sup> The granulocyte product should be transfused as soon as possible, ideally within 6–8 hours of collection, given the limited lifespan of neutrophils. Prior to release from the blood bank, the granulocyte product is crossmatched as well as irradiated to prevent recipient TA-GVHD. Granulocytes should be infused through a 150–260-µm filter, but leukocyte reduction filters must never be used.<sup>101</sup> There are reports of patients developing pulmonary toxicity while simultaneously receiving amphotericin B and granulocyte infusions. Additional studies, however, did not confirm a relationship between the development of pulmonary toxicity due to interaction between amphotericin B and granulocyte transfusions.<sup>102</sup> In vitro studies have shown reduced neutrophil aggregation when liposomal formulations of amphotericin B are used; therefore, these formulations are preferred when simultaneously using granulocyte infusions.<sup>103</sup>

### **Adverse reactions to blood transfusion**

Oncology patients undergoing transfusion therapy are subject to the same set of adverse reactions as are seen in any patients receiving blood components. However, oncology patients are at increased

risk for many more types of adverse reactions. In addition, discerning between an adverse transfusion event and exacerbation of an underlying condition can be difficult.

Oncology patients are often immunosuppressed; therefore, they are at increased risk for TA-GVHD (discussed in Chapter 50) and many transfusion-transmitted infections. CMV is one of the most problematic transfusion-transmitted infections for oncology patients. The virus has a seroprevalence of 50–70% and has been found in the mononuclear cells of both seropositive and seronegative blood donors.<sup>104,105</sup> Although CMV infections are rarely significant in immunocompetent hosts, in immunosuppressed patients infection can lead to severe pathology such as CMV-related pneumonia, gastrointestinal inflammation, and delayed HSCT engraftment. Obtaining CMV-seronegative blood can be challenging given the high seroprevalence in the general population. In addition, even seronegative individuals can harbor infectious viral particles. Leukocyte reduction is also employed to prevent CMV transmission by reducing the number of transfused mononuclear cells potentially harboring the virus. Several studies have found that leukocyte reduction is as effective as the use of seronegative units; and most physicians consider leukoreduced units to be "CMV-safe."<sup>106,107</sup> However, the risk of CMV is not completely eliminated with these maneuvers; thus, while the use of donor screening and leukocyte reduction is beneficial and most often used clinically, oncology patients are still at some degree of risk for CMV exposure from blood transfusion. Use of pathogen reduced apheresis platelets, however, essentially eliminates the risk of CMV transmission by platelet transfusion.

### **Adverse effects of hematopoietic progenitor cell infusion**

The majority of adverse events associated with HPC infusion are associated with the dimethyl sulfoxide (DMSO) content of the progenitor product. DMSO is a chemical modifier used in a majority of cryopreservatives that allows for the controlled freezing and thawing of mononuclear cells without development of membrane lysis.<sup>108</sup> However, *in vivo*, DMSO is a toxic substance and has been linked to fever, nausea, vomiting, and chills during and immediately after HPC infusion. DMSO has also been linked to pulmonary and cardiovascular problems during HPC infusion, including dyspnea, hypotension, bradycardia, and arrhythmia.<sup>108</sup> Due to the amount of DMSO infused, small children and those patients receiving multiple HPC units are more susceptible to DMSO-related problems.<sup>108</sup>

Researchers have also evaluated whether HPC components other than DMSO can lead to infusion toxicity. Several studies have correlated adverse events to the numbers of granulocytes present in HPC products.<sup>109,110</sup> These studies found that even after DMSO depletion, over half of patients undergoing HPC infusion experienced an adverse event such as fever, rigors, or dyspnea. It is therefore possible that the dyspnea associated with HPC infusion is not entirely caused by DMSO, but that granulocytes may contribute through transfusion-related acute lung injury (TRALI)-like mechanisms. These studies argue that a reduction in granulocyte content of the final HPC product would lead to fewer reactions. However, this is technologically challenging to achieve as removal of granulocytes would also remove a percent of the HPCs.

Bacterial contamination of HPC products can occur during collection or processing of the cells.<sup>111</sup> Adequate field sterilization practices during stem collection and the processing of HPC products in sterile areas with air-controlled biosafety cabinets can reduce the risk of bacterial contamination.<sup>112</sup> However, due to the

significant handling of HPC products, bacterial contamination can be a frequent issue, especially during bone marrow collections.<sup>113</sup> Because of this risk, units are routinely cultured and, if bacterially contaminated, provided to patients in conjunction with prophylactic, broad-spectrum antibiotics. Interestingly, one study has demonstrated that, of 33 patients who received bacterially contaminated HPCs, only 6 developed evidence of bacteremia and none had any long-term complications.<sup>114</sup> Thus, bacterial contamination of an HPC product need not be an absolute barrier to infusion.

### **Chimeric antigen receptor (CAR) T-cell therapy**

CAR T-cells contain CAR fusion protein genetically engineered into autologous T cells. After modification to produce cytotoxic immune T cells targeted to tumor-specific antigens, the CAR T-cells are reinfused to the patient. The first use of CAR T-cell therapy was in 2017 for the treatment of acute lymphoblastic lymphoma. Since then, the use has expanded to include other lymphoid malignancies, typically for refractory or relapsed disease. Patients can experience severe adverse reactions to CAR T-cell infusion, most notably a cytokine release syndrome (CRS).<sup>115</sup> Symptoms of CRS include fever, hypoxia, and hemodynamic instability. Studies have shown that the infusion dose, tumor burden, and proliferation of CAR-T cell *in vivo* may correlate with the severity of CRS.<sup>116</sup> The exact mechanism of CRS is still under investigation; however, it is believed to result from cytokine release during the interaction between the immune effector cells and tumor cells. Several cytokines are released during CRS including IL-6, IL-10, TNF, among others. Tocilizumab, an IL-6 receptor blocker, and siltuximab, a chimeric anti-IL-6 monoclonal antibody, are used for the treatment of CRS after CAR T-cell therapy.

### **Summary**

Transfusion support is a major component to the therapy for patients with oncologic disorders. However, given their typically increased transfusion needs and immunosuppressed state, they are often at increased risk for adverse events due to transfusion. Compared to other patient populations, oncology patients are at

increased risk for TA-GVHD, certain transfusion-transmitted diseases, alloimmunization to cellular antigens, as well as disease states altered by immunomodulations. In addition, HPC transplant patients present unique challenges to transfusion services as they require specific assessment for potential ABO incompatibility. Careful selection of blood components and component modification is therefore necessary to provide appropriate therapy to these patients. Significant advances that have been made in transfusion therapy, including more sensitive donor testing, blood component alternatives, and pathogen reduction technologies, continue to improve the safety of transfusion for oncology patients.

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## CHAPTER 42

# Pathogen-reduced blood components and derivatives

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## Introduction

The field of transfusion medicine has made significant improvements regarding blood safety during the last 40 years with the development of methods to screen and identify donors with risk factors for transfusion-transmitted infections (TTIs). These advances include excluding donors with histories of travel to regions endemic for some blood-borne pathogens, requiring blood testing using serologic and nucleic acid assays for specific infectious agents (e.g., HIV, HTLV-I, and Hepatitis), and implementing routine bacterial culture testing of platelet concentrates.<sup>1–3</sup> Despite the success of these efforts, these measures are still just “reactive” in that they are implemented after a new blood-transmissible pathogen is identified. Unfortunately, the pathogen may have already threatened the safety of both the donor and the transfusion recipient populations by the time the infectious agent has been isolated and identified.<sup>2,3</sup> During the HIV crisis, blood transfusions resulted in the transmission of HIV to more than 12,000 recipients and over half of the 16,000 hemophiliacs in the United States in the 1980s.<sup>1</sup> This illustrates how a reactive approach to reducing TTIs may not work quickly enough to prevent the transmission of a novel blood-borne pathogen and thus allow patients to be harmed before effective preventive steps can be implemented.

In contrast, a proactive approach can have a substantive and beneficial impact on donor availability and recipient safety by adding a pathogen-reducing manufacturing step to donated blood components prior to transfusion. The pathogen reduction agent should substantially mitigate the infectious risk caused by a known or unknown pathogen by blocking the pathogen’s blood-borne transmission—even before the new offending agent is identified. Examples of situations in which a reactive approach is not an effective deterrent to blood-borne transmission include scenarios dealing with the following cases:

- Endemic diseases such as CMV, EBV, Chikungunya, Malaria, and Dengue, in which infected donors may escape detection because donor blood screening tests for these pathogens are not required or not routinely performed.
- Pathogens with limited geographic distribution—such as Babesia, Dengue, or Chikungunya—for which preventative measures are not universally in place. This is a critical point since donors today

are much more mobile. Asymptomatic but infected individuals who live in an endemic area may travel and donate in a nonendemic area, and thus their infection may escape detection despite the health history questionnaire performed by donor centers.

- Seasonal pathogens such as West Nile virus or Babesia.

The myriad of possible infectious agents that can be missed by a reactive donor screening and testing approach has motivated the development of a fundamentally different “proactive plan” involving pathogen reduction (PR) technology. This approach has great potential value for improving the safety of the blood supply.<sup>2,3</sup> Across all cellular and plasma-based blood components, PR technology is becoming the central approach for developing a proactive policy for controlling and hopefully eradicating transfusion-transmitted diseases. PR technology utilizes a variety of reagents and techniques to either inactivate or reduce potential pathogens present in blood products with minimal adverse effects on the quality and efficacy of the therapeutic blood component.

## Overview of pathogen reduction technologies

Pathogen reduction technologies encompass techniques and reagents used to inactivate or reduce the infectious potential of pathogens that might be found not only in cellular or plasma-based blood components but in blood derivatives as well. The terms “pathogen inactivation” and “pathogen reduction” are often used interchangeably. Colloquially, both terms refer to decreasing or inactivating the amount of an infectious pathogen in a blood component.<sup>4</sup> We favor the term pathogen reduction and use it exclusively in this chapter because “inactivation” may inadvertently and inaccurately imply complete eradication of the pathogen.

PR technologies fall into two broad categories: (1) methods that target and damage DNA or RNA to prevent microorganisms (e.g., bacteria or viruses) or cells (e.g., lymphocytes) from replicating, and (2) methods that damage primarily microbial lipids. Methods that target and modify single-stranded/double-stranded DNA or RNA reduce the risk of infectious disease transmission in blood components by preventing nucleic acid replication and thus the reproduction of the microorganisms.<sup>5</sup> It

**Table 42.1** Pathogen Reduction Technologies

	Blood Component	Mechanism of Action	Regulatory Status
<b>Amotosalen/UV-A</b>	Plasma Platelets	Nucleic acid crosslinking and monoadduct formation	CE marked FDA approved
<b>Riboflavin/UV</b>	Plasma Platelets Red blood cells Whole blood	Direct nucleic acid damage and oxidation of guanine; ROS	CE marked
<b>Amustaline/glutathione</b>	Red blood cells	Nucleic acid crosslinking and monoadduct formation	Currently undergoing trials
<b>UV-C</b>	Platelets	Pyrimidine dimerization and direct nucleic acid damage; ROS	CE marked; not in routine use
<b>Methylene blue/visible light</b>	Plasma	Nucleic acid damage from photooxidative reactions; ROS	CE marked
<b>Solvent/detergent</b>	Plasma	Lipid membrane damage	CE marked FDA approved

A summary of the major categories of pathogen reduction technology and the applicable blood components, the mechanism of action for inactivating pathogens, and the current regulatory status for each technology.<sup>3</sup>  
ROS: reactive oxygen species.

should be noted that the doses of gamma radiation used to prevent transfusion-associated graft-versus-host disease (TA-GVHD) do not inactivate microbes. Because none of the standard blood components (i.e., red cells, platelets, or plasma) require nucleic acid replication to provide a therapeutic in vivo effect, this mechanism of PR does not significantly interfere with the clinical effectiveness of the blood component. Since all pathogens except prions require nucleic acid replication for reproduction and for infection, these methods are particularly effective for broadly reducing the risk of infectious disease transmission in blood components. Importantly, PR technologies that utilize reactions with nucleic acids also effectively prevent lymphocytes from replicating and thus should markedly restrict the risk of TA-GVHD without need for gamma or X ray irradiation. The PR technologies that fall in this category are amotosalen/UV-A, riboflavin/UV, amustaline/glutathione, methylene blue/visible light, and UV-C alone (Table 42.1).

Chemical treatment methods primarily disrupt lipid-enveloped viruses and also the lipid bilayers of bacteria. The only US Food and Drug Administration (FDA) approved technology that utilizes this technique is a solvent/detergent (S/D) method using tri-*n*-butyl phosphate and octoxynol (Triton X-100, a nonionic surfactant). Because this technique renders most types of cells nonviable by disrupting their plasma membranes, this approach is limited to noncellular blood components such as fresh frozen plasma and blood derivatives such as IVIG and clotting factor concentrates. Pathogen reduction techniques like S/D treatment are most effective for inactivating lipid-enveloped viruses and to a lesser degree disrupting bacteria and fungi. However, S/D treatment is not effective against nonlipid-enveloped viruses or spores.<sup>6,7</sup>

As of writing this chapter, there are four pathogen-reduced blood components approved by the US FDA for human use: (1) single donor platelets, (2) fresh-frozen plasma, (3) cryoprecipitate, and (4) pooled S/D plasma. The first three components are manufactured using amotosalen/UV-A PR technology. Platelets and plasma prepared with riboflavin technology and platelets treated with UV-C technology are only approved for use outside the United States. In this chapter, we review the PR technologies currently under development or in use for the various blood components and derivatives. We discuss their mechanisms of action, efficacy profile against various pathogens, published

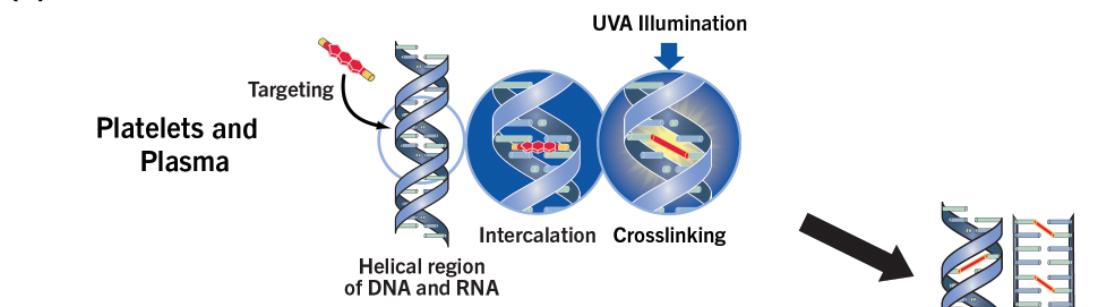
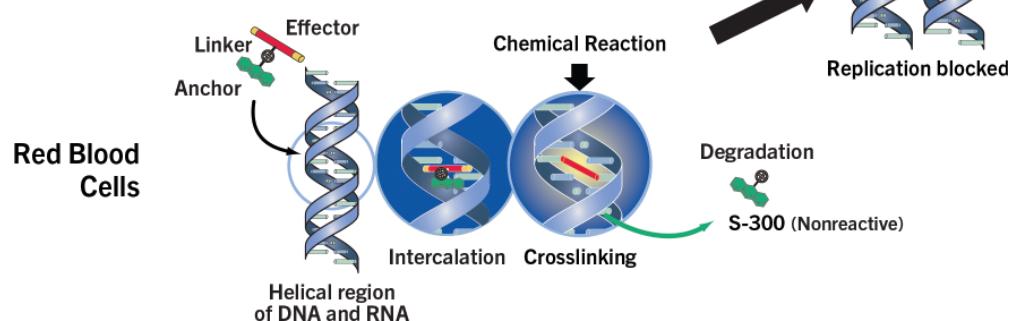
in vitro and in vivo studies, reported immunological effects, product safety, and toxicology.

## Pathogen reduction technologies—mechanisms of action

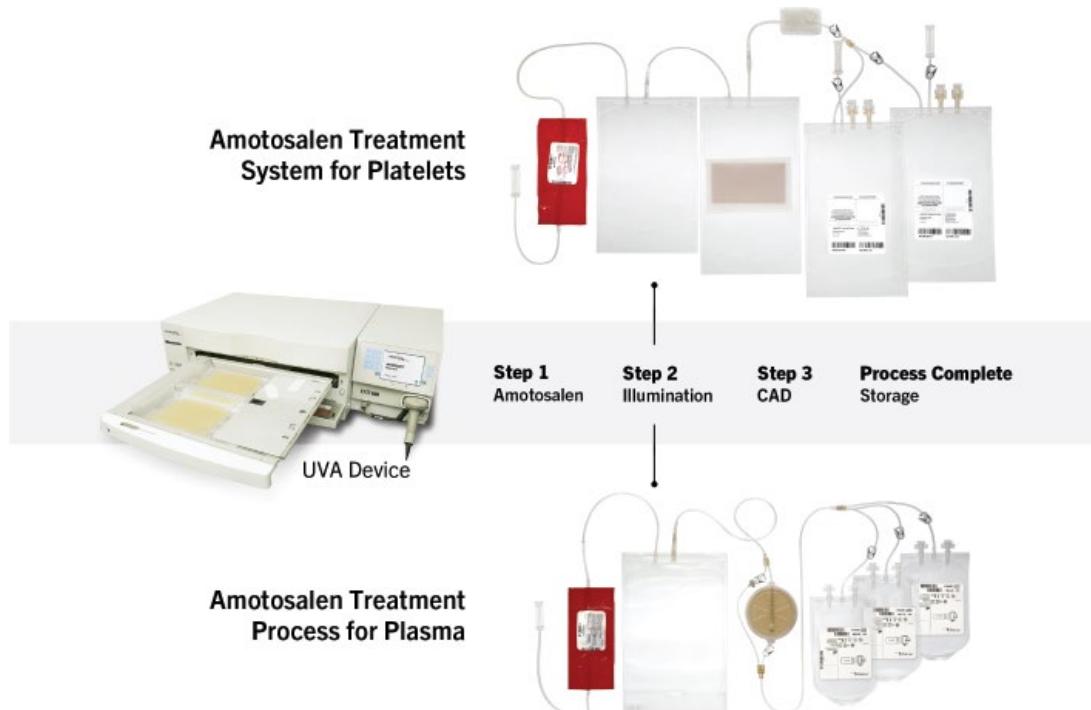
### Amotosalen/UV-A

For the amotosalen/UV-A methodology, a synthetic psoralen compound called amotosalen (S-59, Cerus Corp., Concord, CA) is added to platelets or plasma in the component bag and mixed together by agitation. The amotosalen-treated platelet or plasma component is then exposed to a UV-A light source through a process called photoillumination. This maneuver causes double-stranded or single-stranded nucleic acid damage by the formation of monoadducts and crosslinking of base pairs with the psoralen. Amotosalen belongs to a class of molecules called psoralens, which are a group of naturally occurring chemicals found in foods and plants such as figs, grapefruit, and celery.<sup>8</sup> As mentioned before, amotosalen intercalates into helical regions of nucleic acids, including both single- and double-stranded DNA and RNA. Activation by UV-A light (320–400 nm) causes covalent monoadduct crosslinking (Figure 42.1A). These crosslinks prevent the separation of chromosomal nucleic acids and tertiary RNA structures and thus effectively block nucleic acid replication (Table 42.1). After amotosalen is added to a blood component and photoactivated by UV-A light, a compound adsorption device (CAD) is used to reduce the concentration of residual photoproducts (Figure 42.2). This manufacturing technique is currently approved in Europe (CE Marked) and in the United States by the FDA for single donor platelets, fresh-frozen plasma, and cryoprecipitate.

Amotosalen/UV-A cannot be used to effectively pathogen reduce units of whole blood or RBCs because red blood cells absorb UV-A light, blocking the photoactivation of psoralens and lowering the efficiency of the pathogen reduction process.<sup>9</sup> Amotosalen/UV-A technology is effective against lipid-enveloped viruses, Gram-positive and Gram-negative bacteria, fungi, spirochetes, parasites, and many nonenveloped viruses (e.g., hepatitis A, hepatitis E, parvovirus B19, and poliovirus), bacterial spores (e.g., *Bacillus cereus*), and prions show resistance to amotosalen/UV-A inactivation.<sup>10,11</sup> Nucleic acid crosslinking by amotosalen/UV-A also affects leukocytes and thus effectively reduces the risk of TA-GVHD.<sup>5</sup>

**(A) Amotosalen (S-59)****(B) Amustaline (S-303)**

**Figure 42.1** (A) The intercalation of amotosalen (S-59) into the nucleic acid helical region is seen with the crosslinking occurring following UV-A illumination. (B) UV-A does not effectively pathogen reduce red blood cells. Amustaline (S-303) intercalates and crosslinks without need for UV light. Importantly, the amustaline intercalation occurs more quickly than its degradation. Thus, amustaline can efficiently inactivate pathogens in red cells. (Figure is courtesy of the Cerus Corporation and is used with permission.)



**Figure 42.2** The illumination system used for amotosalen. For platelets, in Step 3, a small bag containing a compound absorptive device (CAD) is used to remove residual photoproducts. For the platelet process, the CAD is incubated with the platelets for 16 hours to ensure adequate photoproduct removal. For plasma, the compound absorption material is contained in a device placed in-line with the fluid path. Since there are no cells in plasma, the adsorption process occurs much more quickly in plasma than in platelets. Amotosalen is light sensitive and is stored in a dark colored bag to protect potency prior to addition to the blood component. Courtesy of the Cerus Corporation, used with permission.

### Riboflavin/UV

Another pathogen reduction approach utilizes the addition of riboflavin (vitamin B2) to the blood component following collection, with subsequent exposure to UV light (290–320 nm). Riboflavin is a photosensitive compound that intercalates with DNA or RNA, and the exposure to UV light causes riboflavin photolysis and induces guanine oxidation, resulting in single-strand breaks (Figure 42.3A).<sup>3,12–14</sup> The mechanism of action is thought to involve the generation of reactive oxygen species (ROS) as well.<sup>12</sup> This process likely causes extensive damage to guanine bases that overwhelms DNA repair and prevents nucleic acid replication (Table 42.1). Riboflavin acts by selectively binding to nucleic acids and lipids but not to proteins. The breakdown products formed after UV light exposure are naturally occurring and are present at low concentrations (Figure 42.3B and 42.3C). Riboflavin breakdown products do not need to be removed from the blood component after photoactivation.<sup>15</sup> Riboflavin/UV shows efficacy against Gram-positive and Gram-negative bacteria, many lipid-enveloped viruses, spirochetes, and parasites, and a number of nonenveloped viruses. Like amotosalen/UV-A, riboflavin/UV has shown to have less efficacy against nonenveloped viruses (e.g., hepatitis A) and spore-forming bacteria, and no efficacy against prions. This technology has been approved for RBCs, plasma, platelets, and whole blood outside the United States (e.g., CE marked in Europe) but has not been approved for use with any blood component in the United States. Riboflavin causes enough damage to DNA in leukocytes to effectively reduce the risk of TA-GVHD.

### Amustaline/glutathione

Amustaline/glutathione technology is currently in development with several Phase-III randomized clinical trials being conducted for red blood cells. Since UV-A light is not effective for ensuring adequate pathogen reduction of RBCs, a different technology suitable for red cell products was developed. Amustaline (S-303, Cerus Corp., Concord, CA) is a small molecule that contains an alkylating component that crosslinks nucleic acids without the need for photoactivation. This pH-sensitive system utilizes amustaline and glutathione (Figure 42.1B).

Amustaline is composed of three distinct moieties that form the basis for its mechanism of action as a pathogen-reduction agent: (1) a nucleic acid acridine anchor that noncovalently binds to the nucleic acids of contaminating pathogens and white blood cells, (2) a bifunctional alkylator group that crosslinks nucleic acids, and (3) a cleavable linker that spontaneously hydrolyzes in blood (Figures 42.1B and 42.4). Amustaline has a half-life of about 25 minutes and decomposes through spontaneous hydrolysis. The hydrolytic reaction is much slower than is the alkylation crosslinking reaction, allowing amustaline to inactivate nucleic acids before being degraded.<sup>16</sup> The main product resulting from decomposition of amustaline is called S-300, and this product has a much lower affinity for nucleic acids than amustaline. Amustaline can also react with molecules such as plasma proteins or RBC membrane proteins. To avoid these unwanted chemical reactions, glutathione is added as a quenching agent to prevent further reactivity.<sup>9</sup> Furthermore, although amustaline crosses cell and viral membranes, glutathione remains in the extracellular compartment and thus does not reduce

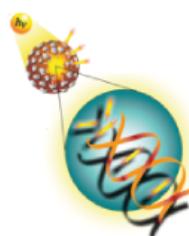
**(A) The Riboflavin/UV system inactivates pathogens by altering their nucleic acids in two primary ways**

**UV light only: reversible inactivation**

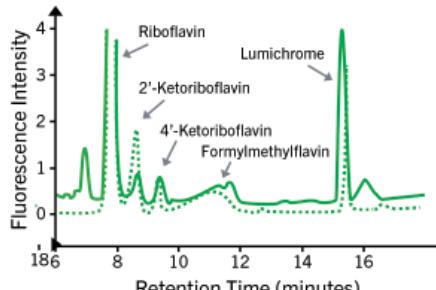
- UV light alone breaks chemical bonds in nucleic acids

**UV light + riboflavin: irreversible inactivation**

- Riboflavin molecules form complexes with nucleic acids
- UV light from the illuminator activates the riboflavin molecule in the complex
- Photoactivated riboflavin induces alterations to nucleic acids, making pathogens unable to replicate



**(B)**

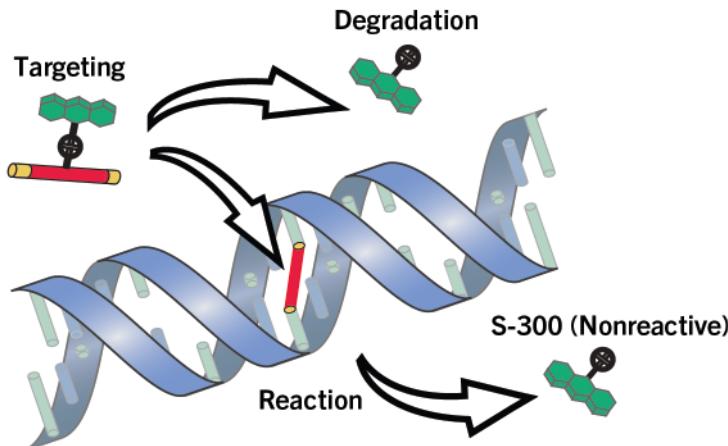


**(C)**



**Figure 42.3** (A) Two mechanisms by which riboflavin/UV inactivates nucleic acids. (B) Riboflavin-related compounds before (solid line) and after (dotted line) exposure to UV light. There is no significant change in these naturally occurring compounds. (C) An example of a riboflavin illuminator. (Figures are courtesy of the Terumo Corporation and are used with permission or are already in the public domain.)

## Amustaline (S-303) Reaction Mechanism



**Figure 42.4** A close view of the mechanism of action of amustaline, showing degradation of the anchor and effector components. The photoproduct S-300 is nonreactive. (Figure is courtesy of the Cerus Corporation and is used with permission.)

the ability of amustaline to crosslink nucleic acids. Amustaline/glutathione has been shown to be effective against a large range of pathogens including Gram-positive and Gram-negative bacteria, many nonenveloped and enveloped viruses, and certain parasites.<sup>17–22</sup> It is also efficient in inhibiting lymphocyte DNA replication and thus effectively prevents TA-GVHD.<sup>23</sup>

### Ultraviolet C Light

Ultraviolet C light technology uses UV-C light (100–280 nm) alone as the pathogen reducing agent to damage DNA without any need for additional compounds. Currently, this technique is only approved in Europe (CE marked) for platelets and not approved for any product in the United States. UV-C light causes the formation of cyclobutene pyrimidine and pyrimidine-pyrimidone dimers, preventing DNA duplication and thus cell replication of contaminating pathogens (Table 42.1).<sup>24,25</sup> The mechanism of action also involves generation of ROS. An ultraviolet C (UV-C) dose of 0.2–0.4 J/cm<sup>2</sup> has been reported to inactivate bacteria and viruses while maintaining cell quality.<sup>24</sup>

UV-C treatment has been shown to be effective against nonspore forming bacteria and nonlipid-enveloped viruses (e.g., hepatitis A and porcine parvovirus, a model for parvovirus B19) with less efficacy against spores and lipid-enveloped viruses, including retroviruses such as HIV.<sup>24,26–28</sup> Evidence suggests that UV-C inactivates T cell replication as efficiently as gamma irradiation and thus can be used to prevent TA-GVHD.<sup>29</sup>

### Methylene blue/visible light

Methylene blue (MB) is a phenothiazinium dye that is used to treat many different conditions—including nitrate poisoning, malaria, and methemoglobinemia—and it also has been utilized as a PR agent.<sup>30</sup> MB causes damage to DNA and RNA in the presence of visible light (390–700 nm)<sup>31,32</sup> by intercalating into DNA or binding to the DNA helix depending on the concentration and ionic strength of Mg<sup>2+</sup> in the blood component (Table 42.1).<sup>33</sup> Exposure to light (with peak absorption at 620–670 nm) causes photo-oxidative reactions to occur and generate ROS, which causes damage to guanine residues on nucleic acids.<sup>34,35</sup> After photoactivation, any remaining MB is removed by a filtration process.<sup>36</sup> MB treatment inactivates enveloped

viruses but has limited activity against bacteria and nonenveloped viruses<sup>30,33</sup> MB does not readily pass through cell membranes and hence is not effective against intracellular viruses.<sup>37</sup> In addition, MB is not used for pathogen reduction of cellular products because it does not cross cell membranes, and it thus cannot affect nucleic acids of bacteria, fungi, and parasites. MB does not inactivate leukocytes and does not lower the risk of TA-GVHD.<sup>30</sup> MB plasma is only approved in Europe and not in the United States (Table 42.1).

### Solvent/detergent

S/D treatment is currently used in the production of pathogen-reduced plasma. The production of S/D plasma involves pooling a large number of plasma units together (up to 1500 ABO-identical donors) and then treating the pooled plasma. The treatment entails filtration with 1-μm filters and then processing with 1% tri(n-butyl) phosphate and 1% octoxynol (Triton X-100) for 60–90 minutes. These reagents are then removed with various extraction steps. The solvent/detergent treatment dissolves the membrane of lipid-enveloped viruses (Table 42.1). Due to this mechanism, nonenveloped viruses are not inactivated.<sup>7,30</sup> The pooling step in the manufacturing process is thought to reduce the risk of infection transmission simply due to the fact that any present pathogens are diluted. This technology cannot be used for cellular products because the treatment would destroy red cells and platelets. As expected from its mechanism of action, S/D treatment effectively disrupts bacteria, fungi, and lipid-enveloped viruses but is not effective against spores or nonlipid-enveloped viruses.<sup>6,7</sup> The only currently approved formulation of S/D-treated plasma is fresh-frozen plasma.

### Efficacy of pathogen reduction

The primary objective of PR technology is to inactivate infectious agents that could be contaminating blood components. Large amounts of data have accumulated showing the inactivation capacity of the major PR technologies as measured by log<sub>10</sub> reduction in pathogens (Table 42.2). The technologies have different effects on different pathogens and not all pathogens are completely eliminated—hence the term pathogen *reduction* and not pathogen *elimination*. Maintaining a balance between pathogen

**Table 42.2** Log Reduction of Pathogen Reduction Technologies

	Log10 Reduction (pfu/mL)				
	Amotosalen	Riboflavin	UV-C	Amustaline	Methylene Blue
<b>Viruses (enveloped)</b>					
HIV-1, cell free	≥5.6 <sup>11</sup>	5.9 <sup>159</sup>	1.4 <sup>159</sup>	—	—
HIV-1, cell-associated	≥5.4 <sup>11</sup>	4.5 <sup>159</sup>	—	>5.9 <sup>137</sup>	≥5.45 <sup>57</sup> ¶
HBV	>5.5 <sup>159</sup>	4.0*	—	—	—
HCV	>4.5 <sup>159</sup>	3.2 <sup>159</sup>	≥5.0 <sup>174</sup>	—	≥3.83 <sup>57</sup> ¶
HEV	—	≥3 <sup>175</sup>	—	—	—
Duck HBV (HBV model)	≥4.8 <sup>11</sup>	—	≥2.8 <sup>174</sup>	>5.1 <sup>137</sup>	≥6.0 <sup>57</sup>
BVDV (HCV model)	≥4.1 <sup>11</sup>	5.8 <sup>159</sup>	—	>4.8 <sup>17</sup>	≥5.44 <sup>176</sup>
HTLV-I	4.7 <sup>11</sup>	—	—	—	—
HTLV-II	≥5.1 <sup>11</sup>	—	—	—	—
CMV, cell free	≥4.2 <sup>11</sup>	6	—	—	≥4.08 <sup>57</sup>
CMV, cell-associated	≥5.9 <sup>159</sup>	6	—	—	—
WNV	≥6.3 <sup>11</sup>	>5.1 <sup>159</sup>	5.4 <sup>159</sup>	>6.0 <sup>177</sup>	≥5.78 <sup>57</sup>
Chikungunya	≥5.7 <sup>11</sup>	3.7*	—	>5.8 <sup>137</sup>	≥9.73 <sup>57</sup>
Influenza A virus	≥5.9 <sup>11</sup>	>5 <sup>159</sup>	—	—	≥5.1 <sup>57</sup>
SARS-CoV	>5.8 <sup>159</sup>	—	—	>6.5 <sup>137</sup>	≥4.9 <sup>57</sup>
Zika virus	10.3 <sup>173</sup> ^	—	>3.80 <sup>178</sup>	>5.99 <sup>18</sup>	≥4.48 <sup>178</sup>
Herpes simplex virus	—	6.09 <sup>179</sup>	≥2.8 <sup>174</sup>	—	≥5.5 <sup>57</sup>
Rabies virus	—	6.3 <sup>180</sup>	—	—	≥4.89 <sup>57</sup>
Dengue virus	≥4.3 <sup>11</sup>	1.8	—	>6.61 <sup>137</sup>	≥4.46 <sup>181</sup>
La Crosse virus	—	≥3.5 <sup>175</sup>	—	—	—
Crimean-Congo hemorrhagic fever virus	2.9 <sup>182</sup> ¶	—	—	—	—
<b>Viruses (nonenveloped)</b>					
HAV	0 <sup>159</sup>	1.8 <sup>159</sup>	—	—	0 <sup>57</sup>
Parvovirus B19	3.5 to >5 <sup>159</sup>	>5 <sup>159</sup>	5.46 <sup>159</sup>	—	≥5.0 <sup>57</sup>
Blue tongue virus	5.2 <sup>11</sup>	—	—	>5.0 <sup>17</sup>	—
Human Adenovirus	≥4.9 <sup>11</sup>	—	—	>7.4 <sup>17</sup>	≥5.33 <sup>176</sup>
Calicivirus	2.1 <sup>11</sup>	—	—	—	≥3.9 <sup>176</sup>
Poliovirus	—	3.36 <sup>179</sup>	—	—	>1 <sup>176</sup>
<b>Bacteria, Gram-negative</b>					
<i>Escherichia coli</i>	≥6.3 <sup>11</sup>	7.3*	>4.0 <sup>159</sup>	>6.7 <sup>17</sup>	—
<i>Enterobacter cloacae</i>	>6.6 <sup>11</sup>	4.8 <sup>159</sup>	>4.3 <sup>159</sup>	—	—
<i>Klebsiella pneumoniae</i>	>6.2 <sup>11</sup>	6	4.8 <sup>159</sup>	—	—
<i>Pseudomonas aeruginosa</i>	≥6.7 <sup>11</sup>	5.4 <sup>159</sup>	>4.9 <sup>159</sup>	4.5 <sup>137</sup>	—
<i>Salmonella cholerasuis</i>	6.2 <sup>11</sup>	2.4	—	—	—
<i>Serratia marsecens</i>	≥6.7 <sup>11</sup>	4.0 <sup>159</sup>	>4.0 <sup>159</sup>	5.1 <sup>17</sup>	—
<i>Yersinia enterocolitica</i>	≥5.9 <sup>11</sup>	3.3 <sup>159</sup>	—	6.8 <sup>17</sup>	—
<b>Bacteria, Gram-positive</b>					
<i>Bacillus cereus</i> (incl. spores)	3.7 <sup>11</sup>	2.7 <sup>180</sup>	—	—	—
<i>Bacillus cereus</i> (vegetative)	≥5.5 <sup>11</sup>	1.9 <sup>159</sup>	4.3 <sup>159</sup>	—	—
<i>Bifidobacterium adolescentis</i>	≥6.0 <sup>11</sup>	—	—	—	—
<i>Clostridium perfringens</i> (vegetative)	≥6.5 <sup>11</sup>	4.3*	>4.7 <sup>159</sup>	—	—
<i>Corynebacterium minutissimum</i>	≥5.3 <sup>11</sup>	—	—	—	—
<i>Listeria monocytogenes</i>	≥6.3 <sup>11</sup>	3.5*	—	>7.1 <sup>183</sup>	—
<i>Cutibacterium acnes</i> (formerly <i>Propionibacterium acnes</i> )	≥6.5 <sup>11</sup>	>2.0 <sup>159</sup>	4.5 <sup>159</sup>	>6.6 <sup>20</sup>	—
<i>Staphylococcus aureus</i>	≥6.6 <sup>11</sup>	4.9 <sup>159</sup>	>4.8 <sup>159</sup>	5.1 <sup>17</sup>	—
<i>Staphylococcus epidermidis</i>	≥6.4 <sup>11</sup>	5.1 <sup>159</sup>	4.8 <sup>159</sup>	>7.1 <sup>137</sup>	—
<i>Streptococcus pyogenes</i>	≥6.8 <sup>11</sup>	4.6 <sup>159</sup>	—	—	—
<i>Lactobacillus</i> species	≥6.4 <sup>11</sup>	—	—	—	—
<b>Spirochetes</b>					
<i>Borrelia burgdorferi</i>	≥6.8 <sup>11</sup>	—	—	—	—
<i>Treponema pallidum</i>	≥6.4 <sup>11</sup>	ongoing	—	—	—
<b>Parasites</b>					
<i>Babesia microti</i>	≥4.9 <sup>11</sup>	>4.0 <sup>134\$</sup>	—	>5.0 <sup>137</sup>	—
<i>Babesia divergens</i>	—	7.4 <sup>134\$</sup>	>6 <sup>174</sup>	—	—
<i>Leishmania major</i>	>4.3 <sup>159</sup>	>5.0 <sup>159</sup>	—	—	—
<i>Leishmania mexicana</i>	≥5.0 <sup>11</sup>	—	—	—	—
<i>Orientia tsutsugamushi</i>	>5 <sup>173</sup>	>5.0 <sup>168</sup>	—	—	—
<i>Plasmodium falciparum</i>	≥6.6 <sup>11</sup>	>3.2 <sup>159</sup>	≥4.9 <sup>174</sup>	>7.9 <sup>137</sup>	—
<i>Plasmodium yoelii</i>	—	>4.4 <sup>43</sup>	—	—	—
<i>Trypanosoma cruzi</i>	≥7.8 <sup>11</sup>	5.8 <sup>134\$</sup>	6.0	>5.3–5.4 <sup>137</sup>	—

¶ Evaluated in plasma, not platelets in PAS

§ Evaluated in whole blood

^ Evaluated in plasma

\* Unclear if these values are from cell-free or cell-associated models; “—” indicates no data available.

(Note: Refs. [173–183] refer to values only listed in this table.)

Inactivation of pathogens by four pathogen reduction technologies as measured by log<sub>10</sub> reduction. Values shown as greater than (>) or greater than or equal to (≥) indicate reduction levels below the limit of detection of the assay, and the actual levels of reduction could be higher for these organisms than indicated. Some of the data presented here were performed on analogous pathogens used as models for the listed pathogens. These details are available in the cited references in the table. Amotosalen/UV-A reduction numbers are assessments in platelets suspended in 65% platelet additive solution-3 (PAS-3)/35% plasma unless otherwise noted. Riboflavin reduction numbers shown are assessments in platelets unless otherwise noted. Riboflavin log<sub>10</sub> reduction results from standard in vitro assays for infectivity (TCID50) were calculated as per Appendix II of European Agency for the Evaluation of Medicinal Products, Committee for Proprietary Medicinal Products—Note for Guidance on Virus Validation Studies. UV-C and methylene blue reduction numbers were assessed in plasma. Amustaline/glutathione reduction numbers were assessed in RBCs. Values indicated with an asterisk (\*) for riboflavin technology were provided by Terumo Corporation.

reduction and preserving blood cell viability limits the ability of PR technologies to fully eliminate pathogens from blood components, and thus PR treatment is unlikely to always sterilize the transfusion product.

Several approaches have been used to determine the inactivation capacity of pathogen reduction technologies. Studies to establish pathogen reduction capabilities have generally utilized *in vitro* or *in vivo* animal models in addition to human trials. One clinical trial demonstrated a reduction in the transmission of malaria from use of riboflavin-treated whole blood in a patient population living in an endemic malarial region.<sup>38</sup> Another study, as part of a national hemovigilance program in several European countries, has reported the monitoring of the decrease in septic transfusion reactions associated with use of amotosalen/UV-A-treated platelets.<sup>39</sup> The results of pathogen inactivation studies are usually given as the number of organisms killed *in vitro* and reported as log<sub>10</sub> reduction values. These numbers are often used to predict *in vivo* effectiveness and to compare technologies. However, this approach tends to be an oversimplification<sup>40</sup> because there are several factors in addition to the virus level which affect the likelihood of infection transmission (Table 42.3).<sup>41</sup> Many results are reported as genome equivalents,<sup>40</sup> but the infectivity of a genome is not known with certainty because the pathogen reduction technology might alter or damage the nucleic acid sequences or even cut out parts of the genome. Thus, segments of a genome could be detected by nucleic amplification testing but not be infectious, and the level of virus detected would not necessarily relate to infectivity.<sup>40</sup> Also, the frequency of replication of competent viruses differs based on the type of virus and stage of infection in the donor.<sup>42</sup> For instance, blood components with the same viral levels may or may not transmit infection. Furthermore, components from the same contaminated unit of whole blood may or may not transmit infection, which is in part related to the lower volume of the derived components and the resulting lower doses of the virus.

**Table 42.3** Factors that can influence the infectivity of contaminated blood components.

<b>Viral factors</b>
Viral load
Viral genotype/clade
Genome mutations
Ratio of detectable nucleic acid equivalents to infectious particles
Stage of viral infection (acute vs. chronic)
Binding of virus to endogenous neutralizing antibodies
Diminishing viral replicative capacity with component storage
<b>Donor factors</b>
Stage of infection
Presence of antibody to the agent
<b>Transfusion factors</b>
Transfusion of other components with neutralizing antibody
Blood component being transfused (see below)
<b>Product factors</b>
Specific component (e.g., RBCs or FFP)
Storage duration of RBCs
<b>Patient-related factors</b>
Existing immunity through natural infection or vaccination (HBV)
Lack of viral receptors (i.e., resistance to infection)
Degree of immunosuppression
Body weight and/or blood volume

All of these factors must be considered when evaluating the efficacy of pathogen reduction since *in vitro* log reduction numbers may not necessarily correlate directly with clinical benefit. Adapted from Kleinman *et al.* (2009).<sup>41</sup>

The pathogen inactivation data in Table 42.2 are useful for providing general information, but for reasons discussed above and in Table 42.3, they do not serve to provide for direct comparisons among the various pathogen reduction technologies. This point is illustrated by the prevention of malaria transmission from donors with higher levels of circulating parasites than was to be expected based on riboflavin *in vitro* data.<sup>38,43</sup>

Bacteria provide a unique threat via transfusion as small numbers of organisms are able to proliferate in the product bag after collection. Furthermore, if any viable bacteria are left after PR, they can quickly grow back to life-threatening concentrations. For this reason, McDonald *et al.* have emphasized the need to assess PR effectiveness for bacteria in terms of sterility at outdate (e.g., for platelets, this is either after five or seven days of storage).<sup>44</sup> Assessment of nine different bacterial strains demonstrated dramatic differences among PR technologies, with amotosalen/UV-A and UV-C showing the highest degrees of efficacy.

## Pathogen reduction for noncellular blood components

Pathogen-reduced plasma products have been available in Europe since the 1990s. Earlier pathogen-reduced plasma components included both methylene blue and solvent/detergent-treated products.<sup>45</sup> Newer techniques using UV photoactivation include amotosalen/UV-A plasma approved for use in the United States by the FDA and riboflavin/UV-treated plasma, which is CE marked in Europe. There are reported changes in levels of various coagulation factors after PR treatment. These changes vary among the different technologies used and among the published reports. Overall, however, the PR-treated plasma components have been shown to be hemostatically effective for use in clinical practice.<sup>46</sup>

### Fresh-frozen plasma

#### Amotosalen/UV-A

Amotosalen/UV-A-treated plasma has been shown to have lower levels of certain coagulation factors compared to nonpathogen-reduced FFP. The treated plasma showed lower levels of factor V, factor VIII, protein S, and protein C with mild increases in coagulation times.<sup>47,48</sup> However, there has been no evidence to suggest diminished clinical efficacy.<sup>47</sup> Amotosalen/UV-A-treated plasma has fibrinogen levels within the reference range for conventional FFP. One study has shown that fibrin clots formed in PR plasma are denser and made up of thinner fibers, but these findings have unclear clinical implications.<sup>49,50</sup> Although amotosalen/UV-A-treated plasma is FDA-approved for use in the United States, it is not being regularly manufactured and is not in regular use in US transfusion services as of writing this chapter. It is being evaluated by researchers such as for a postmarket study of plasma transfusion for severe burns by the Coalition for National Trauma Research (NCT04681638). Amotosalen/UV-A plasma is also CE marked and is in routine use in European countries.

#### Riboflavin/UV

Plasma treated with riboflavin for pathogen reduction has reduced levels of some plasma coagulation factors compared with those levels of untreated plasma.<sup>48,50,51,52</sup> Riboflavin-treated plasma is as effective as conventional plasma and provides improvement in INRs, but slightly more riboflavin-treated plasma was required for a clinical effect.<sup>53</sup> Riboflavin-treated plasma had similar efficacy compared to conventional plasma in patients with an acquired coagulopathy.<sup>53</sup> Riboflavin plasma is CE marked and is used only outside of the United States.

### UV-C

Currently, no plasma products treated with UV-C light are approved for use in any country. UV-C light is known to damage proteins,<sup>26</sup> and although a proof of principle has been demonstrated for plasma, UV-C-treated plasma has been reported to have lower activities of some coagulation factors.<sup>54</sup> In addition, proteins in plasma can “quench” the ability of UV-C to reduce pathogens and thus pose challenges to applying this technology to plasma components.

### Methylene blue

MB-treated plasma is currently available in Europe but is not approved in the United States. Plasma treated with MB has lower levels of fibrinogen, protein S, and factors V, VIII, and XI. Further, the thrombin-generating capacity of MB plasma has been shown to be reduced.<sup>45,56</sup> There is a lack of large, randomized controlled trials evaluating the clinical efficacy of MB-treated plasma, and there are conflicting results from prospective and observational studies of MB-treated plasma.<sup>57</sup> Studies have shown no significant differences in time of in vivo bleeding, and blood loss between MB-treated and S/D-treated plasma in patients undergoing cardiac surgery,<sup>57,58</sup> but others have noted increased volumes of MB-treated plasma used in liver transplant patients.<sup>59</sup> In addition, one retrospective study has indicated that MB-treated plasma may be less effective in treating thrombotic thrombocytopenic purpura compared to fresh-frozen plasma.<sup>60</sup>

Although MB-treated plasma is approved in Europe, concerns due to a reportedly higher risk of severe allergic reactions to MB-treated plasma<sup>61</sup> have resulted in discontinued use in France.<sup>37</sup> Other European countries, however, have not observed increases in allergic reactions,<sup>57</sup> and one prospective, multicenter, nonrandomized study has shown low rate of serious adverse reactions of 0.5 per 10,000 MB-treated plasma units.<sup>37</sup>

### Solvent/detergent

Various S/D-treated plasma products have been available in the market, but currently the only S/D plasma product approved by the FDA for use in the United States is Octaplas (Octapharma, Hoboken, NJ), which is available in all ABO types. In Europe, a “universal” S/D plasma product is available.<sup>62,63</sup> Earlier formulations of S/D-treated plasma were associated with adverse events such as thromboembolic complications and were withdrawn from the market.<sup>7</sup> Current S/D plasma products have not been reported to have these complications.<sup>64</sup> Available S/D plasma products today have coagulation factor levels similar to those of FFP with the exception of lower protein S levels, but the clinical significance of these findings have not been fully assessed.<sup>64–66</sup>

### Cryoprecipitate

Cryoprecipitate is capable of transmitting viruses as well as being a source of bacterial contamination.<sup>67</sup> Cryoprecipitate is often transfused as a pooled component—usually a pool of five whole blood units. Pooling components increases the risk of TTIs proportional to the number of units in the pool. Because cryoprecipitate is stored at room temperature after thawing, conventional cryoprecipitate has a shelf-life of six hours for single units and for products pooled with an FDA-cleared sterile connecting device. The shelf life is four hours for cryoprecipitate pooled without a sterile connecting device. In contrast, for cryoprecipitate derived from plasma treated with amotosalen/UV-A (known as pathogen-reduced cryoprecipitated fibrinogen complex), the FDA has permitted an extension of

the post-thaw shelf life to five days at room temperature.<sup>68</sup> Cryoprecipitate derived from amotosalen-treated plasma is reported to have lower levels of fibrinogen and factor VIII but comparable levels of vWF compared to conventional cryoprecipitate.<sup>69</sup> Amotosalen/UV-A cryoprecipitate is available for use in the United States. For cryoprecipitate derived from riboflavin-treated plasma, decreased levels of factor VIII, von Willebrand factor, and fibrinogen have been reported compared with conventional cryoprecipitate, but these levels exceed the minimal requirements of the Council of Europe.<sup>70</sup> S/D cryoprecipitate made from S/D-treated plasma has been reported to have acceptable levels of factor VIII, vWF, and fibrinogen.<sup>71</sup> Cryoprecipitate derived from MB-treated plasma has also been studied and has been shown to have higher levels of factor VIII, lower levels of factor XIII, and similar levels of fibrinogen activity compared to cryoprecipitate derived from amotosalen-treated plasma.<sup>72</sup>

### Plasma derivatives and factor concentrates

Derivatives manufactured from pools of plasma from large numbers of human donors must be processed to maximize product safety. Pathogen reduction technologies have been used to reduce the risk of infectious disease transmission. Intravenous immunoglobulins (IVIG) are produced from fractionated donor plasma.<sup>73</sup> Methods for reducing pathogenic risk in IVIG products include the fractionation process itself, pasteurization, nanofiltration, and solvent/detergent treatment.<sup>73,74</sup> Factor concentrates are mostly recombinant and do not present a risk of blood-borne pathogen contamination. However, coagulation factor concentrates that are manufactured from pooled human plasma are pathogen-reduced with S/D treatment. Albumin is derived from large pools of human plasma, but the manufacturing steps use processes such as fractionation and pasteurization and do not utilize pathogen reduction technology.<sup>75</sup>

### In vitro and in vivo quality of pathogen-reduced cellular components

#### In vitro platelet function tests—background

In vitro tests of platelet function fall into two categories: (1) indirect tests such as platelet count, metabolism, structure, presence of receptors, or activation markers and (2) direct in vitro measurements of adhesive, aggregating, and procoagulant activities. These in vitro tests have been used to assess platelets that have been pathogen reduced. We provide a brief discussion about these assays and some considerations that need to be made when assessing the function of PR platelets.

Parameters such as platelet counts, metabolic functions such as glucose, pH, and lactic acid are the easiest to measure, and all these parameters are routinely studied to evaluate the quality of platelet concentrates. Glucose consumption by cells and importantly by any contaminating bacteria generate a high level of lactic acid, which reduces the pH of the unit of platelets and contributes to platelet damage. These cellular activities reduce the recovery and survival of radiolabeled platelets in healthy subjects. However, good platelet function can be maintained even with glucose levels of zero, so the absence of glucose does not necessarily imply poor platelet function.

Platelets have a discoid shape which affects light scattering and causes a well-known “swirling effect.” During storage, platelets progressively become activated and undergo a disc-to-sphere

transformation, lose their discoid shape, become spherical, and lose the ability to display "swirl." While the presence of swirling is predictive of a satisfactory pH value, the absence of swirling is less helpful because many platelet concentrates with poor swirl still have an adequate pH.<sup>76</sup> Platelet swirl is thus a poor quality measure.

The hypotonic shock response has been used to evaluate platelet quality as an indication of platelet membrane integrity and the ability of platelets to contract as part of the release reaction. However, these various platelet assays do not provide direct information about platelet hemostatic function, such as adhesive, aggregating, and procoagulant functions. Flow cytometry has limitations since different antibodies will recognize different specific binding sites within antigens. Also, these markers cannot predict the functional effect of the binding of a specific antigen to a specific receptor. Decreased glucose levels and increased lactate production lead to lower pH, alterations in platelet morphology, decrease in surface glycoproteins, enhanced expression of platelet activation markers, and reduced functional or aggregation response. However, none of these changes or combination of changes have been shown to directly relate to *in vivo* hemostasis.

In summary, *in vitro* tests of platelets evaluate limited aspects of the complex hemostatic action of platelets. Tests provide information on different aspects of platelet function, but results from these tests are not necessarily predictive of the clinical hemostatic capacity of platelet concentrates once they are transfused.<sup>77</sup> Thus, results of *in vitro* tests of PR platelets should be evaluated with these issues in mind.

### **In vitro testing of pathogen-reduced platelets**

Extensive testing has been performed on both amotosalen and riboflavin treated platelets. It is important again to note that *in vitro* platelet function tests do not predict impaired hemostatic effects.<sup>78</sup> In general, testing results are similar between amotosalen and riboflavin treated platelets.<sup>77</sup>

### **Amotosalen/UV-A *in vitro* platelet quality**

The changes associated with amotosalen/UV-A treatment of platelets are decreased glucose, increased lactate accumulation, and a falling pH.<sup>79–82</sup> In addition, alterations in platelet morphology, decrease swelling, and a decreased resistance to hypotonic shock also occur.<sup>79–82</sup> There are moderate reductions in membrane glycoproteins with increased expression of P selectin, which suggests platelet activation.<sup>83,84</sup>

### **Riboflavin/UV *in vitro* platelet quality**

Alterations induced by riboflavin are similar to those induced by amotosalen with increased anaerobic metabolism followed by a decline in pH, morphologic alterations, reduction in swirling, and decreased resistance to hypotonic shock.<sup>85–90</sup> There are moderate reductions in membrane glycoproteins with enhanced expression of platelet activation markers indicating apoptosis.<sup>86–88,90–98</sup> Thus, riboflavin-treated platelets have some altered *in vitro* functional properties, but these are similar to amotosalen-treated platelets.<sup>77</sup> One *in vitro* thromboelastography study of riboflavin-treated platelets has been reported to show weaker clot strengths.<sup>99</sup>

### **UV-C *in vitro* platelet quality**

The metabolic and morphologic changes in UV-C-treated platelets are similar to those described for amotosalen and riboflavin. This involves a moderate decrease in glucose, increase in lactate, decrease

pH, alterations in swirling, and reduction in hypotonic shock response. Changes in membrane proteins and expression of P selectin are slightly less pronounced than for amotosalen and riboflavin.<sup>100–103</sup>

### **Summary of *in vitro* quality of PR platelets**

More direct platelet function is measured by adherence, aggregation, and procoagulant generation. Both amotosalen-treated<sup>81,104,105</sup> and riboflavin-treated<sup>76–78,85</sup> platelets have decreased aggregation responses, but these responses are somewhat better preserved with UV-C treatment.<sup>91–93</sup> In studies under flow conditions which more closely evaluate the adhesive function of platelets, amotosalen-treated platelets showed results similar to control untreated platelets.<sup>106</sup> Stivala *et al.* showed reduced adhesion and aggregation of amotosalen-treated platelets to von Willebrand factor collagen substrates.<sup>105</sup> Riboflavin-treated platelets had reasonably well-preserved adhesive and cohesive functions comparable to control untreated platelets<sup>93</sup> and were similar to the results with amotosalen-treated platelets. Overall, these studies suggest that the functional changes noted in *in vitro* tests are less pronounced in the blood flow testing settings, and the changes are similar among all three PR methods. Use of thromboelastogram metric assays that measure the contribution of thrombin, fibrinogen platelet glycoproteins, and cytoskeleton in clot formation has shown reductions in the maximum clot strength of riboflavin platelets and similar results from UV-C platelets.<sup>97,107–109</sup> While many changes have been documented as a result of both amotosalen, riboflavin, and UV-C treatment, these have a weak impact on the overall proteome of platelets and thus do not reveal any remarkable changes of clinical concern.<sup>110</sup> In published studies comparing the effect of gamma radiation versus riboflavin/UV and amotosalen/UV-A, gamma irradiation resulted in changes seen as if there was an acceleration of the platelet storage lesion.<sup>77,111,112</sup> A large number of studies show that different pathogen reduction technologies cause similar modifications in *in vitro* tests, and these studies do not, in general, indicate any specific adverse effect on hemostatic efficacy.<sup>77</sup>

### **In vivo studies in healthy human subjects**

#### **Radiolabeling studies**

Studies of radiolabeled platelets in healthy research subjects showed a lower recovery and shorter survival of both amotosalen<sup>113–115</sup> and riboflavin-treated platelets<sup>92</sup> compared to nontreated platelets (Table 42.4). Thus, it could be expected that post-transfusion platelet count increments would be lower, and the time to the next platelet transfusion from a prior one would be shorter after the transfusion of riboflavin- or amotosalen-treated platelets. This has been confirmed in randomized controlled clinical trials.<sup>116</sup> *In vivo* recovery and survival of UV-C light-treated platelets also were reduced. This finding is similar to that of other PR systems.<sup>100</sup>

#### **In vivo randomized controlled clinical trials of pathogen-reduced platelets**

There have been 10 randomized controlled clinical trials of PR platelets,<sup>116</sup> 7 of amotosalen,<sup>117–123</sup> and 3 of riboflavin platelets.<sup>123–125</sup> In trials of amotosalen platelets, a total of 1922 patients have been studied (954 PR and 968 control). In trials of riboflavin platelets, a total of 862 patients have been studied (434 PR and 428 control).

For the seven amotosalen trials, bleeding was the end point in three and corrected count increment (CCI) in four.<sup>116</sup> For

**Table 42.4** In vivo Recovery and Survival of Radio-Labelled Pathogen-Reduced Platelets

Recovery		
	Amotosalen	Riboflavin
Control Platelets	50.3% ± 7.7%	66.5% ± 13.4%
PR Platelets	42.5% ± 8.7%	50.0% ± 18.9%
Survival		
	Amotosalen	Riboflavin
Control Platelets	144 ± 29 hours (6 ± 1.2 days)	142 ± 26 hours (5.9 ± 1.1 days)
PR Platelets	115 ± 31 hours (4.8 ± 1.3 days)	104 ± 26 hours (4.3 ± 1.1 days)

In vivo recovery and survival of radiolabeled pathogen-reduced platelets. Since both amotosalen and riboflavin platelets have less recovery and shorter survival after being transfused, CCls for pathogen-reduced platelets are expected to be lower.<sup>92,113</sup> Data are expressed as mean ± standard deviation.

riboflavin-treated platelets, bleeding was the end point in two and CCI in one trial.<sup>116</sup> In all of these trials, there was no difference in the portion of patients who experience WHO grade 2 or greater bleeding between patients who received conventional platelets or those who received PR platelets. This indicates that PR platelets are as clinically effective as standard platelets. In most trials, transfusions with PR platelets were associated with lower CCI and shorter time to the next transfusion. These findings are not unexpected based on the studies of radiolabeled platelets in healthy research subjects (Table 42.4). In most trials, there was less than one additional unit of PR platelets transfused compared to conventional platelets, but the duration of platelet support was similar, and the use of red blood cell transfusion was no different.<sup>126</sup> Similar results were found in a quality assurance review of PR platelet usage in pediatric and adult patients.<sup>127,128</sup> These results indicate that patient receiving PR platelets did not experience exceptional bleeding. In contrast, one randomized controlled trial measuring one-hour CCI in patients receiving UV-C-treated platelets compared to conventional platelets failed to show noninferiority.<sup>129</sup>

The frequency and nature of adverse events were not different following the transfusion of either PR or conventional platelets, indicating that there are no unusual safety concerns from these transfusions. Studies from Europe with amotosalen/UV-A platelets showed marked decrease in bacterial contamination in PR platelets compared to conventional platelets.<sup>39</sup> An analysis of data from the hemovigilance programs from Belgium, Switzerland, and France showed, in aggregate, zero probable or definite/certain septic reactions following transfusion of 609,290 amotosalen/UV-A platelet units. These results were significantly lower than those reported with concurrently transfused and/or historic septic reaction rates from the transfusion of non-PR treated platelets for these countries.<sup>39</sup>

### Whole blood and red blood cells

#### Amotosalen/UV-A

Amotosalen/UV-A is not used for RBCs or whole blood pathogen reduction as mentioned above since UV-A light does not efficiently reduce units of red cells. However, riboflavin technology can be used to treat RBCs and whole blood. Amustalen/glutathione technology is actively being studied in several clinical trials for the treatment of RBCs.<sup>130,131</sup>

#### Riboflavin whole blood and red blood cells

Riboflavin technology can be used to treat either whole blood or red blood cells.<sup>132–136</sup> The process is similar to that used for platelets and plasma. Riboflavin is added to the whole blood or red blood cells and exposed to UV light. Cell quality data show satisfactory in vitro results to support storage of the blood for up to 21 days.<sup>132–134</sup> Two in vivo studies of radiolabeled riboflavin-treated red cells in healthy research subjects showed slightly reduced recovery and shortened survival leading to a 21-day storage duration.<sup>135,136</sup> Normal red cell shelf life is 35–42 days depending on the preservative/anticoagulant used for storage. The IMPROVE I trial determined the recovery and survival of RBCs, and in vitro testing of platelets and plasma from nonleukoreduced whole blood in 12 subjects has been performed.<sup>135</sup> Treated whole blood was separated into components, each of which was tested at the beginning and at the end of storage. Autologous RBCs radiolabeled with <sup>51</sup>Cr were transfused into the healthy subjects, and 24-hour recovery and survival half-lives after 42 days storage were determined. Red cell recovery at 24 hours post-transfusion was 82.5% for riboflavin-treated cells and 91.7% for control-untreated cells, which are values that meet US FDA criteria for satisfactory recovery.

One trial performed was a prospective, randomized, double-blind, controlled, single-center study at the Komfo Anokye Teaching Hospital in Ghana.<sup>38</sup> The objective of this study was to determine whether riboflavin-treated whole blood was less likely to transmit malaria. Patients were randomized 1:1 to receive ≤2 transfusions of either riboflavin-treated or untreated (i.e., standard preparation) whole blood. If additional transfusions were required, standard untreated whole blood was used. Subjects were followed for 28 days post-transfusion, and parasite detection was carried out on Days 1, 3, 7, and 28. Transmission of malaria was reduced by 87% in patients who received the riboflavin whole blood compared to standard whole blood.<sup>38</sup> Importantly, this study was able to show reduction of infection from the transfusion of pathogen-reduced red cells.

#### Amustaline/glutathione red blood cells

Red blood cells treated with amustaline/glutathione are currently under active investigation with several randomized controlled clinical trials.<sup>130,131,137</sup> A radiolabeling study of amustaline-treated RBCs reported that these RBCs show sufficient post-transfusion recovery to satisfy FDA criteria.<sup>136</sup> One randomized controlled trial showed no difference between hemoglobin content, RBC usage, or clinical outcomes between amustaline-treated and conventional RBCs.<sup>130</sup> As of writing this chapter, two US clinical trials are currently being performed: the ReCePi trial is studying the efficacy and safety of amustaline-treated RBCs in patients undergoing complex cardiac surgery, and the ReDeS trial is studying the increases in hemoglobin levels in patients receiving amustaline-treated RBCs and the reduction of risk of transmission of the Zika virus and other TTDs.

### Immunological effects of pathogen-reduced blood components

#### Alloimmunization to platelet transfusions

About 20% of cases of refractoriness to platelet transfusions are due to immunological causes. Alloimmunization to different HLA Class I molecules is a frequent underlying etiology. As discussed by Maier *et al.* in Chapter 16, pathogen reduction technol-

ogy has been investigated as a method for reducing the risk of alloimmunization to platelet transfusions. The use of ultraviolet irradiation and/or the treatment with photoactivating agents to damage DNA are thought to reduce the immunogenicity of leukocytes found in the blood component and thus reduce antibody responses to HLA molecules. However, conflicting results among preclinical animal studies and several clinical trials have led to mixed conclusions on the effect of pathogen reduction on platelet alloimmunization.

Observations from several animal studies suggest evidence of reduction in alloimmunization with PR platelets due to various effects in reducing the immunogenicity of contaminating donor leukocytes. These observed effects include inhibition of antigen presentation, reduced survival and expression of HLA Class II in antigen-presenting cells, and induction of tolerance, leading to decreased anti-MHC alloantibody formation.<sup>138–141</sup> These results contrast with often conflicting and unexpected conclusions from data collected from clinical trials showing a range of increases, decreases, and no differences in alloimmunization rates from platelets treated with various different PR technologies. For example, a subanalysis of the PREPARES trial showed increased rates of HLA I class alloimmunization to riboflavin-treated platelets<sup>142</sup> while the SPRINT trial found no significant difference in HLA antibody formation rates from amotosalen-treated platelets.<sup>143</sup> One hospital system reported increased rates of incompatible platelet crossmatch tests concurrent with increased transfusions of amotosalen-treated platelets.<sup>144</sup> However, an analysis of the Italian IPTAS study demonstrated no association of amotosalen-treated platelets with platelet alloimmunization. It was suggested that the observations of increased clinical refractoriness may have been due to intrinsically lower CCIs from PR platelets rather than alloimmunization.<sup>145</sup> As discussed in Chapter 16, these disparate results could be due to differences in clinical trial designs and product preparation unrelated to pathogen reduction. It is likely that resolving these discordant observations will require the completion of ongoing clinical trials and careful data analysis.

### **Prevention of transfusion-associated graft-versus-host disease**

Pathogen reduction techniques that cause nucleic acid damage in pathogens also damage nucleic acids in lymphocytes and prevent their proliferation in blood components.<sup>146–152</sup> Thus, PR blood components should not cause TA-GVHD. This has been established in animal studies,<sup>150</sup> but it is unlikely that a classic randomized controlled clinical trial will ever be conducted in humans. Many centers have discontinued irradiating amotosalen/UV-A or riboflavin/UV PR platelets and have not observed TA-GVHD as a consequence.<sup>153,154</sup> Thus, it seems reasonable that other PR techniques could eliminate the need for gamma or X-ray irradiation for the prevention of TA-GVHD.<sup>155</sup> The package insert for amotosalen/UV-A-treated platelets states that amotosalen/UV-A can be used “as an alternative to gamma irradiation for the prevention of transfusion-associated graft-versus-host disease” and provides FDA-approved language that PR platelets do not need to be irradiated. In fact, irradiating PR platelets could cause a “double hit injury” to the platelets that could potentially decrease post-transfusion platelet recovery and survival. However, early PR trials involving amotosalen/UV-A platelets, including the SPRINT study, did include irradiated amotosalen/UV-A platelets that were shown to be clinically effective.<sup>5,156</sup>

## **Safety and toxicity of pathogen-reduced technologies**

### **Amotosalen**

Amotosalen preclinical toxicity has been evaluated in several studies, including assessments for acute toxicity, reproductive effects, neonatal toxicity, phototoxicity, carcinogenicity, safety pharmacology, genotoxicity, and neoantigenicity.<sup>8,49,157–159</sup> These studies have found no evidence of organ or reproductive toxicity, neonatal toxicity, carcinogenicity, or neoantigenicity.<sup>160</sup> It was determined that dermal phototoxicity occurs at psoralen concentrations that are 1000 times higher than those levels used clinically or found naturally in diets.<sup>161</sup> Animal studies have also shown no toxicological effects in adult, juvenile, and neonatal animals. There have been concerns expressed regarding use of amotosalen/UVA platelets in neonates and infants. Specifically, these concerns relate to photodermatitis—skin rashes—in children who received a psoralen pathogen-reduced platelet and were treated for hyperbilirubinemia with blue light phototherapy.<sup>162</sup> As such, amotosalen-treated platelets are contraindicated in neonates receiving treatment with “phototherapy that emit a peak energy wavelength less than 425 nm or have a lower bound of the emission bandwidth less than 375 nm.”<sup>163</sup> Phototherapy devices used clinically in the United States do not emit energy in these wavelengths. Recent studies have shown no adverse events in adult or pediatric patients attributed to amotosalen/UV-A pathogen-reduced products.<sup>127,128</sup> As such, amotosalen/UV-A pathogen-reduced platelet and plasma products are FDA approved for all populations.<sup>10,11</sup> There had been some early reports of increased risk of acute respiratory distress syndrome (ARDS), but this has not proven to be a clinical problem and these platelets have come into wide use.<sup>11,156,164</sup> Results of a large hemovigilance study of adult and pediatric hematology patients have shown no increased risk of pulmonary complications of PR platelets compared to conventional non-PR platelets.<sup>126</sup> One promising study has shown no increase in adverse effects of amotosalen-treated platelets with seven-day storage compared to conventional platelets with five-day storage, but as of writing this chapter, an extended storage time has not yet been FDA approved.<sup>165</sup>

### **Riboflavin**

Since riboflavin is a vitamin—namely vitamin B2—its toxicity has been well studied, and it is generally regarded as safe. When riboflavin is subjected to UV light, four breakdown products are produced in the circulation that occur physiologically at low concentrations.<sup>166,167</sup> Thus, as no new, nonphysiologic compounds are generated, there is no need for a post-treatment removal process. The toxicity of these compounds and the amounts infused as part of the pathogen reduction process have been well studied and have been shown to be well within accepted safety limits.<sup>167</sup>

### **Amustaline/glutathione**

The systemic toxicity, genotoxicity, and carcinogenicity of amustaline- and glutathione-treated RBCs have been studied in rats, dogs, and mice.<sup>16,168</sup> As described before, amustaline has side reactions with plasma proteins and proteins found on the extracellular surface of the RBC membrane, and glutathione is used to quench these reactions. One report studied the systemic toxicity and pharmacokinetics of amustaline-treated red cells in dogs and rats by administering RBCs with levels of amustaline up to five times higher than those used in RBCs for clinical use and with levels of glutathione

that would not completely quench the amustaline.<sup>16</sup> This study showed no effect on mortality and no evidence of organ toxicity, and pharmacokinetics assessment demonstrated no accumulation of amustaline breakdown products in the plasma. A second study from the same group investigated the genotoxicity and carcinogenicity of amustaline-treated red cells.<sup>168</sup> There were no carcinogenic effects found in a heterozygous p53<sup>+/−</sup> mouse model, and no cytogenetic aberrations were found in an *in vitro* clastogenicity assay in human peripheral blood lymphocytes or in an *in vivo* mouse bone marrow micronucleus assay. These findings support the safety of amustaline/glutathione PR-RBCs.

### UV-C

Evaluations of the toxicity of UV-C treated platelets are limited. The lack of the addition of a photoactive chemical excludes the need for a traditional toxicological assessment.<sup>169</sup> One preclinical study performed repeated transfusions of UV-C-treated autologous platelet concentrates in beagle dogs and found no signs of intolerance to the transfusions.<sup>170</sup> This study assessed immunogenicity with high-resolution proteomics, which detected few modifications in platelet proteins due to UV-C treatment, and did not detect any antibody responses.

### Methylene blue

Methylene blue has been demonstrated to have an overall good safety profile with low toxicity.<sup>32,37</sup> Adsorbent filtration of MB plasma has been shown to reduce residual MB down to undetectable levels.<sup>171</sup> However, there are reports of allergic and anaphylactic reactions from plasma treated with MB, leading to the discontinuation of its use in certain countries as discussed previously.<sup>30,172</sup>

### Solvent/detergent

Residual amounts of solvent and detergents in S/D-treated plasma are at very low levels and are of little concern for toxicity. The European Pharmacopoeia allows for less than 2 µg/mL of trinitrobutylphosphate solvent and less than 5 µg/mL of Triton X-100 in S/D-treated plasma, which are significantly lower than concentrations considered to be toxic.<sup>7</sup> In fact, the majority of S/D plasma products have been found to have levels of these compounds below the threshold of detection.<sup>30</sup> There have also been no

known reports of toxicity or adverse reactions after the transfusion of millions of S/D plasma units.<sup>7</sup>

### Summary

The importance of pathogen reduction for the public health aspects of the blood supply cannot be overstated. Bacterial and viral contamination of blood components is an ongoing concern as transfusion transmitted diseases have serious and even fatal consequences. The additional potential for contamination with unknown yet to be encountered pathogens exacerbates the concerns for a safe blood supply. Despite the upfront costs of pathogen reduction, proactively reducing the overall risk of transfusion-transmitted diseases will prove to be economically and socially invaluable, particularly when a new blood-borne disease arises in the future. Advances in utilization and efficacy of pathogen reduction technologies will play a major role in maintaining and ensuring a safe blood supply.

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## **SECTION VII**

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# **Adverse sequelae of transfusion**

## CHAPTER 43

# Hemovigilance: weighing the risks versus benefits of transfusion

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## Introduction

Transfusion of blood products can be a life-saving procedure. Like all medical procedures, the act of blood transfusion includes risk for the recipient. Even the act of not transfusing a blood product may pose the risk of life-threatening anemia or bleeding to a patient. Although generally considered safe, the donation of blood products also involves risk for donors who can experience injury related to the donation process. Around the globe, a wide variety of legislation, regulations, rules, and voluntary programs exist which are designed to monitor and minimize risk for both the donor and the recipient. While stringent requirements, oversight, and data reporting may increase the safety of blood transfusion or donation, resource limitations often constrain blood safety controls in order to meet demand. In addition, the prevalence of transfusion-associated risk such as transfusion-transmitted diseases can vary among blood donors in different areas due to disease prevalence. As such, the evaluation of the risk versus benefit of blood product transfusion is setting dependent and requires the integration of a significant amount of data to determine whether a transfusion is indicated. Hemovigilance systems can help determine the risk–benefit ratio.

## Risk

Although there is no agreed upon definition of the concept of risk, in general risk is thought of as the likelihood that harms or something untoward occurs because of an intervention.<sup>1</sup> The intervention can be an action, such as giving a blood product, or an inaction, such as deciding to withhold a transfusion. In medicine, risk is often understood and conveyed through both subjective and objective terms.<sup>2</sup> Examples of subjective risk include how impactful or significant an adverse reaction is to a patient, whereas objective examples include how often or frequently an adverse event occurs. Two approaches for reducing risk in transfusion have been widely employed and include quality monitoring systems and standardized reporting and learning systems (RLS).<sup>3,4</sup> Systematic and consistent categorization of adverse events related to transfusion or donation is needed to truly understand and improve the accuracy of our risk assessments.

## Reporting and learning systems in transfusion

The term *hemovigilance* has become widely used over the past decade to describe the systematic surveillance of adverse transfusion reactions and events, encompassing the whole transfusion chain from donor to recipient, or “vein to vein.”<sup>5,6</sup> The concept for RLS in transfusion medicine has been to establish hemovigilance systems to identify, track, and understand risks in blood donation and blood product transfusion in order to improve safety. A wide variety of RLS exist around the world (Table 43.1).

Hemovigilance systems range from voluntary to compulsory. The Canadian Patient Safety Institute (CPSI) found that voluntary systems were best, and some established hemovigilance systems, such as SHOT and the Danish Registration of Transfusion Accidents (DART), are voluntary. Voluntary systems are often started by professional or scientific bodies, and participants may therefore feel more ownership and, hence, more willingness to participate. Compulsory systems are usually initiated by governments and supported by laws, regulations, or directives.

## Hemovigilance methods

### Reporting systems

Hemovigilance systems rely on the reporting of data from members. Both WHO and CPSI recommend nonpunitive and confidential hemovigilance systems to encourage accurate reporting, especially given that many adverse events and near misses involve human error.<sup>7</sup> When reporting systems are used for learning purposes, it is important that details that could be used to identify people or places are removed or changed.

Data can be collected in either a passive or an active manner. Passive systems report only recognized adverse events or near misses, whereas active systems also tend to report when a transfusion or blood donation was uncomplicated. Even with active reporting systems, adverse events related to a transfusion or donation may be missed. One reason for not recognizing transfusion or donation-related adverse events is lack of awareness of the signs and symptoms of a reaction or attributing the adverse event to some other cause. The number of adverse events that are missed could be significant.

**Table 43.1** Examples of Hemovigilance Systems

Country	Date of Initiation, Scope of Reporting (Voluntary at Inception Unless Otherwise Mentioned)	Governance
Japan	1993: Transfusion-associated adverse reactions and infectious diseases	Japanese Red Cross Society
France	1994: Mandatory system created by national legislation; all severity levels	Inspectorate for Healthcare Products (currently: Agence Nationale de Sécurité du Médicament et des Produits de Santé)
United Kingdom	1996: Serious Hazards of Transfusion (SHOT); serious reports	Professional societies
Denmark	1999: Danish Registration of Transfusion Accidents (DART); modeled on SHOT	Society for Clinical Immunology Early emphasis on donor adverse reactions (Danish Donor Society)
South Africa	2000: All severity levels; donor reactions included from 2010	South African National Blood Service
Netherlands	2002: Transfusion and transplantation reactions in patients (TRIP); "all"	Professional societies
Norway	2004: TROLL. Reports on transfusion reactions and complications of blood donation from 2004. Reports on other adverse events from 2007	From 2004 to 2007: The Norwegian Society for Immunology and Transfusion Medicine (Professional Society). From 2007: The Norwegian Directorate of Health is responsible for hemovigilance, but the system is operated by the Norwegian Knowledge Centre for the Health Services
New Zealand	2005: All severity levels	New Zealand Blood Service
United States	2006: National Healthcare Safety Network, Biovigilance reporting system. Gradual increase of participation; incorporated donor vigilance from inception	US Biovigilance Network (public-private collaboration between the US Department of Health and Human Services, including the Centers for Disease Control and Prevention, and organizations involved in blood collection, transfusion, tissue and organ transplantation, and cellular therapies)

For example, careful review of vital signs during transfusion found that up to 75% of transfusion reactions may not be reported.<sup>8</sup> Catching adverse events may be more likely in the future with the implementation of computer health information systems that use algorithms to detect and alert staff to potential adverse events.<sup>9</sup>

Another reason for underreporting adverse events in transfusion is that the adverse event is not temporally adjacent to the transfusion or donation. For example, in donors, adverse reactions that occur while the blood donor still is present in the blood bank or donor center are fairly easy to capture, whereas some serious complications, like faints that occur after leaving the blood bank or donor center, may not be reported until the time of the next donation, if ever. Another example of temporally delayed reactions to transfusion is delayed hemolytic transfusion reactions, which typically are detected days after the transfusion. Due to the presence of hemovigilance networks, strategies are emerging to better capture adverse events that occur days after the transfusion. For example, the use of a hemovigilance network in France has successfully increased awareness and recognition of delayed hemolytic transfusion reactions through the dissemination of information through the network.<sup>10</sup>

### Reported data, investigations, and assessment

Adverse reactions are investigated to establish the diagnosis and likelihood or imputability that the adverse event was related to the transfusion or donation. Reporting to the hemovigilance system requires sufficient information to allow the assessors to verify the type of reported reaction. In addition, the adverse events are typically rated for severity. Most hemovigilance systems have objective criteria for establishing both imputability and severity so that adverse events can uniformly be evaluated.<sup>11</sup>

A range of adverse events are reported to hemovigilance systems. In some countries, legislation requires only reporting of serious adverse events or fatalities. When only serious adverse events are reported, less serious adverse events or near misses are not always captured. Identification of near misses and all adverse events are key to a robust and effective RLS. However, the number of reports on the hemovigilance system dramatically increases if all adverse events and near misses are reported. Therefore, available resources determine the type of data reported to a hemovigilance system.

Analysis of the hemovigilance data from a country or region requires context and background. Typical background data that are registered for reporting donor complications are the donors' age and gender, the donation type (whole blood or apheresis), the date and time of donation, and if it was a first-time donation. Typical background data for transfusion reactions are patient age and gender, the indication for transfusion, department and/or ward, and time from transfusion to reaction. Other key information includes whether universal leukodepletion of blood components is in place and for transfusion-transmitted diseases, the prevalence of those diseases in that area.

Context and background help the users of a hemovigilance system to understand the frequency of an adverse event. For example, vasovagal reactions may be reported more frequently in young women, but if we do not know what proportion of donors are young females, this information has limited value. In certain cases, more background information may be useful, such as a female donor's parity in cases of TRALI and the patient's cardiovascular status when transfusion-associated cardiac overload (TACO) or transfusion-associated dyspnea (TAD) is suspected. Also, follow-up testing for antibodies for human leukocyte antigens or platelet antigens can help confirm a diagnosis.

### Hemovigilance data analysis, assessment, and reporting

Reporting to a centralized system allows for the analysis of systemic risk in order to derive recommendations for the improvement of practice and prevention of future errors and incidents and to accurately determine objective risk. From an analysis of hemovigilance data, risk and likelihood of recurrence can be combined to support prioritization of a particular problem for detailed analysis and preventive measures. This can be performed at a system or national level, but also at a local or institution level. Mistakes or errors at the moment of transfusion may have several latent causes, such as lack of training, poorly designed processes, inadequate software, or understaffing. Facilities should assess such causes and supply sufficient information to the hemovigilance system to allow analysis of recurrent problems and weak "links" in the transfusion chain.<sup>12,13</sup> A number of methods for analyzing and classifying contributing

causes have been developed.<sup>12</sup> A practical toolkit based on techniques recommended by the (former) UK National Patient Safety Agency is available on the SHOT website ([www.shotuk.org](http://www.shotuk.org)).<sup>14</sup>

In order for learning and improvement to take place, the collected information is made available to the professionals in all aspects of the transfusion chain. Recommendations for improvement can be made, and ideally incorporated in practice through changes in blood donor care, the production of blood components, recommended practices laid down in testing or transfusion guidelines, or other mechanisms. Through ongoing reporting, the effects of measures can be evaluated and trends can be tracked. Where possible and relevant, peer-reviewed publication should also be pursued to strengthen rigor and ensure international accessibility of the results for incorporation in meta-analyses.

### **Adverse events not captured by hemovigilance systems**

Table 43.2 lists the typical types of adverse events associated with blood product transfusions that are reported to hemovigilance systems. While monitoring and reducing these typical transfusion complications is important, there are many other adverse events associated with transfusion that are not routinely captured by most hemovigilance systems. Most of these other adverse events were discovered through randomized controlled trials that showed an increase in a particular adverse event in the transfused group as opposed to the control group. Without these trials, most of the adverse events would otherwise be attributed to the patient's underlying disease state. For example, pooling of 21 randomized trials involving 8735 patients showed that the intervention of transfusing red blood cells was associated with serious infection, especially in patients undergoing orthopedic surgery or presenting with sepsis.<sup>15</sup> Without these data, the development of an infection would otherwise be attributed to the patient's underlying condition.

Another example was a randomized controlled trial in patients with liver disease presenting with an upper gastrointestinal bleed which identified that the transfusion of red blood cells at hemoglobin threshold of 7 g/dL versus 9 g/dL significantly reduced mortality and bleeding, presumably by not increasing portal pressures.<sup>16</sup> The link between transfusion and mortality applies to platelet transfusions as well. A prospective randomized trial found that platelet transfusions in patients with intracerebral hemorrhage while on antiplatelet therapy were significantly more likely to have serious adverse events and death.<sup>17</sup> Without either of these studies, the effect of the transfusion on mortality would not have been appreciated.

Though not derived from randomized-controlled data, the analysis of a prospective registry data of patients undergoing surgery found that intraoperative transfusion was associated in a

dose-dependent manner with the risk of a developed postoperative clot that needed an intervention.<sup>18</sup> As we gather more information and study transfusion in more detail, it is clear that adverse events and the risk-to-benefit ratio for transfusion have not been fully appreciated. Unfortunately, many of these significant risks of transfusion are not currently captured by most hemovigilance systems. Ideally, as electronic health records become more sophisticated, reporting of these other adverse events could be captured and reported to hemovigilance systems.

### **What have we learned from hemovigilance systems?**

Hemovigilance system reporting and analysis has increased our understanding of adverse reactions that occur in both blood donors and recipients of blood transfusion. The large data sets from hemovigilance systems are invaluable to identify trends from infrequent events. Many adverse reactions are very rare, and a single-institution would never transfuse enough blood products to experience enough events to identify meaningful trends or information. For example, the analysis of 10 years of SHOT data found that leukoreduction likely does reduce the risk for transfusion-associated graft-versus-host disease, a finding that would be impossible with analysis from a few institutions given the low incidence.<sup>19</sup>

Similarly, even though donating blood is considered safe, the analysis of up to 11 years of data from the International Haemovigilance Network was able to quantitate the risk of adverse events, including very rare complications such as cellulitis and air embolism.<sup>20</sup> As hemovigilance systems for donors become more robust, long-term risks, if any, might be identified and could also be extended to donors of gametes, embryos and tissues.<sup>21</sup>

Hemovigilance data have also made it possible to compare transfusion reactions to differently manufactured blood products. The effect of introducing male-only plasma to reduce the incidence of TRALI is one such example.<sup>22,23</sup> Another study using hemovigilance data accumulated from 23 countries found that there was a statistical difference in allergic reactions among recipients of apheresis plasma versus whole-blood-derived plasma.<sup>24</sup> The effect on transfusion reactions by switching from fresh-frozen plasma to solvent-and-detergent treated pooled plasma in Finland is another example.<sup>25</sup> In a health technology assessment (HTA) in Norway, comparing SDplasma, single-donor pathogen-reduced plasma, quarantine FFP, and FFP, data from different hemovigilance systems proved to be valuable.<sup>26</sup> In this HTA, the data from large countries that have different plasma products in use and a comprehensive hemovigilance system like France's were useful in a field dominated by scientific studies too small to detect differences in infrequent complications.

Overall, hemovigilance systems have demonstrated that blood transfusion is generally safe. However, there are preventable errors that still occur. An example from SHOT proves this while it still points at areas for further improvement. Over a 10-year period, during which time 30 million blood components were issued from UK blood services, SHOT received 3770 reports, of which 2717 (72%) were of an incorrect blood component transfused. Ninety-five percent of these patients survived with no serious effects, but 24 deaths were attributed wholly or in part to avoidable transfusion errors, and 100 patients suffered major morbidity.<sup>27</sup> Through the use of the SHOT hemovigilance system, the risk of incompatible transfusion errors continues to decrease, with the wrong blood in tube highlighted as a major cause, indicating labeling and patient identification of blood specimen samples need to be addressed.<sup>28</sup>

**Table 43.2** Examples of Adverse Events Reported to Hemovigilance Systems

<b>Respiratory Complications</b>	Transfusion-related acute lung injury Transfusion-associated circulatory overload Transfusion-associated dyspnea
<b>Allergic Complications</b>	Urticaria/Hives Anaphylaxis
<b>Hemolytic Complications</b>	Acute hemolytic transfusion reaction Delayed hemolytic transfusion reaction Delayed serologic transfusion reaction
<b>Immune Complications</b>	Transfusion-associated graft-versus-host disease Post-transfusion purpura Febrile nonhemolytic transfusion reaction
<b>Infectious Complications</b>	Transfusion-transmitted infections

With the implementation of pathogen reduction technologies, hemovigilance systems will be very important in detecting and documenting a presumed reduced rate of transfusion-transmitted infections. The United States based National Healthcare Safety Network Hemovigilance Module recently reported 54 cases of transfusion-transmitted infections that met their criteria out of 7.9 million transfused components.<sup>29</sup> Given the low rate of recognized transfusion-transmitted infections, hemovigilance systems will be invaluable to make sure that the benefit of pathogen reduction outweighs any possible risks that have been associated with pathogen reduction such as an increase in platelet refractoriness, an increased exposure to platelet transfusions, and lower corrected-count increments.<sup>30</sup>

## Discussion

Hemovigilance results combined with published research are leading to real improvements in transfusion safety. Both SHOT and the French hemovigilance systems have reported a lower rate of ABO-incompatible red blood cell transfusions in recent years. Comparison of hemovigilance system data can give useful information on local, regional, national, and international levels, which can be used in local improvement work as well as in making national and international guidelines and regulations.

Limitations of hemovigilance systems include not capturing harm to patients because of failure to provide blood or other adverse events such as thromboembolism or infection that could be related to transfusion. Another limitation includes differences in system and in practice or reporting, which restricts the ability to

investigate differences in reported incidences between systems or even regions.

Hemovigilance systems help determine that both blood donation and blood transfusion are generally safe, but that there are opportunities to further improve safety. Knowing the incidence and seriousness of complications is very valuable in this respect. Giving correct information on risks to donors and patients is necessary for donors and patients to trust the transfusion service. In the future, international agreement on definitions and on recommended background data to collect will improve benchmarking as will more sophisticated electronic health reporting systems that have the potential to capture more transfusion-related adverse events.

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# Transfusion-transmitted viral infections (TTVIs)

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## Introduction

Ever since the advent of the global human immunodeficiency virus (HIV) epidemic, transfusion medicine has maintained a heightened awareness of the potential risk of transmitting infection through blood transfusions. This has paradoxically increased despite the actual infectious risks of blood transfusion being lower than ever in developed countries. The enhanced level of safety has resulted from a series of stepwise interventions and policies in blood services throughout the world. Microbial risks were once mainly related to persistent infections such as HIV, hepatitis B virus (HBV), and hepatitis C virus (HCV), where viral carriage and latency were the key factors predisposing an agent to transmission by transfusion. More recently, several acute infections have posed a threat to blood safety when they have occurred at high incidence in certain populations. West Nile virus (WNV) in North America is a good example of this, but other infections such as Chikungunya virus and the specter of emerging respiratory viruses such as SARS-CoV-2 have made transfusion microbiologists widen their areas of vigilance. These issues are reflected in this chapter.

## Hepatitis virus infections (A, B, C, D, and E)

### Hepatitis A virus infections

Hepatitis A (formerly known as infectious hepatitis) is an acute infectious disease of the liver caused by the hepatitis A virus (HAV).<sup>1</sup> The HAV is a nonenveloped picornavirus and contains a single-stranded RNA in a protein shell. Many cases have little or no symptoms, especially in youth. The risk of symptomatic infections is directly related to age, with more than 80% of adults with symptoms of acute viral hepatitis.

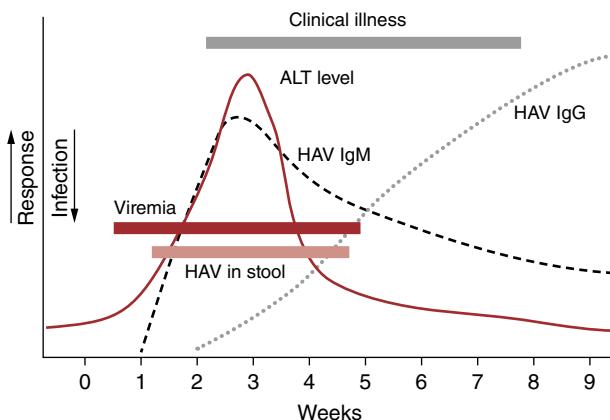
The time between infection and symptom development is between two and six weeks.<sup>2</sup> Clinical symptoms are nausea, vomiting, diarrhea, jaundice, fever, and abdominal pain.<sup>3,4</sup> Acute liver failure occurs rarely, mostly in elderly people. It is usually spread by contaminated food or drinking water with infected feces. It can also be spread through close contact with or blood products from an infectious person. Children are often asymptomatic and can infect

others.<sup>5</sup> After an infection, people develop an immune response for the rest of their lives. The diagnosis requires blood tests for antibodies or nucleic acid amplification (NAT).<sup>6–9</sup> Vaccinations are effective prevention.<sup>10,11</sup> Other preventive measures include handwashing and cooking food properly. A specific antiviral treatment is currently not possible. Patients are treated for nausea or diarrhea. The human body is usually able to eradicate the virus without severe liver disease. The treatment of acute liver failure is a liver transplantation.

Worldwide, approximately 1.5 million symptomatic cases occur each year with probably millions of asymptomatic infections. About 40% of all acute viral hepatitis is caused by HAV. It is resistant to detergent, acid (pH 1), solvents (such as ether and chloroform), drying, and temperatures up to 60 °C. It can survive for months in fresh and salt water. HAV infections are more common in regions of the world with poor sanitary conditions and limited resources of clean water. In developing countries, about 90% of children have already been infected at the age of 10 years.<sup>12</sup> In developed countries, on the other hand, the infection primarily strikes susceptible young adults, most of whom get it when traveling to countries with a high incidence of the disease or by contact with infectious people.

After ingestion, HAV will be transported into the bloodstream through the epithelium of the oropharynx or intestines. The blood carries the virus to its target, the liver, where it multiplies within hepatocytes and Kupffer cells (liver macrophages). Virions are excreted into the bile and bloodstream. HAV is secreted in large amounts from about 11 days before the onset of symptoms or appearance of anti-HAV IgM antibodies in the blood (Figure 44.1). Therefore, donors could be asymptomatic but still viremic. The incubation period is 15–50 days and mortality of less than 0.5%.<sup>13,14</sup> Within the liver hepatocytes, the RNA genome is released from the protein coat and converted by its own ribosome in the cell.

A serotype and six genotypes have been described. The human genotypes are numbered I–III. Genotypes IV–VI are found in non-human primates.<sup>15</sup> A single isolate of genotype VII isolated in man was also described. Genotype III has been isolated from human and monkey.<sup>16</sup> Most human isolates are from genotype I.



**Figure 44.1** Scheme of HAV infections and immune response.

While the virus spreads by the fecal–oral route and infections often occur in areas of poor hygiene and overcrowding.<sup>17</sup> HAV can also pass through the parenteral route via contaminated blood or blood products. Recently, a large producer of plasma-derived therapies reported an increasing incidence of HAV RNAemia among plasma donors in the United States, with a peak incidence of 5.8 cases/100,000 donors in October 2019.<sup>17</sup> A 10 person subset of these donors underwent additional testing and had detectable RNAemia for a mean of 95 days, which was noted to be significantly longer than is generally believed (see Figure 44.1 for a more traditional model).<sup>17</sup>

During the acute phase of infection, the liver enzyme alanine transferase (ALT) is in the blood at significantly elevated concentrations. The enzyme is derived from the liver cells destroyed by the virus.

There is no specific treatment for hepatitis A. Patients are advised to rest, avoid fatty foods and alcohol, eat a balanced diet, and stay hydrated.

In the United States in 1991, there was a low mortality rate for hepatitis A of four deaths per 1000 cases for the general population, but a higher rate of 17.5 per 1000 in those aged 50 years and older. The risk of death from acute liver failure by HAV infection increases with age and underlying chronic liver disease.<sup>18</sup>

### Keynotes: hepatitis A virus

Virus	Region	Transmission	Screening	Inactivation	Vaccination
Picornaviridae, RNA, and six genotypes	South America, Africa, and Asia	Fecal-oral and blood components	NAT and antibody screening	Reduced efficiency	Yes

### Hepatitis B virus infections

The hepatitis B virus (HBV) has a complex coiled genomic structure.<sup>19,20</sup> An envelope embeds the core of the virus. The virus belongs to the virus family Hepadnaviridae. Based on the replication cycles, the DNA virus is close to the retroviridae. Therefore, they are named DNA retroviruses. The surface antigen of HBV was first described in 1963 by B.S. Blumberg as a new serum protein in Australian Aborigines.<sup>21–23</sup> He named the new antigen the Australia antigen. In 1968, a correlation was made between the Australia antigen and the transmission of hepatitis B. Therefore, the antigen was renamed HBsAg for *hepatitis B surface antigen*. HBsAg is embedded by lipid envelopes, and approximately 25 µm round or filamentous subviral particles can be present in huge numbers of up to  $10^{13}$  particles/mL or  $10^6$  ng/mL in serum. The complete HBV particle diameter is from 45 to 52 µm. Inside the HBsAg envelope, the hepatitis B core antigen (HBcAg) is located.<sup>24,25</sup> Inside of that is the HBV genome, with a length of 3200 base pairs. The HBV genome is a complex virus particle with round structure and characteristic properties (Figure 44.2).

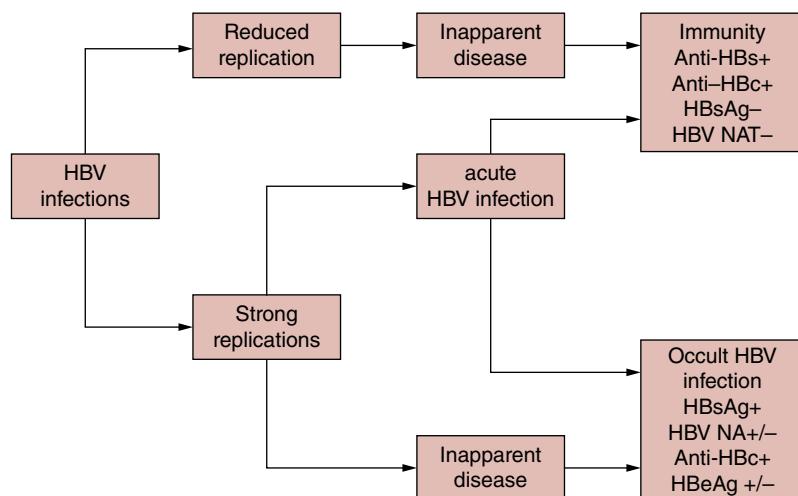
### Characteristics of DNA molecules of hepatitis B genomes

Open circular open-coiled structure with overlapping ends of the double-strand DNA (dsDNA) genome:

- Single-string gap, place for the endogenous DNA-polymerase
- Covalent linkage of the virus protein coding DNA minus strand
- (5'-position) with the virus DNA-polymerase
- Lineages of the DNA plus strand with a RNA primer

### HBV infection pathomechanism

The virus infects liver cells by the bloodstream.<sup>26,27</sup> Via currently unknown receptors, the virus genome is transported into the



**Figure 44.2** Different HBV forms.

liver cells. Cellular repair enzymes build a covalently closed circular DNA (ccc-DNA) that activates the cellular gene expression, producing messenger RNA (mRNA). The mRNA represents the complete genome and works as a matrix for the core protein and the viral DNA-polymerase. Those proteins assemble together to the core particle and embed the mRNA (self-assembly).<sup>28,29</sup> In the next step, the viral DNA-polymerase transcribes the core particles of the RNA into DNA. The RNA is cleaved after producing the viral DNA plus strand. The residual 18 5'-terminal bases of the RNA pregenome represent the primer for the plus strand. In parallel to the genome maturation, subgenomic mRNA molecules are expressed. They are named as 3-DO-carboxy-terminal HB proteins, S (small), M (middle), and L (large), with preS1, preS2, and S protein structures.<sup>30</sup> The HB proteins are released into the endoplasmic reticulum. Within the endoplasmic reticulum, the HB particle embeds the circlet core particle and builds the final HB virus. The HBsAg and the HB virus are released via Golgi apparatus into the bloodstream.

In addition, HBV is coding for two nonstructure proteins, named HBeAg and HBx.<sup>31-33</sup> HBeAg is a nonessential form of HBcAg and has an immune modulating function. HBx has several effects on viral and cellular gene expression. Based on this transcription-regulating mechanism, including a reversed transcription enzyme, HBV is at risk of new genomic variants based on the mutation of viral enzymes. The reason is caused by the missing proofreading function of the reversed transcriptase enzymes. Nevertheless, due to a longtime adaptation between the host and virus without selection pressure, HBV viruses are characterized by eight stable wild-type viruses. Currently, their genotypes are known as HBV genotypes A to H. The most predominant genotypes in Asia are B and C, whereas the most predominant genotypes in Europe are A and D.<sup>34</sup> With an immune selection pressure, HBeAg negative mutants, named *escape mutants*, with mutation within the HB proteins can develop.<sup>35-37</sup>

### **HBV pathogenesis between virus and host**

HBVs are strictly species specific and hepato-autotrophic.<sup>38</sup> Other cells like leukocytes are rarely infected. The reason for the species specificity is currently unknown. HBV and all genome products are usually under nonpathogenic conditions; therefore, HBV-infected persons produce virus copies in a high concentration without showing clinical symptoms or damage of liver cells. HBV is not directly cytopathic, and liver cells are usually a privileged region of the human body, where immune cells can be unable to attack the infection for months or years or indefinitely. Newborns, children, or immunodeficient patients usually cannot produce efficient immune reactions. Therefore, an asymptomatic infection with a high viral concentration can persist.<sup>39</sup> Chronic HBV infections are defined with the detection of HBsAg or HBV-DNA over a period of six months.<sup>40,41</sup> About 10% of all HBV infections become chronic. Seroconversion from HBeAg to anti-HBe antibodies reduces HBV replication and concentration. Nevertheless, patients with anti-HBe antibodies and HBsAg that sometimes still had a high viral concentration were observed. Patients with a chronic HBV infection are at high risk for liver cirrhosis and liver cancer, approximately 10% for each.<sup>42-45</sup>

After an HBV infection, a liver disease of three different phenotypes may be observed:

- 1 Immunotolerance and high infectivity
- 2 Partial immune control with HBsAg positivity and low-level infectivity
- 3 Natural acquired immunity

The humoral and cellular immune response can depress virus replication in most cases, but the ccc-DNA form of HBV is rarely completely eliminated in the hepatocytes. Therefore, patients infected with HBV can reamplify the virus, especially when their immune situation is changed, such as after immunosuppression. Stopping immunosuppression puts patients at high risk of a severe immune reaction including a fulminant hepatitis B reaction. Liver cell carcinoma develops most frequently in HBV-infected middle-aged patients from Asia and Africa after longtime virus persistence. The probability of liver cell cancer is increased with exposition of aflatoxin or by a coinfection with hepatitis C virus.<sup>46-51</sup>

### **HBV transmissions**

Infected patients contain HBV virus in blood, saliva, semen, breast milk, vaginal, and sore secretions, which all can cause infection by percutaneous or transmucosal route. In high epidemic regions, transmissions from mother to child are important, especially because most child infections have no clinical symptoms.<sup>52-55</sup> The vertical infection from mother to child usually accrued in utero, at the end of pregnancy or during nativity. Transmissions by sexual contact are reported. Contaminated, inefficiently sterilized medical devices like endoscopes are possible infection sources as are personal devices such as toothbrushes, razors, and so on. Also, thorns and branches accidentally contaminated with blood have transmitted HBV during sport activities such as orienteering races.

To prevent transfusion of infections by blood components,<sup>56-59</sup> all blood components are screened in most countries for HBsAg, and blood donations in developed countries are in addition screened by HBV nucleic amplification technique (NAT).<sup>9,60,61</sup> In countries with low epidemic prevalence, anti-HBc screening has been implemented for the detection of chronic infected HBV donors (occult hepatitis infections [OBI]).<sup>35,62,63</sup>

Approximately 50% of the world population has been exposed to HBV. World Health Organization (WHO) estimates that approximately 350 million HBsAg-positive people exist. Major epidemic regions are Africa north of the Sahara, Southeast Asia, and major regions of Russia. Middle risk accrued in Southeast Europe, South America, India, North America, Australia, and Northern, Central, and Western Europe. The prevalence of HBV infection is age dependent, approximately 15% of the world population older than 60 is affected.<sup>64</sup> Although exact data regarding the prevalence of HBV exist, data regarding hepatitis B incidents are still limited or missing. Most patients die due to long-term clinical diseases of liver cirrhosis and liver failure.<sup>65,66</sup>

The clinical symptoms of HBV infection depend on the immunopathogenesis and demonstrate a broad range:

- 1 Light liver damage only detectable by a small increase of transaminases and spontaneous healing. Chronic hepatitis decompensated liver cirrhosis and hepatitis cellular carcinoma are possible if viral replication continues.
- 2 Liver disease with extrahepatic manifestation such as arthritis, exanthema, glomeruli nephritis, and polyarteritis nodosa caused by immune complex with high concentration of HBV.
- 3 Acute hepatitis with high levels of liver enzyme (ALT values grossly increased).
- 4 Fulminant hepatitis with complications of liver cirrhosis and liver carcinoma (less than 1%).

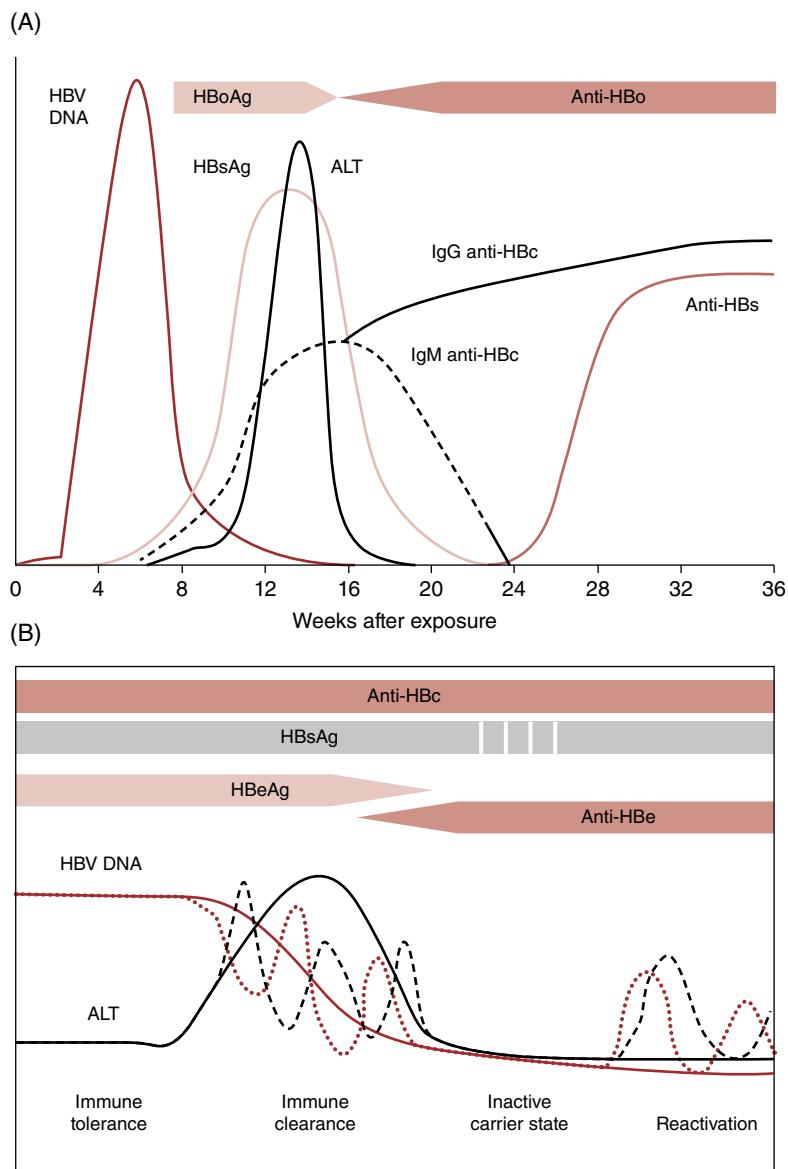
Most cases achieve immunity against HBV within three to six months, but virus replication can continue at a low level over many years. Only highly sensitive NAT assays could detect these low-level carriers (NAT sensitivity should be less than 10 IU/mL). A chronic

HBV infection with only mild symptoms should also be treated, to prevent severe clinical complication such as liver cirrhosis and carcinoma, which can develop after more than 10 years, or to protect others from HBV infections.

### Diagnostic opportunities

Important for HBV diagnostics are HBV antigens (HBsAg and HBeAg), HBV antibodies (Anti-HBc, Anti-HBs, and Anti-HBe) in patient serum and plasma,<sup>63</sup> and HBV detection by NAT,<sup>58,60,67–69</sup> as shown in Figure 44.3.

Finally, histopathological investigations can amend diagnostics of serum/plasma. There is no correlation between the detection of virus parameters (antigens or antibodies) and inflammation activity. Antibodies against HBV, especially anti-HBc, can be detected lifelong in most cases.<sup>24,25,70</sup> The antibody titer against HBsAg (anti-HBs) represents one's immunity status. Vaccinated people should have an anti-HBs titer higher than 100 U/L to have a sufficient response against HBV. Breakthrough infections were reported in the United States with people with a reduced anti-HBs titer of less than 100 U/L.<sup>71,72</sup> The most important parameter is the analysis of



**Figure 44.3** Clinical, serologic, and virologic course of acute and chronic HBV infection. (A) Schematic profile of serologic and virologic markers of a typical acute resolving hepatitis with the average timing of detection of serum HBV DNA, HBsAg, and HBeAg, and their corresponding antibodies, with respect to the ALT elevation reflecting immune-mediated liver cell destruction after HBV replication (as reflected by serum HBV DNA levels) has been mostly controlled by noncytolytic mechanisms. (B) Course of chronic hepatitis B after perinatal or early-childhood-acquired infection. The four distinct phases shown are immune tolerance (high-level HBV replication with normal ALT levels), immune clearance (high ALT level, lower HBV DNA levels, and frequent hepatitis flares, depicted with dashed lines for both HBV DNA and ALT, which may lead to loss of HBsAg and anti-HBe seroconversion), inactive carrier state (normal ALT, presence of anti-HBe, and low or even undetectable HBV DNA), and reactivation phase after many years of inactive carrier state (lower and fluctuating HBV DNA and ALT levels in the presence of anti-HBe), commonly associated with selection of core or precore HBV variant. Reproduced from Figure 46.2 in Alter & Esteban-Mur, Chapter 46, *Ross's Principles of Transfusion Medicine*, 4th Edition.

HBsAg in serum/plasma. Commercial enzyme immunoassays (EIA) for HBsAg are very sensitive (detection limit less than 0.1 ng/mL) and very specific (higher 99.9%). The diagnostic window for HBsAg is approximately 32–38 days.<sup>69,72–74</sup> HBsAg can be detected after vaccination for up to one week in the serum. Recently, the utility of HBsAg testing was questioned, as one study reported that elimination of the testing from the US donor program would have a negligible impact on transfusion transmission of HBV.<sup>75</sup> False negative results due to escape mutants are rare. In some cases, HBsAg will be negative in the acute infection period. Therefore, the detection of hepatitis B should be supplemented by anti-HBc IgM and HBV NAT.<sup>60</sup> Patients with detectable HbsAg for more than six months are considered to have developed a chronic occult hepatitis B infection (OBI).<sup>35</sup> NAT can be positive or negative in OBI patients. The last decade observed improvement in NAT systems, which now have an analytical sensitivity of approx. 2 IU/mL, which represent a diagnostic window of approximately 20 days for HBV infection. Most NAT systems detect HBV, HCV, and HIV-1 simultaneously in a multiplex assay. Therefore, blood donor screening by NAT follows these three parameters, although in some countries, HBV NAT screening is voluntary. As shown by Roth *et al.*,<sup>76</sup> screening of more than 114 million blood donations for HBV by NAT detected 1728 NAT only positive blood donors (negative for HbsAg, Anti-Hbc, anti-HBs, and anti-Hbe). HBV concentration can increase, especially in immunosuppressed patients, up to  $10^{10}$  genome equivalents per mL (geq/mL). In OBIs, the HBV-DNA concentration is reduced to low levels, 1–10 geq/mL, or under the detection limit of the NAT system. The doubling time of HBV in a mouse model is very long, approximately 2.56 days. Therefore, the diagnostic window is much longer compared to hepatitis C (approx. 11 hours) or to HIV-1 (approx. 18 hours).<sup>77</sup>

### HBV therapy

In principle, the eradication of HBV might be possible because the virus infects only the human population and a sufficient vaccine is available. If more than 95% of the human population can be vaccinated, the virus will not be able to survive in order to find nonvaccinated humans. Unfortunately, only monoclonal agents are used for vaccines. They induce an efficient immune protection against genotype A. Therefore, high anti-HBs titers are necessary to protect against other HBV genotypes.

At the end of the 1970s, interferon- $\alpha$  (IFN $\alpha$ ) was detected as efficient against HBV. Treatment with IFN $\alpha$  reduced the viral load within a few days.<sup>78</sup> Unfortunately, it increased again after IFN $\alpha$  therapy was ended. Interferon has nasty flulike side effects; a continuation of therapy can be justified when its antiviral effect is proven by follow-up of HBV DNA. A permanent cure can be assumed if an initially positive HBeAg and HBV NAT has negative six months after the end of therapy. Considerably more tolerable than interferon is the nucleoside analog lamivudine therapy known from the HIV therapy.<sup>79,80</sup> Lamivudine inhibits reverse transcriptase of HBV DNA, causing chain termination. Of special significance is lamivudine for prophylaxis of reinfections after liver transplantation and in case immunosuppression causes HBV reactivation. Nucleoside analogs effective against herpes viruses, such as famciclovir and ganciclovir, have only a small effect on HBV. If the treatment is not perfect or widely applied, the HBV infection can cause liver failure or hepatocellular carcinoma formation. In these cases, a liver transplant might be the only valid therapy, and even then, one must prevent an HBV reinfection of the transplanted organ.

### Keynotes: hepatitis B virus

Virus	Region	Transmission	Screening	Inactivation	Vaccination
Hepadnaviridae, Worldwide DNA, retrovirus, and eight genotypes (A–H)	Blood components, sexual activities, and blood-infected utensils	NAT, antigen (HBsAg) and HbeAg), and AB (anti-HBc, anti-HBe, and anti-HBs)	Possible up to 6 log	Yes, titer should be >100 IU/L	

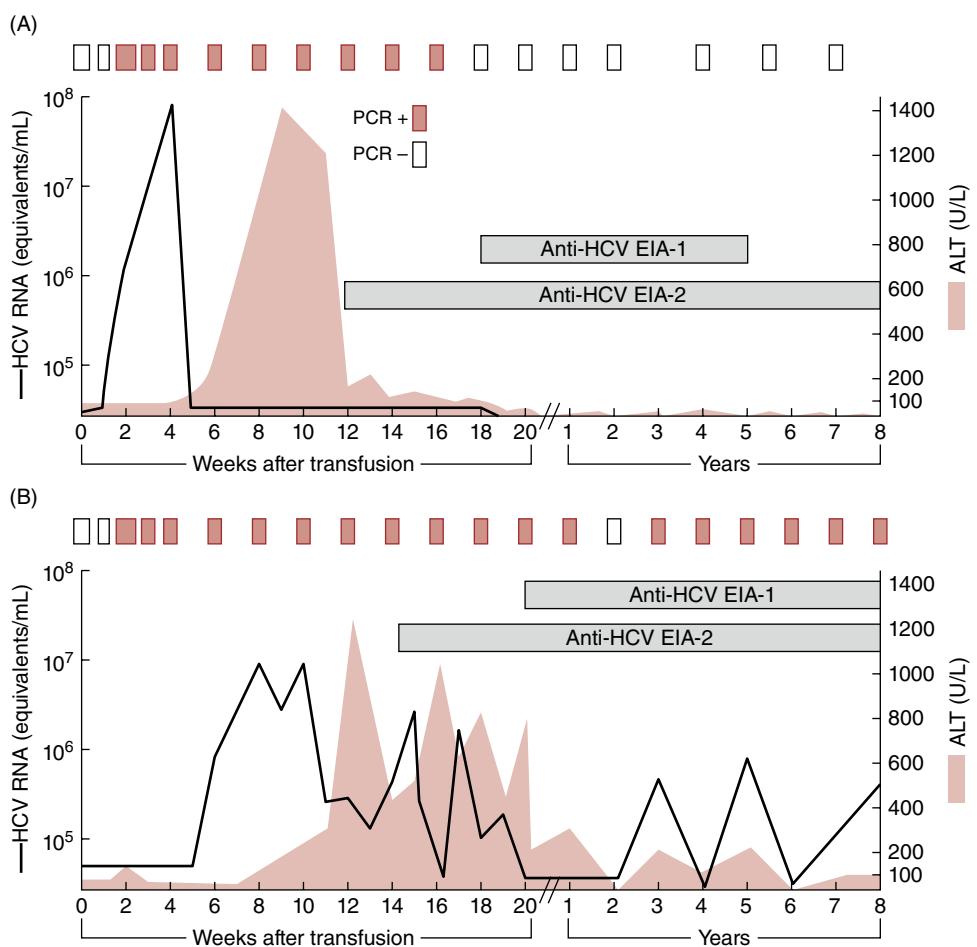
### Hepatitis C virus

The 1989 genetic methods were able to identify hepatitis C virus (HCV), which was shown to be responsible for most blood-borne cases of non-A, non-B (NANB) hepatitis (Figure 44.4). HCV has a genome of approximately 9600 base pairs,<sup>81,82</sup> which is a single-strand RNA (ssRNA) with a plus strand polarity. From the viral RNA, a continuous open reading frame of 3010–3033 amino acids long poly-protein is synthesized. Resulting from a post-translational cellular signal peptidase and two viral proteases, 10 different structural or nonstructural proteins are produced. By sequencing of HCV genome, so far six different genotypes with numerous subtypes can be identified.<sup>83</sup> Due to taxonomic characteristics, the HCV genome belongs to the Flaviviridae family. Worldwide, the incidence of HCV is dropping, approximately 58 million people are currently infected with HCV with about 1.5 million new infections annually.<sup>84–86</sup> Historical epidemiological data indicate a high HCV prevalence in certain risk groups, such as hemophiliacs (60–90%), dialysis patients (30–40%), and intravenous drug users (>80%). Compared to HBV, the risk of HCV transmission through sexual or close family contact is considered low. In approximately 40% of all HCV infections, the transmission pathway cannot be detected. The diagnosis of HCV infection is essentially based on the detection of antibodies against the core protein and the nonstructural proteins NS3 to NS5.<sup>87</sup> However, the presence of antibodies does not provide information about whether a self-limiting or chronic HCV infection occurred. Also, the determination of IgM antibodies cannot differentiate between acute or chronic infection. HCV antibodies usually occur within the transaminase peak of an acute infection which will be in a range of 50–70 days.

The antibodies are stable over 10–15 years after the hepatitis C infection. Screening by NAT and HCV antibodies can differentiate between an acute HCV infection (NAT positive and HCV AB negative) and a chronic HCV disease (NAT positive and HCV AB positive). The quantitative HCV RNA determination is suitable for the control of the antiviral treatment.<sup>88,89</sup>

The efficiency of the HCV antiviral therapy depends on the HCV genotype.<sup>90</sup> Genotype knowledge is essential for recognition of epidemiological correlations and detection of HCV infection chains. The diagnostic window for HCV is long for antibody development, still about 55 days.<sup>91,92</sup> That window could be cut, however, by the introduction of NAT in the blood donor screening. Depending on different NAT methods, the current diagnostic window is between 6 and 8 days if the virus is in the replication process (ramp-up period). Roth *et al.* found, in a worldwide donor screening of more than 300 million investigations,<sup>76</sup> 680 HCV-infected donors with only NAT positive screening.

Hemovigilance data from Germany clearly showed the efficiency of blood donor screening by NAT for HCV (Figure 44.5). Before

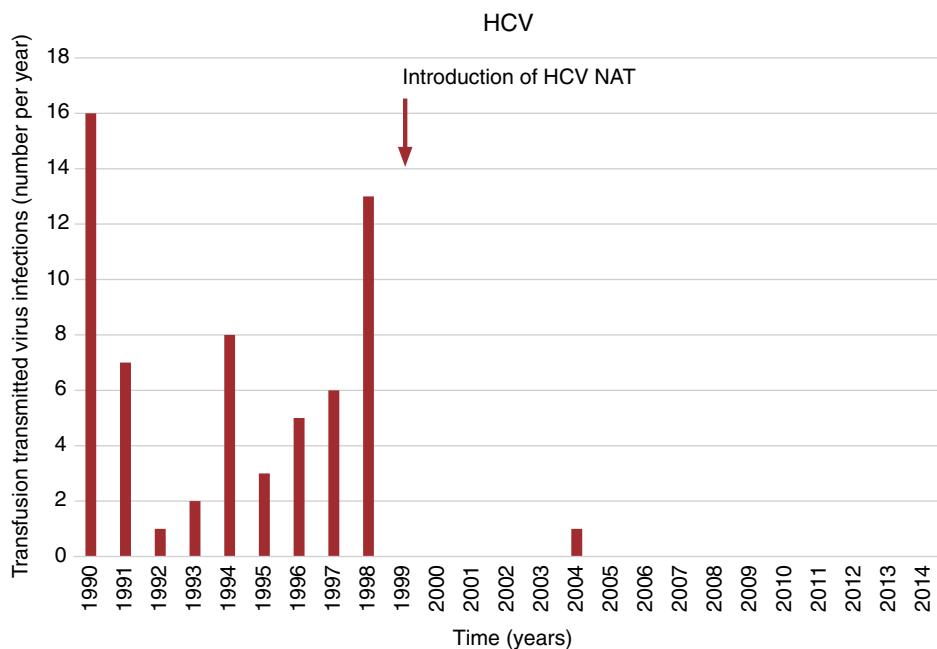


**Figure 44.4** Biochemical, serologic, and molecular biologic profile of acute and chronic transfusion-associated hepatitis C virus (HCV) infection. Acute, resolving hepatitis C is shown in (A) and chronic hepatitis C is shown in (B). Resolving disease cannot be differentiated from progressive disease by the time of onset of detectable HCV RNA by means of polymerase chain reaction (PCR), the magnitude of HCV RNA elevation as measured by means of branched DNA assay, the interval to the first elevation in the level of alanine aminotransferase (ALT), the magnitude of ALT elevation in the acute phase, or the interval between exposure and the first appearance of antibody. Progression to chronic disease cannot be predicted in the acute phase, and the only distinguishing features in these patterns are the persistence of ALT elevation and the persistence of HCV RNA in persons with chronic hepatitis C. The acute, resolving pattern (A) occurs in 10–15% of patients with transfusion-associated hepatitis C and the chronic pattern (B) in 85–90%. Note: (1) HCV RNA is detectable soon after exposure. Here, the PCR results was positive two weeks after exposure, but it can become positive even sooner. (2) HCV RNA may be detected by means of branched DNA assay coincident with PCR reactivity, but the reaction may be delayed, as shown here. (3) The major peak of viral replication (assessed with HCV RNA level) occurs before the first increase in the ALT level and before any clinical or biochemical evidence of hepatitis; it is presumed that persons might be most infectious in this interval before the acute phase. (4) In acute resolving infection, HCV RNA levels increase rapidly before the decline in the serum ALT level. (5) In chronic infection, the HCV RNA level diminishes and can remain low, fluctuate, or become undetectable; HCV RNA levels sometimes show a periodicity that parallels the fluctuations in the ALT level; in (B), the level of HCV RNA increases a short time before the ALT level does and decreases before the decline in ALT. (6) Second-generation anti-HCV assays shorten the seronegative window in HCV infection much more than do first-generation assays; nonetheless, anti-HCV was not detectable for 12–15 weeks after exposure and for six to seven weeks after the first significant rise in the ALT level. Antibody to HCV (detected with second-generation assays) almost always persists in chronic cases and generally persists in acute resolving cases. Antibodies detected with the first-generation assay (anti-C100 and anti-5-1-1) generally disappear in resolving cases. Reproduced from Figure 46.4 in Alter & Esteban-Mur, Chapter 46, *Rossi's Principles of Transfusion Medicine*, 4th Edition.

1999, approximately 6–8 transfusion-transmitted HCV infections were reported annually in Germany. In 1999, all blood donations began to be screened by minipool NAT. Since then, only one additional HCV transmission has occurred, in 2005. The virus concentration in the infective donor was only 10 IU/mL, and below the detection limit of the minipool screening method.<sup>93</sup>

Even a reduction from minipool to individual donation NAT might not have prevented this HCV transmission. Due to the high doubling time in the ramp-up phase of about 11 hours, diagnostic window for HCV differs only about one to two days between blood

donor screening in minipools of 96 samples or testing on an individual NAT basis. The long-disputed question of whether early treatment of acute HCV infections can prevent chronicity of the disease was solved in 2001 when Nakamura *et al.* reported the kinetics of the HCV during interferon therapy.<sup>94</sup> They showed that high concentrations of interferon alpha could prevent a chronic disease.<sup>95</sup> The coupling of interferon with a branched-chain polyethylene glycol molecule (Peg-interferon) leads to a compound with prolonged action compared to unmodified interferon through a longer absorption, a slower clearance, and a correspondingly



**Figure 44.5** HCV transfusion-transmitted infections in Germany. Source: Funk *et al.* Hemovigilance Report 2011, Paul-Ehrlich-Institute, Germany, Frankfurt. Reproduced with permission.

higher half-life.<sup>95–97</sup> In the past several years, numerous oral HCV medications have been approved in the United States, including sofosbuvir, sofosbuvir-velpatasvir, elbasvir-grazoprevir, glecaprevir-pibrentasvir, and ledipasvir-sofosbuvir.<sup>96</sup>

#### Keynotes: hepatitis C virus

Virus	Region	Transmission	Screening	Inactivation	Vaccination
Flaviviridae, RNA, 6 genotypes, 30 subtypes, and residual risk TTID: <1:1 million	Worldwide	IVD drug user, medical instruments, blood components, and sexual activities	NAT, antigen (NS3, NS5), and AB	Possible up to 6 log	Not available

#### Hepatitis D virus infections

Hepatitis D virus was discovered by M. Rizetto 1975 in liver biopsies from patients with chronic hepatitis B infection.<sup>97</sup> By immunofluorescence analysis, he found a new antigen of HBV. At that time, only three were known: HBsAg, HBeAg, and HCcAg. The new antigen was named HBV-D antigen. Later, it was shown that the D-antigen referred to a new hepatitis virus, hepatitis D virus (HDV).<sup>98,99</sup> The D-antigen is embedded in HDV with proteins of HBV. HDV genome contains a small (approximately 1700 bases long), covalently closed, circular, single-stranded RNA that is complementary to itself over a wide range and therefore looks like a partial double-stranded virus.<sup>100–104</sup> Such genomic structure had then been found only in viroids. In addition to this viroid-like behavior, HDV genome segment had a gene that encoded HDAg. HDV are approximately 38 nm large round particles with an HBsAg envelope. HDAg exists in a short form (SHDAg) with 195 amino acids and a longer form (LHDAg) with 214 amino acids. SHDAg is necessary for the regulation of the HDV genome replication, whereas LHDAg inhibits the replication process. LHDAg is required for the translation of the envelope of

HDV ribonucleoprotein. The HDV ribozyme has become an important tool in molecular biology because it can cut RNA molecules precisely. The helper virus HBV essentially determines the host and cell tropism of HDV. HDV can replicate intracellularly without HBV and spread from cell to cell, but cannot develop a complete infection.

Superinfections of HDV in chronic HBV carriers (HDV infection after HBV infection) often lead to persistent chronic HDV infection.<sup>105–107</sup> In coinfecting patients (who have HBV and HDV at the same time), the HDV infection is often mild.<sup>105,108–110</sup> The pathogenicity of HDV is controversial because some studies show a high cytotoxicity of SHDAg, but SHDAg-producing cell cultures are viable. In patients with severe HDV/HBV infection, a microvesicular steatosis is found in liver tissue. Coinfection with HDV and HBV sometimes leads to a biphasic course. Often both viruses multiply in parallel. The HB viremia is reduced by HDV in general. However, the course of acute hepatitis is not necessarily more difficult than for HBV monoinfection. The persistence rate in HBV/HDV coinfections is not increased. HDV superinfection of HBsAg carriers is unfavorable. The HDV genotype is also important for the course of the infection. HDV seems to be dominant in this constellation to HBV. Patients with chronic HDV/HBV infection are three times more likely (60–70%) to develop liver cirrhosis and liver cancer than patients with HBV monoinfection. The overall mortality is about three times higher in superinfected HDV/HBV patients.

HDV virus concentration reaches high levels in the acute infection period:  $10^{11}$  geq/mL (genomic equivalents/mL). In this period, patients are very infectious. In the next phase, the virus titer usually decreases to concentrations of  $10^6$  geq/mL. Prenatal infections are possible if HBV is transmitted. Typical transmission pathways are unsanitary injections (intravenous drug use) and contact with open wounds in tropical regions. Worldwide, approximately 350 million people have HBV, and approximately 12–15 million are coinfecting or superinfected with HDV (relation is 1:23).<sup>111–114</sup> Epidemic areas are the Mediterranean, Southeast and Eastern Europe, Central and East Asia, and local areas in tropical South America and Africa. With intravenous

drug users often coinfections by HDV with HCV and HIV are reported. In many countries, the incidence rate of HDV has decreased. Since blood donations are tested for HBV infections in general, a special screening for HDV for blood products is not required because HDV infections are observed only in conjunction with HBV. The most sensitive and reliable detection of HDV is done by NAT.<sup>115</sup> No antiviremic therapies for HDV yet exist. Its treatment with interferon and/or lamivudine has little effect on its chronicity.<sup>116–119</sup> For fulminant hepatitis D cases with terminal liver failure, a liver transplantation is the final therapeutic approach.<sup>120–122</sup> After transplantation, a graft reinfection with HBV is lower than with HBV mono-infections compared to HDV/HBV infections. When HBV reinfection is suppressed by lamivudine and/or hyperimmune globulin, the overall prognosis is favorable. Otherwise, reinfections of HDV are also possible.

#### Keynotes: hepatitis D virus

Virus	Region	Transmission	Screening	Inactivation	Vaccination
Delta virus and RNA	Worldwide	Blood components and sexual activities	NAT in development and antibody screening	Possible up to 6 log	Not available

#### Hepatitis E virus infections

In 1980, hepatitis E virus (HEV) infections were first reported in studies of oral–fecal transmitted hepatitis in India.<sup>123</sup> The experimental transmission in nonhuman primates and the virus visualization and characterization became possible in 1983. In the early 1990s, the HEV genome sequenced and serological and molecular screening tests were developed.<sup>124–128</sup> HEV is an ssRNA with a diameter of 32–34 nm. It is a nonenveloped spherical particle with a genetic heterogeneity. The importance of these genetic variants of clinical courses is currently not clear. Type 1 genotype was found in Asia and North Africa, Type 2 was isolated in the United States, Type 3 was found in Europe, and Type 4 was detected in isolates from China and Taiwan. To date, the taxonomic classification of HEV is not entirely clear. Similarities exist to the caliciviruses.<sup>129–131</sup>

HEV is generally transmitted via the fecal–oral route, and mainly through contaminated drinking water. According to WHO, the

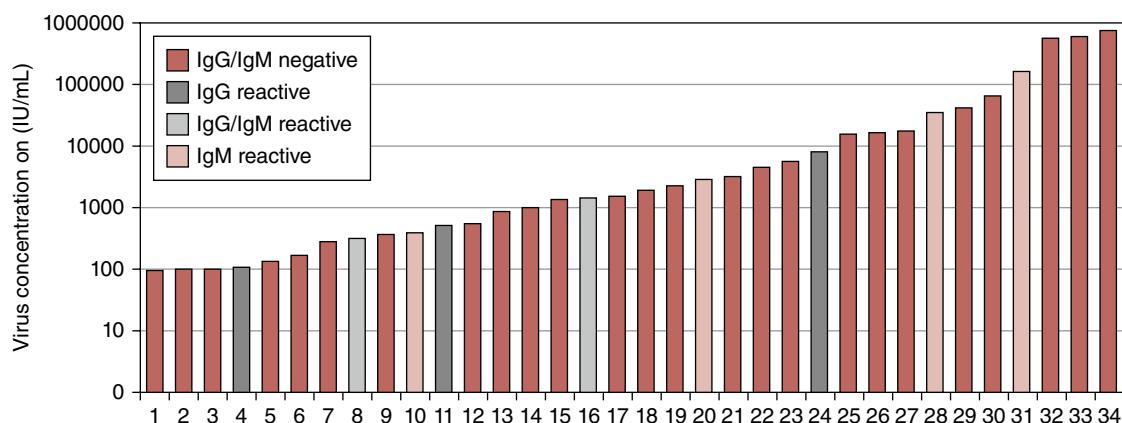
incubation period ranges from three to eight weeks, with a mean of 40 days. The period of communicability is unknown. HEV causes acute sporadic and epidemic hepatitis, which is usually self-limiting. Symptomatic infection is most common in adults between 15 and 40 years. It is frequent in children, but then mostly asymptomatic. In rare cases, fulminant hepatitis with liver failure and death can occur. Severe clinical courses with mortality between 0.5% and 4% mainly occur during pregnancy.<sup>132–135</sup>

WHO estimates 20 million HEV infections, 3 million acute cases of HEV, and 56,000 HEV-related deaths annually worldwide. Highest seroprevalence rates are observed in regions with low development index. WHO estimates indicate that over 60% of all HEV infections and 65% of all HEV related deaths occur in East and South Asia, where seroprevalence rates of 25% are common in some age groups; in Egypt, half of the population aged over five years is serologically positive for HEV. Investigations by Baylis *et al.* show high incidence rates in Japan, the United Kingdom, Germany, and Sweden, between 1:4500 and 1:8500.<sup>136–138</sup> HEV antibodies are usually developed in infected patients after 3–4 months (Figure 44.6). Because neutralizing antibodies' concentration can wane over time, reinfection with HEV might be possible.

In addition to the nutritional infection pathway, HEV transmission by blood products is clearly confirmed in several publications.<sup>3,139–143</sup> However, general blood donor screening for HEV is not universally performed, although some countries, notably Japan, started in 2014 with a 100% blood donor screening for HEV by NAT. Reassuringly, a recent report identified that HEV viremia was only 0.002% in the US source plasma donor population, suggesting that additional mitigation measures may only have a negligible effect on transfusion safety.<sup>144</sup> Pathogen reduction of HEV is limited because the virus is not protein embedded or lipid coated. A licensed vaccine has recently been developed in China.

#### Keynotes: hepatitis E virus

Virus	Region	Transmission	Screening	Inactivation	Vaccination
Caliciviridae, RNA, and four genotypes in humans	Worldwide	Fecal-oral and blood components	NAT and antibody screening	Reduced efficiency	Vaccine recently licensed in China



**Figure 44.6** HEV NAT and HEV antibody status in 34 donor samples. Virus concentration was in range between 100 and  $10^6$  IU/mL. In 25/34 cases (73.5%), no HEV-specific antibodies were detected. Source: Prof. M. Schmidt, German Red Cross, Frankfurt. Reproduced with permission.

## Hepatitis F virus

Indian scientists found virus particles that could not associate with the known hepatitis A through E. Therefore, it could be a new standalone virus (hepatitis F) or a mutation and new genotype of the hepatitis B virus. It is believed that hepatitis F can cause liver inflammation. Since the existence of the hepatitis F virus is currently not clearly understood, it is only assumed that the main transmission pathway could equal that of a hepatitis A or hepatitis E infection via the fecal–oral route through contaminated drinking water or food. (Note to reader: Keynotes for hepatitis F are currently not available.)

## Hepatitis G virus/GBV-C

Hepatitis G virus or GBV-C is an enveloped RNA virus and a member of the Flaviviridae family (which also includes HCV).<sup>145–147</sup> The viral genome is similar to HCV in structure but has only 25% homology with it. Several genotypes are reported, mainly restricted to the various continents. GBV-C was discovered in patients with sporadic “non-A, non-B” hepatitis. This led to the conclusion that GBV-C was the cause of viral hepatitis, but further study found it was not, nor did it have any other hepatotropic or pathological role. It was just a “passenger” virus isolated at random. Although parenteral transmission is highly efficient for GBV-C/HGV (58% of recipients of GBV-C/HGV RNA-positive blood products and 32% of aplastic anemia patients), it appears that sexual and vertical transmission are the most common transmission routes.<sup>148</sup>

The prevalence of GBV-C RNA in blood donors ranges from 1% to 5% depending on geographic location. Antibodies, such as anti-E2, are usually three to four times more likely than viral RNA.<sup>149–152</sup> Blood screening is not recommended.

### Keynotes: hepatitis G virus

Virus	Region	Transmission	Screening	Inactivation	Vaccination
Flaviviridae and RNA	Worldwide	Fecal-oral and blood components	NAT in principle possible and antibody screening	Possible	Not available

## Retroviruses

Retroviruses are lipid coated and widely distributed in nature, with examples in insects, reptiles, and almost all mammals. There are four main pathogenic human retroviruses—namely, HIV-1 and HIV-2, belonging to the lentivirus group of the retrovirus family, and human T-cell lymphotropic viruses (HTLV-I and HTLV-II) belonging to the oncoa group. It is generally accepted that HTLV-I and HTLV-II evolved from simian T-lymphotropic retroviruses that were transmitted to humans centuries ago. HIV-1 is also thought to have derived from a simian ancestor as simian immunodeficiency viruses are endemic in chimpanzees in Central Africa. HIV-2 originated from sooty mangabey monkeys in West Africa. It is thought that transmission to African natives occurred only over the last century.

Retroviruses are membrane-coated, ssRNA viruses that require the essential enzyme, reverse transcriptase, to transcribe their RNA to complementary double-stranded DNA that will be integrated into the host cell chromosome. The host cell enzymes aid the virus to complete its life cycle by synthesizing virions that bud from the plasma membrane to infect other cells or organisms.

## Lentivirus diseases

Lentiviruses constitute the basis of their genetic features of a subset of the family of retroviruses and are responsible for a variety of neurological and immunological disorders. Typical representatives are visna-maedi virus of sheep (VMV), infectious anemia virus of horses (EIAV), arthritis-encephalitis virus of goats (CAEV), and the immunodeficiency viruses of monkeys (SIV), bovine (BIV), and cats (FIV).

### Human immunodeficiency virus-1

Human pathogenic lentiviruses are the human immunodeficiency viruses of types 1 and 2 (HIV-1 and HIV-2). Due to a distinct genetic entity in the variable Env gene (V3 loop) and the Gag protein (p17), they are subdivided into groups and subtypes. HIV-1 is classified into four groups with a total of 12 known subtypes (Figure 44.7):

- Group M (major): subtypes A–K
- Group O (outlier): subtypes 1 and 2
- Group N (new)
- Group P (pending the identification of further human cases)

In 1983, an unusual accumulation of rare skin tumors (Kaposi's sarcoma), and atypical pneumonia (pneumocystis pneumonia carni, PCP), was observed in homosexual men in the United States.<sup>153–155</sup> It turned out that the cause of these diseases was a reduced cellular immune defense, probably caused by a viral infection. This hypothesis was confirmed in 1983 by Luc Montagnier<sup>156,157</sup> and 1984 by Robert C. Gallo,<sup>158,159</sup> who isolated a novel human retrovirus from affected patients. In addition to the genetic information for the structural proteins, Gag, Pol, and Env genes encoded a number of proteins with regulatory and accessory functions.<sup>160</sup> In HIV-1, the following proteins are included:

- Tat
- Rev
- Nef
- Vif
- Vpr
- Vpu

After activation of the integrated proviral DNA by cellular transcription factors that bind to the 5'-LTR, a de novo synthesis of viral mRNAs starts. These transcripts are spliced twice in the nucleus and transported into the cytoplasm for translation to the regulatory proteins Tat, Rev, and Nef.<sup>161,162</sup> After the splicing process, Tat is transported again into the nucleus and increases the viral RNA synthesis rate by a factor of 100–1000.

The Nef protein can interact with different kinases to bind to the lambda chain of the T-cell receptor and cause a downregulation of the CD4 receptor on the surface of the host cell. Thus, the Nef protein has multiple interactions in signal transduction and in the

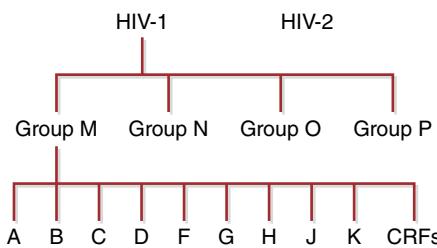


Figure 44.7 Taxonomic of HIV-1 and HIV-2. Source: Prof. M. Schmidt, German Red Cross, Frankfurt. Reproduced with permission.

metabolism of the host cell. Experiments show that HIV-1 can infect a variety of cells: other B lymphocytes, natural killer cells, endothelial cells, megakaryocytes, astrocytes, and glial cells. Nevertheless, CD4 T-lymphocytes, macrophages, and dendritic cells (DCs) are its primary targets. Besides the CD4+ surface marker, which acts as the primary HIV-1 receptor, other coreceptors are necessary for a successful infection. The CCR5 molecule was identified as an HIV-1 coreceptor on CD4+ positive macrophages and dendritic cells.<sup>163,164</sup>

Acquired immunodeficiency syndrome (AIDS) was first described in 1981 in the United States and later in Western Europe as striking at-risk male homosexuals and drug users. These groups were early excluded from blood donation due to indications for transmission of AIDS by blood transfusions.

Indeed, HIV strongly challenged transfusion medicine and the safety of blood products. In addition to blood component recipients, patients with coagulation disorders (hemophilia A and B) were infected in the 1980s (and 1990s) by contaminated factor concentrates.<sup>165–167</sup> This led nations like Germany to classify blood products as medical products, subject to the regulatory requirements of the Medical Act. Even after introduction of highly sensitive blood testing for HIV, lifelong exclusion of men who have sex with men from blood donation persisted for decades. Recently, the FDA has changed its guidance for the first time since 1992, allowing some men who have sex with men to enter the blood donor pool.<sup>168</sup>

Blood products can be screened for antibodies to HIV-1/2, the p24 antigen, and in addition to HIV NAT. Roth *et al.*<sup>148</sup> reported more than 270 million investigations in which 244 samples turned up only NAT positive, preventing HIV-1 transmission by blood components. The diagnostic window depends on the analytical sensitivity of the detection method and is calculated for NAT systems between 6 and 8 days. Despite the high level of blood safety, a calculated residual transfusion HIV-1 infection risk of 0.5–1:1,000,000 remains in the literature. Schmidt *et al.*<sup>169</sup> described an HIV-1 transmission by blood components after introduction of blood screening of HIV-1 by NAT. The cause of the transmission was a false negative HIV-1 NAT test result based on mutations in the primer/probe region of the detection method. The mutations reduced amplification efficiency. In 2012, Chudy *et al.*<sup>170,171</sup> reported further cases with false negative NAT screening results. One case was an HIV-1 transmission by a red blood cell concentrate. The reason for these negative HIV NAT results is that the reverse transcriptase from RNA into cDNA does not have a proofreading function. Errors in transcription are therefore neither registered nor corrected. Blood donor screening for HIV-1 by NAT should be done on a dual target basis, with amplification in at least two independent genome regions, to reduce the risk of mutations in one of the primer/probe areas.

A recent area of emerging concern is regarding the impact of highly active antiretroviral therapy (HAART) on blood donor behavior and education. It has recently been reported that HIV+ individuals on HAART, as well as individuals taking antiviral medication as pre exposure prophylaxis, have been donating blood, potentially increasing the risk of transfusion transmitted HIV.<sup>172</sup> The blood collection industry will need to continue to develop strategies to ensure that donation procedures and testing keep pace with these developments.

### HIV-1 therapy

The current applied antiretroviral therapy (ART) focuses on the inhibition of viral protein reverse transcriptase (RT) and protease (PR).<sup>173–175</sup> Furthermore, various drugs are emerging that pursue

the inhibition of various viral and cellular functions. With the use of a combination therapy (highly active antiretroviral therapy [HAART]), the rate of severe illness has decreased remarkably.

Patient discipline is necessary because drug side effects are frequent. Incomplete medication leads viruses to build up resistance; this leads in time to treatment failure.

### Human immunodeficiency virus-2

Most HIV-2-infected people are living in Central and West Africa. The first case in the United States was reported in 1987.<sup>176</sup> There are eight different subtypes known for HIV-2 (A to H). Subtypes A and B are epidemic. Subtype A is particularly common in West Africa, but also in Angola, Mozambique, Brazil, India, and very limited in Europe or the United States. Subtype B is mainly restricted to West Africa.<sup>177</sup>

HIV-2 is closely related with the simian immunodeficiency virus in sooty mangabeys (*Cercocebus Atys Atys*) (SIVsmm), a species of monkeys that inhabit the forests of the coast of West Africa. Phylogenetic analyses show that the SIVsmm is most related to the two strains of HIV-2, which are connected to spread in humans (A and B HIV-2 groups). SIVsmm is found in sooty mangabeys of the Tai Forest, west of Ivory Coast.

HIV-2 was found to be less pathogenic than HIV-1. The pathomechanism of HIV-2 is not clearly understood, but its transfer rate is much lower than HIV-1. Both infections can lead to AIDS in individual patients, and both viruses can mutate to resist drugs. HIV-2 infections had lower mortality than HIV-1 infections, but coinfection is also possible. The progress of coinfections depends on which virus was first. Coinfection seems to be a growing problem globally as time progresses, with most cases identified in West African countries, but some in the United States.<sup>178,179</sup>

### Keynotes: HIV-1

Virus	Region	Transmission	Screening	Inactivation	Vaccination
Lentiviridae, RNA, 4 groups (M, O, N, and P), 12 subtypes, TTID: <1:1 million	Worldwide and Africa	Blood components and sexual activities	NAT, antigen (p24), and antibody screening	Possible, 4–6 log	Not available

### Keynotes: HIV-2

Virus	Region	Transmission	Screening	Inactivation	Vaccination
Lentiviridae, RNA and eight genotypes (A–H)	Worldwide and West Africa	Blood components and sexual activities	NAT, antigen, and antibody screening	Possible, 4–6 log	Not available

### Human T-cell lymphotropic virus

Human T-cell lymphotropic virus (HTLV-I) was identified and isolated in a Japanese patient in 1978.<sup>180</sup> The virus was found that causes adult T-cell leukemia and lymphoma (ATL) and tropical spastic paraparesis (TSP), which can also be found as HTLV-I-associated myelopathy. The virus is now known worldwide, but endemic in Japan, the Caribbean, South America, and West and

Central Africa, where infection rates are often more than 1%. HTLV-II was originally identified in 1982 in a patient with hairy cell leukemia.<sup>181,182</sup> HTLV-I and HTLV-II show 65% homology. HTLV-II is found in Native American populations and in some populations of injecting drug users.

The overall prevalence in blood donors in the United States is about 9.6/100,000 person years and the incidence is 0.239/100,000. HTLV-II is more common than HTLV-I in blood donors.<sup>183</sup> Current HTLV donor screening tests offered only on antibody detection by ELISA or chemiluminescent (ChLIA). The more sensitive assays use recombinant HTLV proteins in a sandwich assay format. Assays test both HTLV-I and HTLV-II in parallel.

Confirmatory testing of reactive HTLV donation is more complex than a confirmatory test for HIV, in part because of the relatively poor Western blots, which are commercially available. NAT could be an alternative to confirm reactive HTLV-1/HTLV-2 antibody screening results, but no commercial test is currently available.<sup>73,184</sup>

Most British HTLV-positive blood donors have been shown to be of Afro-Caribbean origin or sexual partners of someone Afro-Caribbean origin. Therefore, it is important that sexual partners and other family members are also tested for HTLV in a clinical follow-up.

The diagnostic window for HTLV antibodies is about 51 days. A study by the US transfusion recipients estimated a residual risk of HTLV infections at 1:128,000.<sup>185</sup> In general, transfusion-transmitted infectious risk for HTLV is transmitted by cellular components (leukocytes). Therefore, leukocyte depletion is able to improve blood safety.<sup>186</sup>

Although most HTLV-positive donors show no clinical signs of disease, a small percentage (2–4%) can develop an acute T-cell leukemia (ATL) up to 40 years after infection.<sup>187,188</sup> HTLV-I is also associated with uveitis.

### Keynotes: HTLV-1 and HTLV-2

Virus	Region	Transmission	Screening	Inactivation	Vaccination
Retroviridae and RNA	Japan, Caribbean, and South America	Blood components and sexual activities	Antibody screening	Possible, 4–6 log	Not available

## Flaviviruses

### Dengue virus infections

Dengue virus infections pose a major international public health concern. According to WHO, approximately 2.5 billion people in more than 100 countries are at risk of infection, 50–100 million dengue virus infections occur every year, and approximately 1.25 million people may die due to dengue fever, dengue hemorrhagic fever (DHF), or dengue shock syndrome (DSS).<sup>128,189–191</sup> A very recent study suggests that dengue infections are underreported and that annual infection rates may be even three- to fourfold higher than estimated by the WHO. Dengue fever can therefore be considered to be the most important mosquito-borne viral disease.<sup>192–194</sup> As an arthropod-borne infection, the major transmission pathway occurs by mosquitoes from the *Aedes* genus;<sup>195</sup> however, transmission by blood components or organ transplants has also been documented.<sup>196–198</sup>

Dengue virus is an ssRNA virus belonging to the Flaviviridae family. The mature virion has three structures (core, membrane, and envelope) and seven nonstructural (NS1, NS2a, NS2b, NS3, NS4a, NS4b, and NS5) proteins. There are four serotypes classified according to their immunological properties: DEN-1, DEN-2, DEN-3, and DEN-4.<sup>199–202</sup> The primary vector is *Aedes aegypti*, but other vectors, like *Aedes albopictus* or *Aedes polynesiensis*, are also possible. Female mosquitoes acquire the virus by biting infected humans during the viremic period and become infective after an incubation period of 7–14 days. Subsequently, infected mosquitoes can transmit the virus during every feeding.

Laboratory diagnosis can be carried out using NAT, antibody screening, or dengue antigen tests. The diagnostic window is 3–5 days for IgM antibodies and 2–3 days for NAT.<sup>203,204</sup> The diagnostic window for the NS1 antigen test may also be very short. IgG antibodies are detectable after approximately 9–10 days. Crossreactivity with other flavivirus infections is possible and can be explained by sharing common antigenic epitopes. Commercial NAT systems for blood donor screening are available with a 95% level of detection of 14.9 geq/mL.

Fever is the predominant symptom in adults, whereas people younger than 15 often have asymptomatic infections or an undifferentiated febrile illness with maculopapular rash. Dengue fever (DF) and dengue hemorrhagic fever (DHF) are characterized by severe headaches, sudden onset of high fever, hemorrhagic manifestations, thrombocytopenia, and evidence of plasma leakage. Dengue shock syndrome has a high mortality rate and often acts as a pathway to disseminated intravenous coagulation (DIC). There are no specific antivirals currently available for dengue; therefore, its management is symptomatic and supportive.

In Germany, 594 total imported cases of dengue fever were reported in 2010; 75% were imported from Asia, and 21% came from South and Central America.<sup>205</sup> The last dengue epidemic in Continental Europe occurred from 1927 to 1928 in Greece. Since then, Europe has been the only populated continent without autochthonous dengue virus infections until in 2010, when cases of autochthonous dengue virus infections were reported from southern France and Croatia.<sup>206,207</sup>

In both countries, *Aedes albopictus* mosquitoes have become domestic and were the implicated vectors in the transmission cases. *Aedes albopictus* has become increasingly established in the European Union. It is assumed that the import of mosquito larvae during the transport of car tires, in combination with rising average temperatures in Europe due to climate change, is an important factor of this process.

Today, *Aedes albopictus*, the so-called Asian tiger mosquito, is established in many European countries, such as Greece, Albania, Italy, Croatia, Montenegro, and Spain, and it is spreading further. During mosquito monitoring activities in 2011, *Aedes* species were also detected in the Upper Rhine Valley in Baden-Württemberg.

*Aedes albopictus* is not only a very efficient vector for dengue virus, but also for numerous other arboviruses, such as the Chikungunya virus. Consequently, if travelers with arboviral infections acquired abroad return home, where *Aedes albopictus* has become domestic, there is the risk of causing a local outbreak. Likewise, infected tourists could import infections, as happened during the Chikungunya outbreak in northern Italy in 2007, which was probably caused by a tourist from India.

Although the primary pathway for dengue virus is through the mosquito vector, vertical transmission through intrapartum transmission, nosocomial transmission through needlestick injury,

transmissions by organ and bone marrow transplantation, and transfusion of blood components have all been reported. Even though the diagnostic window is assumed to be very short, asymptomatic donors could be the source of such transmissions. In some cases, however, the differentiation between nonmosquito transmission and mosquito-borne infection is difficult in endemic areas where the vector is widespread.

Transfusion-transmitted dengue virus infection has been confirmed in two cases from Hong Kong and Singapore.<sup>208–212</sup> As a first step to improve blood safety, with regard to dengue virus, many countries have implemented a temporary donor deferral program for travelers returning from highly endemic areas. These deferral strategies might be helpful if only a few donors will be affected (e.g., less than 1%). If more than 5% of the donor population will be involved, there is a high risk of unnecessary donor deferral because donors might not come back, even if the deferral is temporary.

Testing for dengue virus is also an option. In the most recent outbreak of dengue fever on Madeira, in the Atlantic Ocean, Portuguese authorities implemented blood donor screening by NAT for dengue virus RNA. Additionally, an ELISA test for the NS1-antigen of dengue virus is available that allows early detection of an infection. Another approach is the implementation of pathogen-reduction methods.

For Germany, however, the main strategy should be the implementation of strict vector control measures, such as insecticiding and reducing potential breeding sites, in order to avoid the spread of *Aedes* species throughout the country.

#### Keynotes: dengue virus infections

Virus	Region	Transmission	Screening	Inactivation	Vaccination
Flaviviridae, RNA, and genotypes 1–4	Japan, Caribbean, and South America	Vector-transmitted (arbovirus) blood components	NAT and antibody screening	Possible, 4–6 log	Not available

#### West Nile virus infections

The WNV is known since 1937. It has a structure similar to dengue fever virus, and both belong to the genus *Flavivirus* within the family *Flaviviridae*. WNV is an enveloped RNA virus with a positive-sense, single strand of RNA, which is between 11,000 and 12,000 nucleotides long.<sup>213,214</sup> The virus occurs in tropical as well as temperate areas. It mainly infects birds, but can spread to humans, horses, and other mammals.<sup>215,216</sup> The vector of the virus is through mosquitoes of different genera, such as *Culex*, *Aedes*, or *Ochlerotatus*. The European/Asian tiger mosquito, *Aedes albopictus*, can transfer it to human and birds.

From 1999 to 2003, there was a large spread of a new strain (strain New York, lineage 1) of West Nile virus in the United States from the East Coast to the West Coast.<sup>217,218</sup> A huge number of infections, some fatal, led the FDA to mandate blood donor screening for West Nile virus by NAT in 2003. The virus concentration in humans was in a range between  $10^1$  and  $10^5$  geq/mL. Thus, a blood donor screening in small pool sizes (1–8 donations per minipool) was required to reduce the diagnostic window to a minimum of three days.<sup>210,211,219–224</sup> After about 11–12 days, infected patients developed IgM antibodies and got a natural protection against the virus infection. In recent years, the incidence of WNV disease in

Europe increased from year to year (data by ECDC), particularly in Greece, Russia, Hungary, and northern Italy.<sup>225–227</sup> All donors who visited a country with WMV infections have to be deferred from blood donations for 28 days. As an alternative, blood donors can be screened for WNV by NAT with a sensitive method or antibody assays. But the diagnostic window for antibody assays is 11–15 days. Therefore, they are not feasible to detect infected blood donors in the early infection period, and the specificity of the commercial antibody tests is limited. Crossreactivities with other flaviviruses cannot be ruled out.

#### Keynotes: West Nile virus infections

Virus	Region	Transmission	Screening	Inactivation	Vaccination
Flaviviridae, RNA and five lineages	USA, Greece, Russia, and Hungary	Vector-transmitted (arbovirus) blood components	NAT and antibody screening	Possible, 4–6 log	Not available

#### Alphavirus infection

##### Chikungunya virus infections

Chikungunya virus (CHIKV) is an enveloped ss (+) RNA virus and belongs to the genus Alphavirus in the family Togaviridae, a group of arboviruses. Infection is mostly transmitted by the bite of arthropods. The pathogen was discovered in 1953.<sup>228</sup> In humans, the virus causes chikungunya fever.<sup>229,230</sup> The virion has a diameter of about 60 nm, making it one of the smaller viruses. It has a length of about 11,700 base pairs that code for 2474 amino acids. The protein NSP4 takes presumably over the function of an RNA polymerase.

Chikungunya occurs in Africa, Asia, and the Indian subcontinent. In 2005/2006, there was a CHIKV epidemic on the island La Réunion, with a total of 270,000 infections (about 35% of the population) and a total of 254 documented, related deaths.<sup>231,232</sup> A year later (2007), an epidemic occurred in northern Italy in Ravenna.<sup>233,234</sup> Here, 197 suspected cases of chikungunya fever were registered. Currently, there is a local epidemic of CHIKV in the Caribbean, Latin America, and the northern part of South America. WHO reports indicate that by October 2014 over 776,000 suspected cases of Chikungunya have been recorded in the Caribbean islands, Latin American countries, and some south American countries.<sup>152</sup> 152 deaths have also been attributed to this disease during the same period. In El Salvador, the Food and Drug Administration reported more than 30,000 suspected cases this year, and about 1700 have been registered in Venezuela.<sup>235–237</sup> Most infections are in the Dominican Republic. According to the reports of the Pan-American Health Organization OPS, CHIKV has spread to at least two dozen countries and territories in the Western Hemisphere since the first case was detected on the Caribbean island of St. Martin at the end of 2013.<sup>238</sup>

Survivors develop usual neutralizing IgG antibodies that protect them from a second infection for their lives. In the early infection period, an asymptomatic viremic phase may occur; therefore, CHIKV epidemics represent a challenge for blood donor services in endemic countries. Blood donor screening can be made by NAT, and the performance characteristics appear to be favorable, either in the detection of CHIKV alone or in combination with DENV or Zika.<sup>239,240</sup>

### Keynotes: chikungunya virus infections

Virus	Region	Transmission	Screening	Inactivation	Vaccination
Chikungunya virus and RNA	Africa, Asia and the Indian subcontinent, Caribbean Island, and northern part of South America	Mosquitos and blood components	Commercial NAT in development	Yes	Not available

### Herpesvirus infections

Herpesviruses are spread all over the world and can infect all creatures. Humans are known as the host for various herpesviruses. All human herpesviruses (HHVs) have the ability to rest in tissues after acute infection. Depending on the immune system and stress factors, the replication of herpesviruses can start again and cause a reinfection. Human cytomegalovirus (CMV or HHV-5) is the most transfusion-relevant herpesvirus infection for humans.<sup>241–243</sup> Herpes simplex (HHV-1 and HHV-2) and herpes varicella zoster (HHV-3) have limited target cells, but CMV can manifest itself in many different organs. CMV represents a major challenge in immunosuppressed patients exposed to transfusion therapy. Special patient groups at risk are the following:

- Transplant recipients
- Patients with severe immune deficiency
- Fetuses (intrauterine transfusion)
- CMV-negative pregnant women
- Low-birthweight premature infants and neonates
- Multitransfused patients

It is important to distinguish between CMV infection by serological tests or viral isolation and CMV disease by laboratory evidence of infection in connection with specific symptoms that identify the virus. Most CMV infections are asymptomatic, but high fever, sore throat, and symptoms similar to flu and glandular fever occur. Reactivation of CMV can occur when the immune system is weakened.

The target cells of CMV are monocytes and macrophages. Therefore, leucocyte depletion is effective at reducing residual infection risk. The main infection risk is during childhood or the early adult years via sexual activities. At the age of 40, about 60–70% of all donors in Western Europe have anti-CMV IgG antibodies as a sign of a past CMV infection.

Most blood transfusion services are using ELISA-based assays to test blood for the presence of anti-CMV.<sup>244–249</sup> Hemagglutination assays can detect both classes of antibodies, and some commercial tests can now be performed on automated blood group analyzers (PK7300 systems).

CMV transmission by transfusion is now relatively rare in developed countries, particularly those in which all blood components are leukocyte reduced. It is believed that only 12–62% of CMV-seropositive blood units infect CMV-seronegative recipients depending on the interval to the last seronegative donation.<sup>250</sup> The incubation time of the primary CMV infection varies between four and eight weeks. The residual risk of CMV transmission from properly leukoreduced blood products is difficult to establish with certainty, but is sufficiently low that many transfusion services have abandoned the historical practice of providing CMV seronegative products in favor of leukoreduction.<sup>251</sup>

In marrow transplantation, CMV is one of the most important infections and responsible for about 15% of mortality before the introduction of good antivirals.<sup>252–254</sup>

Antiviral drugs, active against CMV, include ganciclovir, foscarnet, cidofovir, and valacyclovir.<sup>255–258</sup> Drugs may be prescribed for prevention, suppression, or treatment.

Epstein–Barr virus (EBV or HHV-4) is responsible for infectious mononucleosis, Burkitt's lymphoma, and nasopharyngeal cancer, and may also be transmitted by transfusion.<sup>259</sup>

*Human herpesvirus 6* effects (HHV-6) is *roseola infantum* or *sixth disease*—a childhood rash. Primary infection in adults is rare.

*Human herpesvirus 8* (HHV-8) is the causative agent for Kaposi's sarcoma and other tumors such as Castleman disease. Infections with HHV-8 are also associated with polyneuropathy, organomegaly, and endocrinopathy. As with CMV and EBV, HHV-8 appears to cause a persistent infection within the host immune cells.<sup>260–262</sup>

Transfusion-transmitted EBV infections are usually asymptomatic, but some present as infectious mononucleosis with a slight fever, loss of appetite, and nausea. Most HHV-4 infection is benign with a low frequency of disease development.<sup>263–265</sup>

### Keynotes: herpes virus infections

Virus	Region	Transmission	Screening	Inactivation	Vaccination
Herpesviridae, DNA, and eight genotypes (HHV 1–8)	World wide	Human-to-human contacts and blood components	NAT and antibody screening	Possible, 4–6 log	Not available

### Parvovirus B19 infections

The parvoviruses still form the only family of viruses whose genome consists of single-strand, linear DNA, and the only known human pathogen is parvovirus B19. It was isolated in 1975 by Yvonne Cossart and employees from blood associated with the clinical picture of erythema infectiosum, also known under the names of *fifth disease* or *slap-cheeked syndrome* in young children.<sup>266–268</sup>

Parvoviruses, as the name indicates, are among the smallest known viruses. The virus capsids have a diameter of only 18–26 nm and are not surrounded by a membrane or an envelope. They have an iso-octahedral structure and consist of 60 capsomeres, which are 95% the structural protein VP2 and 5% VP1 protein. VP2 is identical to the carboxy-terminal region of VP1 sequence. The capsids mediate the absorption of the parvovirus B19 to its target cells. The linear DNA single strands of parvovirus B19 is 5600 bases long. The genome has two large open reading frames: the first location is for the nonstructured protein NS1 (MW 71,000). The protein activates the promoter and has important functions in the replication of virus DNA. Furthermore, after the replication, the protein induces apoptosis of the infected cells. The second reading frame is located in the 5' half of the genome and is coding for the structural proteins VP1 and VP2.

Parvovirus B19 infects erythropoietic precursors<sup>269,270</sup> (red blood cells in different stages of differentiation in the BFU-E [erythrocyte-forming unit], CFU-E [erythrocyte colony-forming unit], and the erythroblasts in the bone marrow, as well as pronormoblasts in the fetal liver) by binding to the P antigen, a glycosphingolipid, which is the membrane receptor on the target cells.<sup>271</sup> Various other cell types also contain this receptor explaining the widespread manifestations of parvovirus B19 infection. Individuals lacking the P blood group antigen are naturally immune to parvovirus B19 infection.

Parvovirus B19 is usually transmitted by droplet infection. In the bloodstream of infected persons, the virus concentration can increase to very high concentrations of  $10^{11}$ – $10^{13}$  geq/mL.<sup>272–275</sup>

Infections by parvovirus B19 mainly occurred in the early childhood. Therefore, 40–50% of adolescents aged 15 years already have virus-specific antibodies. The percentage grows up to 80% at the age of 40, which could explain why few transfusion-transmitted cases are reported.

After parvovirus B19 infection, the body forms IgM and IgG antibodies directed against the capsid proteins VP1 and VP2. The first IgM antibodies appear approximately 6–10 days after infection with B19. From the 12th day, IgG antibodies are detectable. The virus concentration will be diminished under the NAT detection limit within 3–4 weeks after infection in about 80% of all cases. Low B19 virus concentration can be detectable up to 12 months. CD4-positive T lymphocytes are in addition involved in the elimination of B19 virus.

The clinical symptoms of B19 infections are manifold. The course of the infection and severity of the disease depend on the patient's hematological and immunological status. Possible clinical manifestations include acute anemia, arthritis, transient aplastic crisis, chronic arthritis, thrombocytopenia, neutropenia, pure red cell aplasia, hydrops fetalis, pancytopenia, chronic anemia, myocarditis, pericarditis, hepatitis, acute liver failure, meningitis, acute liver failure, uveitis, vasculitis, and pneumonia. About 33% of children have no symptoms.<sup>276–279</sup> Generally, clinical symptoms are mild also. In adults, however, complications can occur, especially in women. In 50% of adults, pains in the small joints of both hands and feet can occur. In immunosuppressed patients, such as those with congenital immunodeficiency, transplant and cancer patients, and HIV-infected persons, parvovirus B19 can cause a long, life-threatening infection persistent over months or years. B19-associated mortality in transplant patients is estimated to be about 7%. Before parvovirus-B19-specific antibodies are formed, large amounts of B19 virus (up to  $10^{13}$  geq/mL) can be detected in the blood.

Despite the high virus concentration, many infections are asymptomatic so that infected people can come to a blood donation and be accepted as donors. The virus may also be present in the final blood components products such as factor VIII and IX in relevant concentrations because chemical or physical pathogen inactivation methods are less efficient since B19 virus has no lipid or protein membranes. Commercial NAT systems are available and should detect all three genotypes of B19 viruses. Some blood transfusion services have introduced screening for plasma products by NAT.<sup>138,280–283</sup> Pools of source plasma for fractionation are only acceptable with less than 10,000 geq/mL of parvovirus B19. This level of activity is ascertained by considering the level of parvovirus antibodies that are also present in the pool and by their ability to neutralize small concentrations of the virus.

At present, there is no effective vaccine against parvovirus B19 or antiviral chemotherapeutic agents for the treatment of the infection.

### Keynotes: parvovirus B19 infections

Virus	Region	Transmission	Screening	Inactivation	Vaccination
Parvovirus B19, DNA, small virus, and three genotypes	Worldwide	Droplets and blood components	NAT antibody screening	Yes, but less efficient	Not available

### Filoviridae

#### Ebola virus infections

Marburg virus (MBG) and Ebola virus (EBOLA) are nonsegmented negative-stranded RNA viruses and belonged to the family of Filoviridae within the order Mononegavirales.<sup>284–286</sup> The species Ebola virus can be subdivided into subspecies from Reston, Sudan, and Zaire. The virions are filamentous and extremely polymorphic. The total length is up to 14 μm compared to the uniform diameter of about 80 nm. The lipid envelope surrounding the nucleocapsid consists of genomic RNA and four structural proteins.

- Nucleoprotein
- Viral structural protein (VP) 30
- Viral structural protein (VP) 35
- Viral polymerase

The viral polymerase controls the transcription and replication in the cytoplasm of the cell. VP 35 appears to be a cofactor of the viral polymerase.<sup>287</sup> The nucleoprotein envelopes the RNA and interacts with VP30, which in the case of Ebola acts as a transcription factor.

Filovirus-related hemorrhagic fever was first diagnosed in 1967 in Europe (Marburg, Frankfurt, and Belgrade). The first patient was a lab worker who had contact with monkeys imported from Uganda (*Cercopithecus aethiops*). A total of 26 primary and six secondary infections were registered, with seven death cases. The Ebola virus hemorrhagic fever was first described in 1976 during two outbreaks in Sudan and the Democratic Republic of Congo (Yambuku).<sup>288,289</sup> A total of 602 cases were registered, and the mortality rate was 88%. The natural hosts are humans and other primates. Experimental hosts, including guinea pigs, hamsters, and mice, are often the primary infection. The initial transmission pathway (zoonosis) is currently unclear. The transmission from humans to humans occurs through close contact with infected people and their excretions (often nosocomial). Sexual, neonatal, and airborne transmissions are possible, but epidemiologically of minor importance. The incubation period is usually between 1 and 21 days.<sup>290,291</sup> Animal studies indicate that Ebola viruses are very infectious, so small viral concentrations might be sufficient to infect new people. The disease progresses with severe clinical symptoms such as high hemorrhagic fever and loss of fluid (up to 10 L/day). Therefore, patients will have numerous medical complications, including renal insufficiency. Secondary bacterial infections can be added. In the acute period, patients need intensive medical care. Since clinical symptoms begin immediately at the time of viremia, Ebola infection is believed to be avoidable through donor deferrals, including travel deferrals to affected regions, as well as routine donor assessment. Asymptomatic infectious patients have not been observed. Of particular importance to medical transfusion is the care of Ebola patients since crossmatching must be carried out under high secu-

rity and safety conditions. A challenge for blood transfusion service is to produce convalescent plasma from patients with neutralizing antibodies against Ebola virus infections, although demand for this service in the future may be lessened by data showing that this therapy is ineffective.

#### Keynotes: Ebola virus infections

Virus	Region	Transmission	Screening	Inactivation	Vaccination
Filoviridae, Ebola virus, and RNA	West Africa	Human-to-human contacts, droplets, and blood products	Commercial NAT available	Probably yes, no data available	Not available

#### Pandemic influenza

Influenza viruses are lipid enveloped negative-stranded RNA viruses of the orthomyxoviridae family. There are three types of influenza virus, A, B, and C, which give rise to each their type of influenza. Influenza C gives few clinical symptoms and infects only humans. Influenza B generally only infects humans, whereas influenza A can infect a number of species. Influenza A and B can cause serious disease and are the two virus types that cause epidemics each winter.

Influenza pandemics are a persistent threat due to frequent mutations in the genetic code of the virus. Therefore, a persistent efficient immune response is not possible. The lethal influenza viruses that have infected humans have so far been managed by the existing public health measures. This was helped by the relative inability of the “bird flu” virus (H5N1) to infect people.<sup>292–294</sup> However, there is concern that eventually a mutation or mixed infection of these deadly viruses will spread into the public. Since there have been a series of bird flu cases in humans involving H5N1, it is assumed that any new influenza threat will probably be closely related. Vaccines have been prepared; but like normal human flu vaccines, they have good efficacy in immunocompetent individuals, but are of limited use to those at highest risk from such a pandemic (the old and the very young). It is believed that transfusion-transmitted influenza is relatively rare but theoretically possible. But the timeframe of an asymptomatic viremia is very limited, probably only a few hours. At the height of each epidemic, the use of blood is probably severely limited because of the cancellation of planned operations, but also because of the shortage of health workers and blood donors. Commercial NAT detection systems are available, but a routine blood donor screening is not recommended. Only in the case of a global pandemic, this question should be raised again to improve blood safety.

#### Keynotes: influenza infections

Virus	Region	Transmission	Screening	Inactivation	Vaccination
Orthomyxo virus, and influenza virus, and RNA	Worldwide	Human-to-human contacts, and human contacts with birds, pigs, and other animal	Antibody screening and commercial NAT available	Possible	Yes, but vaccination has to be updated annually

#### Other viruses

##### Lymphatic choriomeningitis (LCMV)

LCMV was first described in 1931 as one of the main causes of meningitis.<sup>295,296</sup> LCMV is a lipid enveloped ssRNA virus and one of the Arenaviruses. Its main reservoir is mice, but it can infect other animals or humans. Eight solid organ transplants have been infected with LCMV from two organ donors, raising the concern that LCMV can also be a transfusion-transmitted virus.<sup>297,298</sup> A French study found a low prevalence in the blood donor population, suggesting a low risk of transmission. However, the researchers suggest that the history of the last rodent contact should lead to a temporary deferral of potential donors. This could have an important impact on donor deferrals. Based on the low prevalence rate, a general blood donor screening is currently not recommended.

#### Keynotes: lymphatic choriomeningitis virus

Virus	Region	Transmission	Screening	Inactivation	Vaccination
Arenavirus, LCMV, and RNA	World wide	Contacts with rodents and mice, and blood components not proven	In development	No data available	Not available

##### TT virus (TTV)

TTV was discovered in the search for the elusive reason for the smaller (non-C) component of the “non-A to E” post-transfusion hepatitis. The virus was discovered in a post-transfusion hepatitis patient with the initials TT and first reported by T. Nishizawa in 1997.<sup>299,300</sup> TTV is a small (18–50 nm), nonenveloped DNA virus and belongs to the family Anelloviridae. Isolates have been classified into five main clades numbered 1–5. TTV genogroup 3 also includes the eight virus strains known as SENV-A to H.<sup>301</sup> TTV can be transmitted by transfusion and has a prevalence of TTV RNA between 5% and 80%; there are again significant geographical differences in infection rates with higher prevalence in Africa (up to 80%). It is assumed that 50% of African children of one year have been infected with TTV. Sensitive NAT assays demonstrate a high percentage of viral infections in blood donors. The relevance of TTV in transfusion is limited due to the absence of clinical symptoms.

#### Keynotes: TT virus

Virus	Region	Transmission	Screening	Inactivation	Vaccination
Anelloviridae, TTV, and RNA	Worldwide	Blood components possible	In development	No data available	Not available

#### Summary

Blood safety is an important topic in transfusion medicine with subtopics such as prevention of the incorrect blood transfusion,<sup>302</sup> prevention of transfusion-related acute lung injury (TRALI),<sup>303</sup> and of course prevention of transfusion-transmitted virus infections.<sup>304</sup> After the AIDS scandal, blood safety was improved regarding transfusion-relevant viruses such as HIV-1, HBV, and HCV. The introduction of NAT and serological screening assays comprised big milestones in hemovigilance and reduced the residual infectious risk to less than 1:1,000,000 for HIV-1 and HCV.<sup>305</sup>

But viruses are not stable. They change their genetic information and sequence due to mutation pressure. RNA viruses have no proof-reading function for reverse transcriptase. Therefore, risks of mutation within the primer and probe binding region exist and were the cause for several cases of transfusion-transmitted HIV-1 with NAT-negative blood. Therefore, manufacturers of diagnostic assays must be on alert to adapt their system to new genotypes.

Second, people travel the world. A new virus can be spread around the world within 48 hours. Therefore, new pathogens can become relevant for all countries or for some regions like West Nile virus for the United States and probably for Europe within the next years.

Nevertheless, blood safety is a global challenge for all transfusion services. The scientific exchange within international societies is inspiring, and cooperation within WHO is a necessity. Safe blood products should be available on the same safety level to all patients in the world.

## Disclaimer

The authors have no conflicts of interest.

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A full reference list for this chapter is available at: <http://www.wiley.com/go/simon/Rossi6>

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## CHAPTER 45

# Transfusion transmission of parasites and prions

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### Summary

Blood recipients in the United States (US) face low risk for transfusion of a component contaminated with parasites. Quantifying the risk was historically challenging given several unknown or poorly characterized parameters. Estimates from 15 to 30 years ago suggest one transmission of parasitic infection per million transfusions.<sup>1,2</sup> Enhanced diagnostics and mitigation efforts have delineated the risk while simultaneously reducing it considerably.

A 2009 review of 68 pathogens by the AABB's Transfusion Transmitted Diseases (TTD) Committee<sup>3</sup> identified four protozoan parasites among the agents of greatest concern. For *Babesia microti* and other *Babesia* species, recent data suggest that transfusion transmission was historically undercounted. Screening for *Trypanosoma cruzi*, the etiological agent of Chagas disease, significantly curbed the risk of transmission associated with immigrants from Latin America, while raising questions about autochthonous transmission within the US. Transfusion of malaria parasites continues to be rare, with prevention based on deferral of at-risk donors.

Prions, the causative agent of transmissible spongiform encephalopathies, represent a small but nonzero risk for transfusion recipients, and like parasitic agents require the attention of blood collectors and regulatory authorities. This chapter discusses parasitic and prion agents potentially found in blood donors, associated risks, and strategies for mitigating these risks.

### Babesiosis

Concern for transfusion-transmitted babesiosis (TTB) recently culminated in regulatory guidance in the US after years of documenting expanded transmission and refined estimates of transfusion risk. *B. microti* is primarily responsible for clinical cases and transfusion transmission events in the US, but is one of at least three distinct genotypes transmitted to humans. In Europe, *Babesia divergens* causes most human illness. Multiple *Babesia* species have been documented in Asia, which together with isolated reports from Africa, Australia, and South America indicate the emergence of human babesiosis as a global phenomenon.

Transmitted primarily by ixodid ticks, over 100 species of these intraerythrocytic protozoan parasites infect many vertebrate species

worldwide.<sup>4</sup> *Babesia* infection is often clinically silent, with symptomatic infections more common with advancing age.<sup>5</sup> Clinical infections begin 1–6 weeks following tick bite and are frequently nonspecific, presenting with fever, headache, and myalgias. Risk for severe disease is higher in the elderly, the immunocompromised, and the asplenic; complications include thrombocytopenia, hemolytic anemia, and renal, heart, or respiratory failure.<sup>6</sup> The case fatality rate ranges from 5% to 10% for *B. microti*<sup>6</sup> to 42% for *B. divergens*.<sup>7</sup> That nearly all reported cases of *B. divergens* occurred in asplenic hosts<sup>7</sup> contributes to this disparity.

Human babesiosis is an emerging disease within the US and globally. Since the first US case of human babesiosis in 1966,<sup>8</sup> fewer than 2000 cases were reported over the next four decades, concentrated in seven states considered "endemic" (Massachusetts, Connecticut, New York, Rhode Island, and New Jersey in the Northeast, and Minnesota and Wisconsin in the Upper Midwest).<sup>9</sup> Since babesiosis was made a nationally notifiable disease in 2011, the number of states reporting cases and the number of reported annual cases have doubled (see Table 45.1), reflecting its expanding endemic range.

The primary agent responsible for human babesiosis in the US is *B. microti*. It is transmitted by the black-legged deer tick (*Ixodes scapularis*), the vector of Lyme disease. Heightened awareness and scrutiny, together with increased molecular characterization of patient isolates of *Babesia* parasites, present a complex picture of *Babesia* transmission in nature. *B. microti* is considered a species complex composed of three clades with identical morphology but partly distinct vertebrate hosts and potentially differing levels of pathogenicity for humans.<sup>10</sup> *Babesia duncani*, first recognized in the Western US in the early 1990s, is morphologically identical to *B. microti* but antigenically and genetically distinct.<sup>11</sup> *Babesia* sp. MO1 was originally thought to be the primary European parasite *B. divergens*, but it is distinguished by genetic sequence difference and in vitro characteristics and is often referred to as "divergens-like." Its isolation from asplenic patients in five mostly noncontiguous states (Missouri, Kentucky, Washington, Arkansas, and Michigan) portends future documentation elsewhere.<sup>12–14</sup> Finally, the first human case in Tennessee, found in an immunocompromised hunter with heavy tick exposure, has been characterized as "genetically distinct" from previously identified zoonotic

**Table 45.1** History of Babesiosis Reporting in US before and after Being Made Nationally Notifiable (2011)\*

	1966 – 2010	2011	2012	2013	2014	2015	2016	2017	2018	2019
Number of states reporting	~7	18	22	27	31	33	35	37	40	40
Number of states with cases	Varies	15	14	22	22	24	27	26	28	25
Number of cases reported	<2000	1124	911	1762	1744	2074	1909	2358	2161	2418
Number transfusion cases	159**	10	10	16	6	9	7	3	2	2

\* Data from Spencer (2009)<sup>9</sup> and CDC (2011–2015, 2016, 2017, and 2018).<sup>17–21</sup>

\*\*Reported in Herwaldt et al. for 1979–2009.<sup>22</sup>

agents,<sup>15</sup> but detailed characterization of the patient's isolate remains unpublished.<sup>15,16</sup> Outside of *B. microti*, little is known about the geographic distribution, vertebrate reservoirs, tick vectors, or virulence in humans of other *Babesia* species.

A similar picture of increasing complexity is seen elsewhere. In Europe, reports of human babesiosis are rare, with about 50 human cases reported since 1957 and a fatal case in a splenectomized Yugoslav farmer.<sup>7</sup> European cases are mostly attributed to *B. divergens*, a bovine pathogen.<sup>23</sup> Risk for human infection correlates geographically with the presence of infected cattle and areas infested by the *Ixodes ricinus* tick; little is understood about the role that spleen-intact human populations and silent carriage of *B. divergens* might play in terms of TTB risk.<sup>23</sup> A variant isolated from asplenic patients in Italy, Austria, and Germany has been designated *B. venatorum*<sup>7</sup> and is associated with three human cases. Two cases of autochthonous *B. microti* have been identified in Europe, one likely by blood transfusion,<sup>7</sup> although entomologic<sup>24</sup> and serologic<sup>25</sup> evidence suggests that transmission is much more common than recognized.

In Asia, autochthonous human babesiosis has been confirmed in Japan (due to transfusion-acquired *B. microti*-like infection),<sup>26</sup> Taiwan (also *B. microti*-like),<sup>27</sup> and Korea (designated KO1).<sup>28</sup> More than 100 cases have been newly reported from China, in both healthy and immunocompromised populations, primarily from *B. microti* and secondarily from a *B. crassa*-like organism.<sup>29,30</sup> Isolated reports from Africa, Australia, and South America reflect the ubiquity of *Babesia* worldwide.<sup>31</sup>

The two most important characteristics of *B. microti* infection driving transfusion risk are the preponderance of clinically silent infections<sup>32</sup> and the ability of immunocompetent persons to carry infection for lengthy periods.<sup>33</sup> There is little direct evidence for the proportion of infections that are asymptomatic, but one review suggests that one-half of children and one-fourth of immunocompetent adults have no symptoms with *B. microti* infection.<sup>5</sup> Confirming early studies,<sup>33</sup> a large follow-up study in blood donors documented parasite carriage of more than two years in 2 of 56 subjects; across all subjects the median time to PCR-negative sample collection was 4.7 months and 86% had cleared infection within a year's time.<sup>34</sup>

Together with prolonged parasitemia, storage of red cells contributes to year-round risk despite the overwhelming majority of tick-borne cases reported in June through August.<sup>20</sup> In vitro studies indicate that while cold storage reduces viability, some parasites survive storage at 4 °C and remain infective for 21–31 days.<sup>35–37</sup> A review of 162 transfusion cases in the US from 1979 to 2009<sup>22</sup> showed all but 3 were due to *B. microti*. Most occurred in the seven “endemic” states, but 13% occurred in 21 other states due to interstate movement of blood donors or blood components. Overall mortality was 18%, consistent with the advanced age (median age 65) and the compromised immune status of recipients. The median range from transfusion to diagnosis was six weeks but extended up to seven months. Nearly all implicated components were red cells,

with a median storage age at the time of transfusion of 16 days and four cases where red cells were between 35 and 40 days old. Three cases were caused by *B. duncani* and occurred in California and Washington. Subsequent reports show consistency in epidemiologic patterns with several dozen additional cases reported by CDC or blood collectors from 2010 forward.<sup>38,39</sup> Additional transfusion cases of *B. microti* have been documented once each in Canada, Germany, and Japan.<sup>40</sup>

Traditional diagnosis of *Babesia* infection relied on microscopic exam of Giemsa-stained blood films. Serological methods including immunofluorescent assay (IFA) diagnosis are both sensitive and specific for measuring exposure, and automated IFA assays improved throughput.<sup>41</sup> Real-time polymerase chain reaction (PCR) is highly sensitive and a valuable complement to serological methods which has received regulatory licensure.<sup>42–45</sup>

Human babesiosis was historically treated with clindamycin and quinine (CQ), but atovaquone with azithromycin is now the first-line recommendation in the US given the fewer collateral effects.<sup>5</sup> Treatment with CQ continues to be recommended for severe cases. In severe cases, partial or whole blood exchange transfusion might be indicated, especially for severe infections of *B. divergens*.<sup>5</sup>

Until licensure of a screening assay, the prevention of transfusion-transmitted babesiosis long relied on a health history question asking whether the donor ever had babesiosis. In 2019, the US Food and Drug Administration (FDA) released guidance recommending year-round nucleic acid testing (or pathogen reduction) in 14 states at highest risk, plus Washington, DC. A two-year deferral is recommended for NAT-reactive donors as is maintenance of a screening question in areas not performing NAT testing.<sup>46</sup>

As with other protozoan infections, pathogen inactivation methods hold promise in reducing the risk from transfusion-transmitted babesiosis. Photochemical treatments currently under development indicate parasite reductions of 5 logs in whole blood.<sup>47</sup> Equivalent efficacy has been shown in platelets and plasma.<sup>48</sup> Given that transfusion risk of *Babesia* parasites is associated almost entirely with red cell components, eventual regulatory approval of red cell/whole blood pathogen inactivation technologies will be a hallmark milestone in risk mitigation.

## Chagas disease

Risk for transfusion transmission of *Trypanosoma cruzi* infection declined in the United States following the implementation of donor screening in 2007. Concern had grown from the late 1980s forward with documentation of a small number of transfusion transmissions along with recognition of the potentially large human reservoir population of Hispanic residents. Endemic in Central and South America and in Mexico, this protozoan hemoflagellate is transmitted in nature by reduviid bugs or triatomines. These bugs often bite on the face (and hence are called *kissing bugs*) but do not directly inject the parasite in their bite. Rather, the insect vector

deposits the infective metacyclic forms of trypanosomes in its feces during or after the blood meal, which is rubbed into the wound or transferred to susceptible membranes in the eye or mouth by the sleeping host. Triatomids competent for transmitting *T. cruzi* are widespread in the Americas, including in the US, where autochthonous transmission occurs.<sup>49</sup> The agent is readily transmitted by blood transfusion, solid organ transplantation, and congenital transmission.<sup>50</sup> Occasional outbreaks from contaminated food or drinks have been reported, especially in Brazil.<sup>51</sup> Symptoms during the acute phase last four to six weeks and are characterized by fever, malaise, edema of the face, lymphadenopathy, and hepatosplenomegaly.<sup>52</sup> The fatality rate in the acute phase is usually less than 5%, and many cases go unrecognized. During this period, parasites are readily detectable in peripheral blood.<sup>50</sup> The ensuing chronic phase is lifelong, with infections that remain asymptomatic and parasitemia that is low grade and intermittent.<sup>52</sup> Ten to thirty years later, 20–30% of carriers develop serious sequelae involving cardiac and/or digestive features (megacolon and megaesophagus). Chagas is estimated to kill about 14,000 people annually, mostly due to cardiac complications.<sup>53</sup> Acute stage treatment with benznidazole or nifurtimox leads to cure in 80–99% of cases, but treatment during the chronic phase is less effective at eliminating infection. Therapies for slowing disease progression once symptomatic are under active investigation, but indications vary with the patient's age and severity of morbidity.<sup>54,55</sup>

Public health authorities have achieved considerable success in reducing risk and morbidity from Chagas disease. In the 1980s, an estimated 17 million residents of Latin America were infected and 100 million lived in areas with transmission risk; current estimates are roughly 6 million infected and 70 million at risk for insect transmission.<sup>53</sup> Annual incidence of cases has dropped more sharply, from 700,000 new cases annually to 30,000, a 96% decline.<sup>52,53</sup> This success began in 1991 with the Southern Cone Initiative, which sought to eliminate domestic infection by *Triatoma infestans* and to interrupt transfusion transmission through universal donor screening.<sup>56</sup>

The adoption of universal blood screening in Latin American countries dramatically lowered the risk for transfusion transmission in endemic countries. In the 1990s, only 4 of 17 Latin American countries with available data screened all donors, but now all 21 have universal testing.<sup>53</sup> Estimates within the last 20 years were that 2000 transfusion transmissions occurred annually,<sup>57,58</sup> but universal screening using high sensitivity assays<sup>59</sup> suggests a sharply lower risk today.

Clearly, the risk in the United States and other receiving countries of Latin American immigrants depends on their immigrants' risk for acquisition of infection extending decades into the past. Recent census figures estimate that 60.5 million Hispanics live in the US, of whom 37.4 million are of Mexican origin,<sup>60</sup> 5.6 million are Central American, and 4.0 million are of South American origin. Roughly one-third of these residents are foreign born, and more than 25% of them arrived to the US prior to 1990,<sup>60</sup> before the intensification of control programs in endemic countries. Current estimates project that up to 350,000 Hispanic immigrants in the US may be infected with *T. cruzi*.<sup>55</sup> Canada and Australia are estimated to have more than 1000 *T. cruzi*-infected immigrants each,<sup>57</sup> and Europe between 68,000 and 123,000, nearly entirely undiagnosed.<sup>56,61</sup>

Until licensure of a screening test in December 2006, there were seven reported cases of *T. cruzi* transfusion transmission in the United States and Canada,<sup>62</sup> and five cases of transmission associated with solid organ transplantation.<sup>63,64</sup> The actual number of transfusion cases was undoubtedly higher, in that all seven cases

were detected in immunocompromised patients.<sup>65</sup> Although platelet products were implicated in six of these seven events, robust estimates for infectivity of different blood components were not available until recently. Prior estimates of ≈20% risk of transmission from a *T. cruzi*-infected donor were based on studies conducted in endemic countries where the transfusion of fresh whole blood is common.<sup>57</sup> Lookback studies based on large numbers suggest that the risk of transmission by blood is less than 2% overall, ranging from 0% in PRBCs or plasma to 13% in platelets.<sup>65</sup> A higher inoculum of parasites in platelet concentrates compared to plasma and whole blood might partially explain this difference.<sup>66</sup>

The approach for screening blood donors for *T. cruzi* is different in endemic countries with active transmission than those where risk is associated with an immigrant population. All endemic countries in the Americas have adopted universal screening. In nonendemic countries, a recent survey of blood centers from 22 countries showed that 17 countries employed one or more mitigation strategy, with 15 countries using health history questions to identify risk and 10 using *T. cruzi* serology.<sup>67</sup>

In the US, testing following licensure of the first serological screening test clarified the prevalence and distribution of Chagas. A 2009 FDA Blood Products Advisory Committee (BPAC) meeting<sup>68</sup> resulted in an endorsement of selective testing for US donors where one negative donation would qualify a donor for all future donations and also a recommendation to discontinue requiring the health history question on Chagas disease. These recommendations were formalized in guidance issued by the FDA in 2010 and finalized in 2017.<sup>69</sup> An online AABB Chagas Biovigilance network that was in operation from January 2007 to 2019 evaluated more than 2400 confirmed positive cases from 15,000 reactive donations. Cases were widely distributed but concentrated in California, Florida, Texas, and other states with large Hispanic populations.<sup>70</sup>

Interesting findings triggered by investigations of seropositive donors included documentation of several presumed autochthonous cases. Of 11 reduviids found across the southern US, 4 major and 7 minor triatomine species maintain enzootic cycles of *T. cruzi*.<sup>55</sup> Although domestically transmitted cases had previously been documented,<sup>54</sup> they were rare and the number has now increased from 7 to 42.<sup>55,71</sup> Hence, unlike in other countries that have embraced selective testing strategies, risk in the US includes that from local transmission in addition to that from migrants. A study of nine million donations from 2007–2015 identified an overall confirmed-positive prevalence of 1:15,000 (up to 1:2700 in southern California), with only 5 of 585 radioimmunoprecipitation assay-positive donors classified as due to presumed autochthonous transmission.<sup>72</sup> The same report found zero incident cases from six million person-years of follow-up, which together with low estimates of transmissibility from seropositive donors<sup>73</sup> supports the updated guidance from FDA.

Aside from testing, additional options for risk reduction include donor exclusion, pathogen removal, and pathogen inactivation. Exclusion of donors on the basis of residence and travel history in endemic areas is not specific and is feasible only where these donors constitute a small portion of the donor pool. Leukoreduction filters have been shown to lower the concentration of trypanosomes in blood,<sup>74</sup> but at least one case of transfusion transmission in platelets was from a leukoreduced, and irradiated, product.<sup>62</sup> A variety of photochemical treatment methods in platelets,<sup>75</sup> plasma,<sup>76</sup> and red cell components<sup>77,78</sup> all show promise in reducing the parasite load by several logs.

## Malaria

Human malaria is caused by five species of protozoan parasites: *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae*, *Plasmodium ovale*, and *Plasmodium knowlesi*.<sup>79,80</sup> There are ≈229 million clinical cases annually and 400,000 malaria deaths, and it is especially severe among young children in sub-Saharan Africa.<sup>79</sup> Natural infection occurs through the bite of an infective female *Anopheles* mosquito. Parasites are less frequently transmitted through blood transfusion, organ transplant, parenteral exposure, or mother-to-child transmission.<sup>81</sup> The US recorded an average of three cases of transfusion-transmitted malaria (TTM) per year between 1963 and 1989.<sup>82</sup> More recently, there were 14 cases recorded during the 1990s and 11 from 2000 to 2017.<sup>83</sup> The current risk of 1 per 20 million donations compares favorably to historical estimates of 0–2 cases per million donations in nonendemic areas and 50 cases per million in endemic countries.<sup>84</sup>

In malaria-endemic settings, where frequent boosting by infective mosquito bites helps induce and maintain protective immune responses, severe infection is rare for those who survive early childhood.<sup>85</sup> In a nonimmune population, such as in the US, clinical malaria presents as a febrile illness with paroxysms, possibly at regular intervals, with accompanying flulike symptoms. Complications can include severe anemia, hepatic involvement, cerebral alterations, renal failure, and shock.<sup>86</sup> The case fatality rate for malaria is generally low (less than 1% in developed countries)<sup>87,88</sup> but can surpass 10% in recipients of blood products.<sup>82</sup>

Once endemic throughout much of its territory, malaria in the US is now almost exclusively due to imported infections. The most recent year with published surveillance data (2017) reflects a decade-long trend of increasing cases, with 2161.<sup>89</sup> More than 98% were found in US civilians who visited endemic areas and in foreign civilians from endemic countries. A review of TTM in the US from 1963 to 1999 indicates that many of these infections were preventable, with >60% occurring due to deviations from prevailing deferral guidelines.<sup>82</sup> *P. malariae* has a disproportionate effect, accounting for 65% of transfusion cases where donor exclusion guidelines were followed compared to representing only 4% of cases diagnosed in US travelers. This reflects the ability of *P. malariae* to remain at sub-clinical levels for lengthy periods, even up to 40 years.<sup>90</sup> Because malaria parasites from the four primary *Plasmodia* remain viable in blood stored at 4 °C for at least one week, there exists risk from these species,<sup>81</sup> and presumably from *P. knowlesi* as well. *P. falciparum* malaria has been transmitted in blood stored for 19 days.<sup>91</sup> Interval to onset in transfusion cases varies by species, with *P. falciparum* developing in about 10 days, *P. vivax* taking 16 days, and *P. malariae* taking 40 days.<sup>81</sup> Where a donor has been implicated, recent history in the US implicates semi-immune visitors from endemic areas. Over a 40-year period, only one US civilian without previous residence in malaria-endemic countries has been implicated in transfusion transmission, in contrast to 40 former residents of endemic countries.<sup>83</sup>

In the absence of an FDA-approved blood-screening assay, the prevention of TTM in the United States relies on exclusion of presenting donors with elevated risk for malaria infection. The criteria are oriented to determining risk on the basis of prior infection with malaria, prior residence in a malarial country, or recent travel to a malaria risk area. In 2013, the FDA issued new guidance.<sup>92</sup> Continuity of policy included deferral for

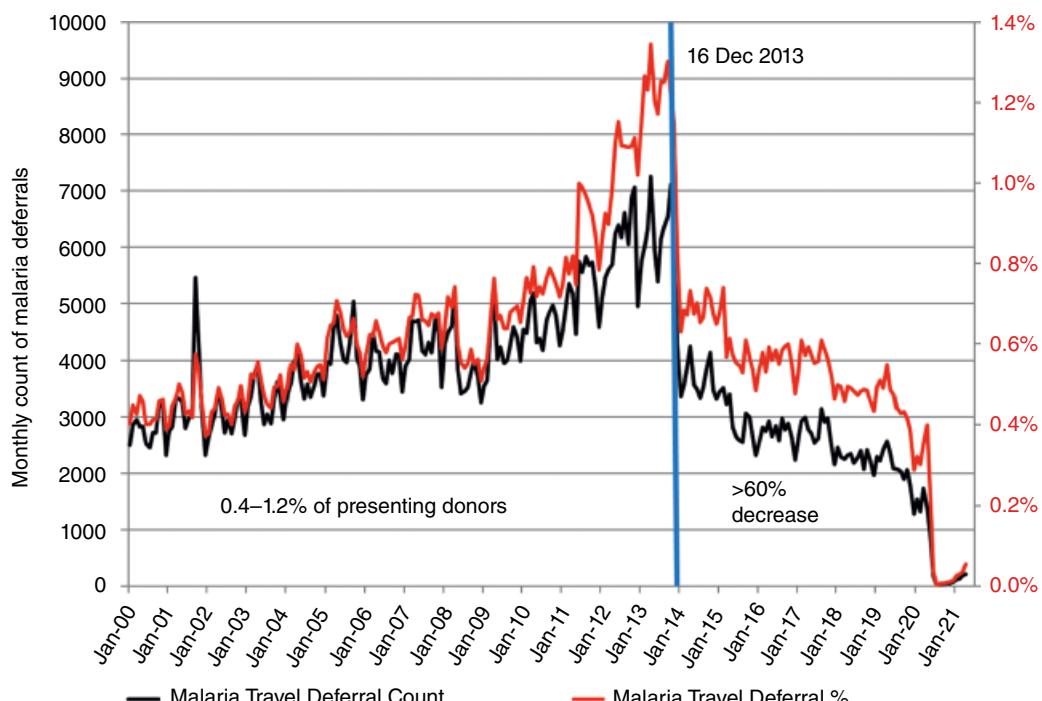
- 1** history of malaria within three years;
- 2** residence in a malaria-endemic country within three years; and
- 3** travel to a malaria-endemic area within one year.

Important clarifications or definitions included the following:

- 1** A *malaria-endemic* area was defined as any area with malaria where CDC recommends antimalarial chemoprophylaxis in travelers in *The Yellow Book*, whereas previously any nonzero risk was considered *endemic*.
- 2** *Travel to a malaria-endemic area* was now defined as travel of duration between 24 hours and 5 years, whereas previously even the briefest exposure—including land travel through a risk area—could trigger deferral.
- 3** *Residence in a malaria-endemic country* was now defined as a continuous stay of longer than five years in a country or countries having any malaria-endemic areas.
- 4** Donors who meet the definition of a *resident in a malaria-endemic country* will be deferred for three years following travel to a malaria-endemic country unless they have lived for three continuous years in a nonendemic country.

These first two changes followed published evidence showing a vanishingly small risk for infection for most donors receiving the one-year travel deferral, but a significant impact on availability equivalent to ≈1% of presenting donors.<sup>93,94</sup> The third change acknowledges the lengthy exposure typically required to acquire partial immunity that allows for asymptomatic carriage, whereas the final change reflects caution against the possibility that partial immunity may be boosted and sustained by return to endemic areas.<sup>85</sup> During the COVID-19 pandemic, an April 2020 update was published that reduced the deferral period to three months for residents of nonendemic countries who traveled to a malaria-endemic country.<sup>95</sup> Figure 45.1 shows the dramatic impact on malaria deferrals from the new guidance in 2013 following implementation at the American Red Cross, compounded further by the COVID-19-related changes in April 2020.

Advances in diagnostics have created opportunities for nonendemic countries to implement strategies that lessen the impact of malaria deferrals while minimizing risk of transfusion transmission. Council of Europe regulations endorse the use of validated serological tests to shorten the deferral period of donors with potential malaria risk who test negative.<sup>96</sup> Donors who lived for six months or more in a malaria-endemic area are acceptable as blood donors if they test antibody negative at least four months after their return from a malaria area. Likewise, donors with less than six months duration in an endemic area can shorten the deferral period from one year if they test antibody negative at least four months after their return.<sup>96</sup> The lengthy use of serological testing in England, France, and Australia indicates a strong safety profile resulting in TTM incidence of approximately 1 per 10–20 million RBC units transfused despite background rates of imported malaria at least fourfold greater than the US (25–40 per million residents, vs. 6 in US). With selective testing algorithms that have been refined over time, donor loss has been kept acceptably small.<sup>97,98</sup> Direct methods for detection of malaria infection include Giemsa- or Wright-stained blood films, although their use is not practical for mass screening in nonendemic areas. Assays detecting circulating parasite antigens such as histidine-rich protein 2 (HRP) and lactate dehydrogenase (LDH) offer sensitivity equivalent to microscopy for *P. falciparum* but are less sensitive for *P. vivax* below a parasite density of 200 parasites per microliter,<sup>86</sup> still insufficient to prevent transfusion transmission. Nucleic acid tests (NATs) have considerably higher sensitivity than microscopy and may be suitable in certain endemic areas,<sup>99</sup> but with an infectious dose as low as 1–10 parasites per unit of blood may not reduce the risk to zero. NAT testing technologies



Unpublished data. Vertical line shows date of implementation of 2013 deferral guidance.<sup>92</sup>

**Figure 45.1** Trends in malaria travel deferrals at American Red Cross, 2000–2021 (Q1).

successfully deployed for *Babesia* might prove suitable to *Plasmodium* as well.<sup>45</sup>

Pathogen reduction technologies have shown promise against *P. falciparum* in both in vitro studies<sup>100</sup> and in a field-based randomized trial in Ghana.<sup>101</sup> While phase 3 trials are in process, no pathogen-reduction platform for red cells or whole blood is licensed in the US.

### Leishmaniasis

*Leishmania* species are protozoan parasites with broad distribution worldwide. *Leishmania* are transmitted in nature by the bite of a female phlebotomine sandfly, but can be transmitted in blood components and, rarely, congenitally or sexually.<sup>102,103</sup> Clinical manifestations can vary widely, ranging from asymptomatic infection to severe illness with visceral, cutaneous, or mucosal involvement. The visceral form (kala-azar) is characterized by fever, wasting, hepatosplenomegaly, and pancytopenia, and if untreated is usually lethal.<sup>103</sup> Cutaneous forms involve progressive skin lesions that become ulcerative, sometimes with mucosal involvement. Although endemic in about 90 countries, the public health burden is hardly uniform. New visceral cases are concentrated in Brazil, South Asia, and East Africa, while the heaviest burden of cutaneous leishmaniasis (CL) is in the Americas, Mediterranean, Middle East, and Central Asia.<sup>104</sup> The World Health Organization estimates that 0.6–1.0 million new cutaneous and 50,000–90,000 visceral leishmaniasis (VL) infections occur annually and 12 million people worldwide are infected.<sup>104</sup> The United States is home to both phlebotomine sandflies and *Leishmania* parasites, but autochthonous transmission is limited to infrequent and isolated outbreaks.<sup>105,106</sup>

The infective form of *Leishmania* parasites in the sandfly is the flagellated promastigote; in humans and other vertebrates, the parasite takes the form of an oval amastigote found in phagocytic vacuoles of macrophages and other mononuclear phagocytes.<sup>103</sup> Diagnostic methods include microscopic visualization of amastigotes in tissue aspirates or biopsies from spleen, bone marrow, or lymph nodes, or in the peripheral blood buffy coat. The duration of parasite circulation in the blood can vary across the >20 *Leishmania* species, but rarely lasts more than one year.<sup>107</sup> The immunological response differs according to the clinical syndrome, with VL leading to a stronger and more enduring antibody production than CL.<sup>108</sup> The conventional treatment of leishmaniasis has been pentavalent antimony, but new strategies include lipid formulations of amphotericin B, injectable paromomycin, and miltefosine.<sup>108</sup>

The risk for transfusion transmission of *Leishmania* may be underreported, with only 14 probable or confirmed cases reported over 60 years.<sup>102,109</sup> About half of the cases were in infants and 80% in children six years old or younger.<sup>109</sup> The average incubation period was over seven months, with fever and hepatosplenomegaly being most common symptoms.<sup>109</sup> Studies from endemic areas and of US military personnel returning from southwest Asia highlight the potential risk. Viscerotropic *L. donovani* and *L. tropica* isolated from US servicemen were shown to survive 25 days in whole blood or PRBCs stored at 4 °C, five days in platelet concentrates at 24 °C, and 35 days in glycerolized RBCs frozen at -70 °C. Further, *L. tropica*-spiked whole blood stored at 4 °C for 30 days retained infectivity to healthy mice. Fresh-frozen plasma did not support parasite survival.<sup>110</sup>

Because transfusion transmission would likely be mistaken for sandfly transmission in endemic areas, and because infection may be subclinical and intermittent in immunocompetent individuals, assessing transfusion risk is difficult. Studies performed on blood donors across Southern Europe and Brazil indicate rates of detectable parasitemia reaching 10% or more,<sup>111</sup> whereas a recent review compiles several reports documenting asymptomatic infection in healthy populations.<sup>112</sup> Numbers of this magnitude suggest that the actual transfusion risk might be higher than indicated by published reports, which represent cases detected in largely immunosuppressed individuals. In contrast, one group in the Balearic Islands has found that despite active surveillance and high prevalence of infection in donors, they have yet to confirm transfusion transmission to patients.<sup>109</sup>

The options to blood banks to prevent distribution of *Leishmania*-contaminated blood are limited. Targeted donor exclusion was practiced selectively in the United States during military operations in Iraq, but the 12-month deferral has been retired. Donors in the US are now currently deferred indirectly via malaria deferral requirements for areas coendemic for malaria. Because serological status correlates poorly with asymptomatic infection,<sup>107</sup> antibody tests hold little promise for donor screening. A recent survey of 28 countries showed that no country employed laboratory testing to mitigate risk, while several employed risk factor questions relating to travel, birth, vector exposure or history of disease.<sup>113</sup> Photochemical inactivation of different *Leishmania* species has shown 4-log reduction of amastigote and promastigote forms in platelets,<sup>114</sup> and another study demonstrated 5-log to 6-log reductions in both platelets and plasma.<sup>115</sup> Another study utilizing riboflavin and ultraviolet light was only partially effective in whole blood, with a 2.3-log reduction in parasite load.<sup>116</sup> Furthermore, the filtration of leukocytes at both the point of collection and the bedside has been shown to dramatically lower free and intracellular *Leishmania* parasites.<sup>117</sup>

## Toxoplasmosis

*Toxoplasma gondii* is an obligate, intracellular protozoan that has felines as a definitive host but can grow in any mammalian or avian organs or tissues.<sup>118</sup> Felines become infected in nature by consuming intermediate rodent hosts with infective *Toxoplasma* cysts in the brain or skeletal muscle. The parasite is ubiquitous in nature and can infect a wide variety of animals, developing into cysts that remain infective for years. Human infection occurs through multiple modes of transmission. Most common are consumption of raw or undercooked meat containing *Toxoplasma* cysts, and accidental ingestion of *T. gondii* oocysts from soil or cat litter contaminated with excreted oocysts. Increasingly, water is implicated as a vehicle for transmission.<sup>118</sup> There are rare reports of infection acquired via solid organ transplantation and blood transfusion.<sup>118</sup> Finally, congenital transmission can occur when the mother acquires primary infection during pregnancy, but the probability of transmission to the fetus depends on the timing of infection.<sup>118</sup>

Up to one-third of the world's population is infected with *T. gondii*. About 90% of primary infections are subclinical, and they appear to be lifelong. Symptomatic infections are usually nonspecific and self-limiting, typically involving fever and isolated swollen lymph nodes. More rarely, infection can lead to myocarditis, pneumonitis, hepatitis, or encephalitis.<sup>118</sup> Toxoplasmosis is often severe

in immunocompromised hosts, and it is a common opportunistic infection of persons with AIDS. Estimates of seroprevalence across countries vary from 10% to 90%, and the acquisition of infection depends on local and household factors that include hygiene and sanitation, source and preparation of food, exposure to felines, and climatic factors influencing the survivability of oocysts in nature.<sup>118</sup> In the US, a representative study from 1999 to 2004 indicated an age-adjusted seroprevalence <10%, with foreign-born residents three times as likely as US-born residents to be antibody-positive, 24.8–8.2%.<sup>119</sup>

Transfusion transmission of toxoplasmosis was reported 50 years ago, in a case where patients with acute leukemia were transfused with leukocytes from donors with chronic myelogenous leukemia.<sup>120</sup> Despite the scarcity of reports of transfusion transmission, the risk is well documented in heart, liver, and kidney recipients.<sup>118</sup> Seroprevalence studies in healthy blood donors indicate antibody prevalence ranging from 7.4% in Durango, Mexico,<sup>121</sup> to 75% in northeastern Brazil.<sup>122</sup> Antibody presence is long-lived and does not necessarily denote infectivity. Little information is available, however, on the long-term kinetics of antibody development and patent parasitemia, and parasite isolation or detection by PCR is generally not useful in immunocompetent patients.<sup>123</sup> The ability of the parasite to survive 50 days at 4 °C<sup>120</sup> and isolated reports of transfusion transmission in the literature both establish an element of risk.

Prevention of transfusion-transmitted toxoplasmosis is not feasible with either donor exclusion or serologic screening.<sup>124</sup> In most places, discarding units from seropositive donors would heavily prejudice blood availability, with unclear indications that positive antibody status of the donor implies risk for parasitemia. In high-prevalence countries, many blood recipients are likely to have been previously exposed. In an immunocompetent recipient, transfusion transmission is likely to go undetected. Given the parasite's ability to readily invade and replicate in leukocytes,<sup>125</sup> leukocyte filtration might diminish the risk in similar fashion as with cytomegalovirus. Whether inactivation treatments being evaluated for other protozoa might be of use for *Toxoplasma* remains unknown.

## Microfilariasis

Filarial worms are arthropod-borne macroparasites that include several different organisms. *Wuchereria bancrofti* and *Brugia* spp. cause lymphatic filariasis; *Loa loa*, *Onchocerca volvulus*, and *Mansonella streptocerca* cause nonlymphatic, subcutaneous filariasis; and *Mansonella ozzardi* and *Mansonella perstans* cause nonlymphatic infections of different body cavities and are typically asymptomatic or mild.<sup>3</sup> The filariases occur in more than 72 countries, and the health and socioeconomic burdens from lymphatic filariasis and *O. volvulus* are particularly severe, with an estimated prevalence of 51 million and 21 million infections, respectively.<sup>126,127</sup> These organisms share similar life cycles and are all transmitted by hematophagous arthropods. In each, adult female worms produce larvae called *microfilaria*, which for most species circulate in the bloodstream, sometimes with periodicity timed to their primary vector's feeding habits. The microfilaria are the infective form for insects, but when transmitted by transfusion they are incapable of propagating further.<sup>128</sup>

The lymphatic forms of filariasis, which cause elephantiasis, have been targeted by the World Health Organization as potentially eradicable, primarily by eliminating the human reservoir of microfilaria that infect the mosquito vectors through repeated mass administration of curative drugs.<sup>126</sup> Estimates indicate a 75% decline in prevalence of human infections, including elimination from several countries and reversing the pathology associated with infection.<sup>127</sup>

There is little published information on the risk for transfusion transmission of filariasis. Limited studies from Nigeria showed prevalence of microfilaria of 3.5% with *Loa loa*,<sup>129</sup> 15.6% with *M. persans* and 1.3% with *L. loa*,<sup>130</sup> and 1.3% with unspecified microfilaria.<sup>131</sup> One report found an American blood donor with microfilaria.<sup>128</sup> Isolated case reports of transfusion transmission exist from Italy<sup>132</sup> and India,<sup>133</sup> in both cases indicating that the outcome in blood recipients might be no more severe than mild allergic reaction to microfilarial antigens. The microfilaria of both *W. bancrofti*<sup>134</sup> and *L. loa*<sup>135</sup> survive routine storage conditions for blood. Those from *M. ozzardi*<sup>136</sup> and from *B. malayi* and *W. bancrofti*<sup>137</sup> have been recovered following cryopreservation. Given the modest clinical consequences even with direct transfusion, most countries appear to consider the risk to blood recipients too small to merit donor or component screening.<sup>138</sup>

### Prion diseases

The term prion was originally coined by S.B. Prusiner to denote a small proteinaceous infectious particle, which is resistant to most procedures that inactivate nucleic acids.<sup>139</sup> Prions are the causative agent of transmissible spongiform encephalopathies (TSEs), a group of fatal neurodegenerative disorders characterized by a long incubation period, short clinical duration, and transmissibility to susceptible mammalian species. Prions can be transmitted through several routes of infection, which determine the length of the silent incubation period in an infected host. Usually, peripheral extra-neuronal exposures result in incubation phases that are longer than direct intracerebral routes. TSEs can cause infections of epidemic proportions in humans (kuru) and animals (bovine spongiform encephalopathy [BSE]) and be endemically present in domestic (scrapie) and wild animals (chronic wasting disease [CWD]).

Human TSEs have different etiologies: sporadic, genetic, and environmental (i.e., infectious). Sporadic Creutzfeldt–Jakob disease (sCJD) is the most common TSE, occurring with a frequency of one case per million people per year worldwide.<sup>140</sup> Genetic TSEs are inherited in an autosomal dominant fashion and are associated with more than 30 pathogenic mutations in the prion protein gene (PRNP);<sup>141</sup> they represent 10–15% of all TSEs and include familial CJD (fCJD), Gerstmann–Sträussler–Scheinker syndrome (GSS), and fatal familial insomnia (FFI).<sup>142,143</sup> The infectious forms include variant CJD (vCJD), resulting from dietary exposure to meat unknowingly sourced from BSE-infected cattle,<sup>144</sup> iatrogenic CJD (iCJD),<sup>145</sup> and kuru, an almost eradicated disease described in cannibalistic tribes of New Guinea.<sup>146,147</sup> iCJD has been linked to therapeutic treatments with human pituitary hormones (growth hormone and gonadotropin), dura mater, cornea or pericardial grafts obtained from CJD-afflicted individuals, and to neurosurgical procedures performed with ineffectively decontaminated instruments previously used on CJD-afflicted patients.<sup>145</sup> The most common TSEs in animals are scrapie in sheep and goat, BSE

(commonly called “mad cow disease”) in cattle, and CWD in cervids. BSE is the only animal form of TSE that has been causatively linked to a human disease, vCJD.<sup>144,148</sup>

TSEs are a clinically heterogeneous group of diseases, each presenting with a distinctive phenotype characterized by differences in the age of onset, clinical symptoms, brain pathology and disease duration, and by different patterns of PrP<sup>TSE</sup> deposition.<sup>140,143,147,149</sup> Polymorphisms in the host PRNP gene influence the phenotypic differences and individual susceptibility to certain forms of the disease<sup>150,151</sup> and partially explain the origin of biochemically distinct PrP<sup>TSE</sup> strains exhibiting a range of strain-specific phenotypes in infected humans<sup>152–154</sup> and animals.<sup>155</sup>

### Molecular mechanisms of prion conversion

These diseases share a common mechanism that involves a conformational change in the structure of the membrane-bound prion protein (PrP<sup>C</sup>) leading to self-replicating propagation and accumulation within the central nervous system (CNS) of a misfolded form, termed PrP<sup>TSE</sup>. Specifically, PrP<sup>TSE</sup> results from conformational changes in the secondary structure of the PrP<sup>C</sup> protein leading to an increase in the proportion of beta-pleated sheet from 3–4% to 40%, and a decrease in the proportion of alpha-helix from 40% to 30%, with the concomitant acquisition of new biochemical properties, namely, insolubility in nonionic detergents and resistance to proteinase K (PK) degradation.<sup>156</sup> This transition occurs under unknown circumstances, but once initiated, PrP<sup>TSE</sup> propagates in a self-catalytic way by imprinting its aberrant conformation onto new molecules of PrP<sup>C</sup> via a seed-mediated polymerization process.<sup>157</sup> Temporal and spatial deposition of PrP<sup>TSE</sup> aggregates, which are devoid of detectable informational nucleic acid,<sup>158</sup> coincide with a series of pathological events in the brain, resulting in spongiform degeneration, neuronal loss, and gliosis (proliferation of glial cells), which constitute the diagnostic markers of these diseases.<sup>159</sup>

### The nature, concentration, and distribution of infectivity in blood and tissues

PrP<sup>C</sup> is a highly conserved cell-membrane sialoglycoprotein that is expressed in all tissues in mammals. In blood, PrP<sup>C</sup> is primarily found in plasma, but it is also present in cellular components, specifically, white blood cells and platelets.<sup>160,161</sup> Our current understanding of the pathogenesis of TSEs is largely predicated on animal studies. Biochemical and biological assays link the accumulation of high concentrations of PrP<sup>TSE</sup> and infectivity in the CNS with the neurodegenerative changes observed in all TSEs. Animal models of peripherally transmitted prion diseases<sup>162,163</sup> point to follicular dendritic cells (FDCs)<sup>164,165</sup> in the germinal centers of lymphoid tissue as the key cell in the establishment of infection, and not B lymphocytes as previously suggested.<sup>166,167</sup> The presence of PrP<sup>TSE</sup> accumulation in the FDCs in tonsil, spleen, and lymph nodes of 89 vCJD-afflicted patients examined postmortem suggests a similar pathophysiology for CJD in humans following primary (e.g., cattle BSE to humans), or secondary, and higher order (human-to-human) transmission.<sup>168</sup> Although less frequently, PrP<sup>TSE</sup> and/or infectivity can also be found in non-neuronal tissues from sCJD patients, including the spleen, muscle,<sup>169</sup> and bone marrow.<sup>170</sup> A survey of 32,441 de-identified archival appendix tissue samples that

had been removed during operations performed in the United Kingdom (UK) between 2000 and 2012 revealed an estimated prevalence of PrP<sup>TSE</sup> accumulation of 1 per 2000. This finding was thought due to exposure of the community to beef contaminated with BSE prions and to indicate a vCJD carrier status in the general population.<sup>171</sup> Multiple studies showed that TSE infectivity and PrP<sup>TSE</sup> are present in the blood of experimental rodents, sheep with scrapie and experimental BSE, and deer affected with chronic wasting disease (CWD).<sup>172–180</sup> PrP<sup>TSE</sup> has been successfully detected in buffy coats and plasma of hamsters and sheep experimentally infected with scrapie,<sup>181–183</sup> as well as in plasma of mice with scrapie and white-tailed deer naturally and experimentally infected with CWD.<sup>184–187</sup>

The exact nature of TSE infectivity in peripheral blood and tissues is uncertain. Likewise, it is still not fully understood in what blood component infectivity and/or PrP<sup>TSE</sup> reside and whether and how blood contributes to the spread of the disease from the periphery to the brain. Recent work in this area, particularly relating to the physicochemical characteristics of the infectious prion in plasma, is of relevance, with one study providing evidence of the presence of PrP<sup>TSE</sup> in plasma-circulating extracellular vesicles (EVs) isolated from the blood of preclinical and clinically sick mice infected with mouse-adapted vCJD,<sup>188</sup> lending additional support to the concept that EVs contribute to the spread of misfolded proteins.<sup>189–194</sup>

The spatial distribution of infectivity also varies between models. Brown *et al.*<sup>195,196</sup> mimicked clinical blood separation processes and demonstrated a four- to fivefold higher concentration of infectivity in the buffy coat (per unit volume) compared to plasma. Gregori *et al.* studied purified blood components and concluded that red cells and platelets have very little infectivity, and that approximately 40% of infectivity is associated with leukocytes, with the remainder residing mostly within the plasma.<sup>197,198</sup> In contrast, studies in sheep<sup>174,199–201</sup> and in humans<sup>202</sup> suggest that infection can be transmitted by all blood components.

The temporal development of infectivity in peripheral tissues during the incubation period is similarly uncertain. In rodent models, a variety of patterns of change in infectivity and PrP<sup>TSE</sup> concentration have been observed.<sup>182,203</sup> In humans, the pattern of development of infectivity in peripheral blood and tissues during CJD infection is unknown, although abnormal PrP has been detected in an appendix sample removed two years before the development of clinical variant CJD.<sup>204</sup>

Overall, the current working assumption is that the level of infectivity in the peripheral blood of donors during the subclinical phase of variant CJD infection is likely to be in the order of 1 ID per unit of infected red cells prior to leukodepletion.<sup>205</sup>

## Transfusion Transmissibility of variant and sporadic CJD

Blood and plasma from sCJD and iCJD patients have been shown to harbor infectivity and cause disease following intracerebral inoculation into animals, including humanized transgenic mice.<sup>202,206,207</sup> Likewise, infectivity was demonstrated to be present in bone marrow isolated from sCJD patients.<sup>170</sup> PrP<sup>TSE</sup> can be detected in whole blood of patients with vCJD,<sup>208–210</sup> and similar levels of PrP<sup>TSE</sup> have been found by Western blot in spleen, tonsils, and lymph nodes of patients afflicted with vCJD and sCJD.<sup>211</sup> Based on the ubiquitous expression of PrP<sup>C</sup> and the accumulation of PrP<sup>TSE</sup> and infectivity in non-neuronal tissues, it was speculated that prion diseases could be

transmitted via blood transfusion. In the UK, vCJD was confirmed in four transfusion recipients of nonleukoreduced red blood cell concentrates prepared from 18 donors who subsequently developed vCJD.<sup>212–214</sup> A hemophilia patient, who died of unrelated causes five years after transfusion, showed PrP<sup>TSE</sup> deposits in lymphoid tissues but not in the brain, and probably represents a case of preclinical or subclinical infection.<sup>215</sup>

The data on peripheral blood infectivity in sCJD are open to interpretation,<sup>216</sup> although recent studies suggest that infectivity can be detected in the peripheral blood of patients with sCJD using transgenic mice overexpressing bovine PrP.<sup>202</sup> Even though experimental evidence indicates that blood from some sCJD patients harbors infectivity, epidemiological data from several case-control<sup>217,218</sup> and lookback studies<sup>219</sup> show no evidence of transfusion-transmission of sCJD. The American Red Cross, in collaboration with the US Centers for Disease Control and Prevention, is conducting a lookback study on the risk of transmission of the nonvariant forms of CJD through blood transfusion. The study has been active since 1995 and recently extended to 2024, with the most recent results published in 2017.<sup>220</sup> At the time of the last report there were 65 CJD donors who gave 1816 components to the blood supply. Eight-hundred and twenty-six traceable recipients were identified for the study. Of these, 27 had insufficient information to track using the National Death Index (NDI) search. Of 799 other recipients, a total of 154 were alive and 645 were deceased. Recipients contributed 3934 person-years of follow-up in the study and no transfusion-transmitted cases of CJD have been recognized.<sup>220</sup> The Transfusion Medicine Epidemiology Review (TMER) study from the UK<sup>219</sup> similarly demonstrates a lack of evidence for transfusion transmission of CJD. Together, these two studies represent more than 5100 person-years of follow-up of patients exposed to blood from donors who subsequently manifested CJD. To date, neither study has found any recipient who has died of CJD. A recent study utilizing the SCANDAT<sup>221</sup> database in Denmark/Sweden added an additional 7617 person-years of follow-up and evaluated potential clusters of sCJD cases among recipients of blood components that could be attributed to a single donor, even if that donor was never diagnosed with sCJD, of importance since TSEs continue to be underdiagnosed and underreported. No aggregation of sCJD cases among blood recipients was found that could be attributed to an individual donor. The results of these three studies strongly demonstrate a lack of evidence for transfusion transmission of sCJD.

## Regulatory measures to reduce the risk of transfusion transmission of sCJD and vCJD

There is currently no donor screening method that can identify individuals who will later develop TSEs. The data summarized above indicate that of all TSEs, vCJD poses the highest risk to the blood supply. In most cases, the likelihood of vCJD in a country that has no BSE in cattle depends upon the extent to which people were either exposed outside the country or consumed BSE-contaminated imported beef products.<sup>222</sup> Therefore, it is essential that measures implemented to reduce TTvCJD be proportionate to the risk.

In Europe, the region with the largest number of vCJD cases, accounting for 220 of the 229 cases worldwide, the current directive as regards technical requirements for blood and blood components recommends that persons who have a family history which places

them at risk of developing a TSE, or persons who have received a corneal or dura mater graft, or who have been treated in the past with medicines made from human pituitary glands be permanently deferred from donating blood. For vCJD, further precautionary measures are recommended.<sup>223</sup> To consider appropriate measures authorities should seek two kinds of information regarding the risk of vCJD:

- Prevalence of BSE infection in native and imported cattle populations and potential BSE agent contamination of products.
- Potential human exposures to the BSE agent.

With respect to plasma-derived medicinal products, recommendations to minimize the risk of transmission are based on the country of residence. As such, residence in the UK is a recognized risk factor for vCJD. Consequently, donors who have spent a cumulative period of one year or more in the UK between 1980 and 1996 are excluded from donating blood/plasma for fractionation. No requirement for leukoreduction of plasma is recommended since animal models fail to provide clear evidence that leukoreduction of plasma significantly reduces the risk of TSE transmission. Moreover, available data indicate that the manufacturing processes for plasma-derived medicinal products would reduce TSE infectivity if present in human plasma.

In the US, the first recommendations to reduce the possible risk of transfusion transmission of sCJD and vCJD by blood and blood products issued by the Food and Drug Administration (FDA) date back to 1987. In its first memorandum, the FDA recommended the deferral of individuals who received human cadaveric pituitary growth hormone injections. Following the publication of the first guidance in 1999, the Transmissible Spongiform Encephalopathy Advisory Committee held several meetings to review the newly published scientific evidence and the risk assessment of donor deferrals and TTvCJD and issue new recommendations, including the following:

- Donor deferral for receipt of human growth hormone (hGH), dura mater transplant, or injection of bovine insulin, the latter since 1980, and the potential risk of iCJD development.
- Donor deferral for having a blood relative with CJD and the potential risk of fCJD development.
- Donor deferral for geographic risk of BSE exposure, including deferral for potential exposure to UK-sourced beef on US military bases, and the associated risk of vCJD development.
- The unproven value of leukoreduction based on experimental data showing that a substantial portion of blood-borne infectivity remained in plasma following filtration.

In April 2020, the FDA released final guidance<sup>224</sup> that takes into consideration the most recent experimental and epidemiological findings highlighted above and data collected using FDA risk assessment models. The main revisions involved changes to deferral recommendations for geographical risk of exposure to BSE prions, including potential exposure to UK-sourced beef on US military bases. Under the new guidance, indefinite deferral is restricted to US donors who spent extended periods of time in the UK, Ireland, and France. Risk-ranking models indicated that this change in deferral combined with voluntary leukoreduction would reduce the TTvCJD risk by 89.3% while allowing blood donations by approximately 100,000 currently deferred donors.<sup>225</sup> Given the absence of reported cases of vCJD among 4.4 million military personnel and civilians who might have ingested UK-sourced beef on US military bases in Europe, the FDA no longer recommends deferral for time spent in US military bases in

Europe. The new guidance also recommends the removal of hGH from the medication deferral list following the determination that the prevalence of patients who may have been treated with hGH extracted from cadaveric pituitary glands among blood donors is very low and the transmission risk is theoretical. Similarly, due to the absence of vCJD cases in recipients of bovine insulin, the FDA allows the removal of this drug from the medication deferral list. Likewise, under the new guidance donors who have a blood relative with CJD are no longer deferred since most cases reported are sCJD and not familial TSEs, and blood relatives of individuals with sCJD are not at increased risk of developing the disease.

A comparison between the numbers of CJD-related deferred blood donors at the American Red Cross a year prior and a year after implementation of the new FDA guidance showed a 47% reduction in donor loss or 3000 more donor presentations. This number represents 3% of the FDA-projected reduction in donor deferral or 7.5% assuming that the American Red Cross collections represent approximately 40% of the US blood supply.

## Summary

The current risk for transfusion transmission of parasites in the United States is not high, but it may be greater than the prevailing estimate of one per million units. Risk for transfusion transmission of *Babesia* was almost certainly higher than one per million preceding regulatory intervention, but year-round NAT testing in areas of highest endemicity curtails the risk considerably. The mobility of both people and blood products implies nonzero risk under a selective testing paradigm. Licensure of *T. cruzi* screening assays has brought the incident risk to near-zero. Silent infection in semi-immune donors constitutes a small but ineradicable risk for malaria transmission in blood products in the absence of serological and nucleic acid screening.

Other parasites with wide global distribution, including *Leishmania* parasites, *Toxoplasma gondii*, and microfilaria, are of scientific interest and theoretical transfusion risk. Concern is insufficient by the general public or competent authorities to mandate specific interventions given the perceived level of risk and attendant benefit in relation to cost.

For prions, compelling epidemiological data from three national studies strongly indicate that the transfusion transmissibility of sCJD remains theoretical. If sCJD is transmissible via blood, it is undoubtedly less infectious than vCJD. Altogether, the data summarized above highlight the relevance of lookback studies to inform policy changes and the importance of continuing surveillance of human prion diseases to ensure the safety of the blood supply.

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- A full reference list for this chapter is available at: <http://www.wiley.com/go/simon/Ross16>
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## CHAPTER 46

# Bacterial contamination of blood components

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## Introduction

Bacterial contamination of blood components is a pervasive but often neglected risk of blood transfusion. Historically, public attention has focused disproportionately on transfusion-transmitted viral infections. With improved donor selection (e.g., risk-based deferral) and advances in laboratory screening, the risk of the major transfusion transmitted viruses (e.g., HIV, Hepatitis B [HBV], and C viruses [HCV]) has diminished, at least in the United States (US) and other high-income countries (HICs).<sup>1</sup> Given the reduction of viral transmission via allogeneic blood, the risk of bacterial contamination has emerged as a leading threat to blood transfusion safety. Adoption of routine measures, such as standardized skin preparation, use of diversion pouches, and bacterial culture of platelets in HICs, has interdicted most cases of bacterial contamination of blood products (notably platelets) and associated septic transfusion reactions. Nonetheless, residual risk remains, particularly for platelets. The latter account for the majority of cases of septic transfusion reactions given their associated storage conditions. Recognition of the potential risk posed by bacterial contamination has led to the development and adoption of novel strategies to contend with this risk. This chapter provides an overview of bacterial contamination of blood components with a focus on approaches to minimize or eliminate that risk.

## Bacterial contamination of platelets

### Epidemiology

Sepsis resulting from transfusion of bacterially contaminated platelets is the most common transfusion-transmitted disease. Platelets are stored at room temperature (20–24 °C), making them an excellent growth medium for bacteria. Historically, aerobic culture surveillance studies have demonstrated that 1 in 1000–3000 platelet units were bacterially contaminated.<sup>1</sup> The observed rates of septic transfusion were much lower at approximately 1 in 25,000 platelet units (range 1 in 13,000–100,000).<sup>1</sup>

The reported incidence of contamination widely varies by location, collection type (e.g., apheresis vs. whole blood derived), and the extent to which mitigation measures have been implemented. For example, the Australian Red Cross reported a rate of confirmed culture positive

and indeterminate results of 0.22% for pooled platelets and 0.11% for apheresis units, respectively; by contrast, Canadian Blood Services documented a frequency of 0.09% and 0.04% confirmed-positive results for platelet pools and apheresis units, respectively.<sup>2,3</sup>

The French National Hemovigilance Program utilizing active surveillance in the period 2000–2008 reported rates of sepsis and fatality of ~1:40,000 (24.7 per million) and ~1:200,000 (5.14 per million), respectively.<sup>4</sup> Following the introduction of measures to limit and detect contamination in 2004 in the US, the American Red Cross described an ~70% decline in sepsis reported to their passive surveillance program, with current rates of 1:107,000 for sepsis and 1:1,016,000 for fatalities.<sup>5</sup> From 2014 to 2018, 22 fatalities caused by blood components contaminated with bacteria were reported to the FDA.<sup>6</sup> From 2010 to 2016, the US National Healthcare Safety Network (NHSN) Hemovigilance Module reported rates of septic reactions of 2.5/100,000 and 0.9/100,000 for apheresis and whole-blood-derived platelets, respectively, based on data collected from 195 facilities.<sup>7</sup> In Canada, the Transfusion Transmitted Injuries Surveillance System Programme Report documented 31 transfusion reactions attributed to bacterially contaminated blood components during the period 2014–2018.<sup>8</sup> Since the implementation of platelet screening for bacterial contamination in 2011, the United Kingdom's Serious Hazards of Transfusion (SHOT) program has documented only one confirmed septic transfusion reaction involving contaminated platelets.<sup>9</sup> Nonetheless, even with culture screening in place, Jacobs *et al.* estimated that at least 550 contaminated units are still transfused in the US each year, based on their bacterial testing on the day of transfusion.<sup>10</sup>

### The challenges surrounding case ascertainment and definitions

The true extent of bacterial contamination of blood products is somewhat uncertain. In large part, this is attributed to definition, whereby disparate risks are compared despite being inherently different.<sup>11</sup> Indices that are frequently reported in the literature include the (1) incidence of positive bacterial culture, (2) transfusion of a blood product that was later found to be culture positive, and (3) septic transfusion reactions and (4) deaths attributed to a contaminated blood product. The risk of each of these entities is enormously

variable, is impacted by the environment in which collection and testing is undertaken, the safeguards in place to mitigate that risk (e.g., arm cleaning and diversion pouches), the blood product type (e.g., RBC vs. platelets), the presence of comorbid factors that may mask (e.g., antibiotics) or confound clinical presentation, and the rigor of follow-up investigation to attribute cause. Collectively, these factors impact the imputability of the findings. An attempt to capture the strength of ascertainment is reflected in contemporary hemovigilance modules such as those published by the US Centers for Disease Control and Prevention (CDC), in which imputability is graded from “definite” to “not determined.”<sup>11,12</sup> While classification of risk is improving and estimates are becoming more specific, periodic changes to classification schemas complicate the epidemiology.<sup>11</sup> Bacterial contamination of blood products may occur anytime from collection to transfusion. Independent of the mode or timing of contamination, as well as the identity of the organism, the presence of detectable bacteria should be viewed with extreme caution. One transfusion of a large inoculum of bacteria into a patient poses high risk for severe sequelae. This is particularly the case with platelet transfusion recipients, given overrepresentation of immunosuppressed and critically ill patients in this population. Even bacteria that are typically perceived to be commensal organisms of low virulence have the ability to seed lines and valves.

### Mode of contamination

Platelets pose the major risk of transfusion associated sepsis. The reasons for this include storage at room temperature (20–24 °C) in oxygen permeable bags with agitation for 5–7 days, thus offering excellent growth conditions for many aerobic and facultative bacterial species. Bacteria most commonly enter the collected blood at the time of phlebotomy, whereby the contamination of the needle with skin commensals and contaminants occurs during venipuncture (Table 46.1). More rarely, contamination occurs through blood collection from a donor with asymptomatic donor bacteremia (e.g., with oral or enteric commensals). Investigation of the donors involved in the donation of contaminated components rarely identifies the origin skin commensal organisms such as *Staphylococcus epidermidis* and the aerotolerant anaerobic organism *Cutibacterium acnes*, which are the organisms most often detected as contaminants using culture methods.<sup>13,14</sup> Prior to the introduction of routine culture screening processes, septic fatalities caused by platelet contamination were predominantly caused by enteric Gram-negative organisms.<sup>15</sup> In blood centers where platelet screening is performed using culture systems, the majority of septic transfusion reactions are caused by Gram-positive bacteria.<sup>2,3,7,16,17</sup> Platelet culture is effective in detecting fast growing Gram-negative species; however, Gram-positive bacteria are slower growers and are therefore more likely to be missed.

### External contamination

Very rarely, cases of contamination of the collection bag, tubing, or anticoagulant have occurred due to improper sterilization during manufacturing or contamination of products after collection due to failure of the closed storage system (e.g., defects in the bags or tubing); these have involved environmental contaminants such as *Serratia* spp. and *Acinetobacter* spp.<sup>18,19,21–23</sup> Reports of transfusion reactions involving platelets contaminated with *Acinetobacter calcoaceticus-baumannii* highlight the potential harm associated with external contamination of platelet units. Investigation of two septic transfusion cases involving pathogen-reduced apheresis platelet units contaminated with *Acinetobacter calcoaceticus-baumannii*

**Table 46.1** Bacterial Species Isolated from Platelet Concentrates<sup>2,3,7,16,18–20</sup>

Species	Potential Origin
<b>Gram positive</b>	
<i>Cutibacterium acnes</i>	Skin flora
<i>Propionibacterium</i> spp.	Skin flora
Coagulase negative staphylococci	Skin flora
<i>Staphylococcus aureus</i>	Skin and upper respiratory mucosa
Diphtheroid bacilli	Pharynx, mouth, and skin
<i>Streptococcus pyogenes</i>	Upper respiratory tract, mouth, skin, and GI tract
<i>Streptococcus bovis</i>	Pharynx, skin, GI tract, and female genital tract
<i>Streptococcus agalactiae</i>	GI tract and female genital tract
<i>Streptococcus dysgalactiae</i>	Pharynx, skin, GI tract, and female genital tract
<i>Streptococcus gallolyticus</i>	Alimentary tract of ruminants
<i>Streptococcus viridans</i>	Upper respiratory tract and oral flora
<i>Streptococcus mitis</i>	Upper respiratory tract and oral flora
<i>Gemella</i> spp.	Upper respiratory tract, oral flora, and GI tract
<i>Bacillus cereus</i>	Environment
<i>Clostridium perfringens</i>	GI tract
<i>Lactobacillus</i> spp.	GI tract
<i>Enterococcus faecium</i>	GI tract
<i>Listeria monocytogenes</i>	GI tract
<b>Gram negative</b>	
<i>Serratia marcescens</i>	GI tract
<i>Escherichia coli</i>	GI tract
<i>Morganella morganii</i>	GI tract
<i>Klebsiella pneumoniae</i>	GI tract
<i>Klebsiella oxytoca</i>	GI tract
<i>Citrobacter freundii</i>	GI tract
<i>Salmonella</i> spp.	GI tract
<i>Pseudomonas aeruginosa</i>	Environment
<i>Acinetobacter calcoaceticus-baumannii</i>	Environment

concluded that the contamination occurred after pathogen reduction treatment due to damage of the platelet containers.<sup>18,19</sup> In the US, *Acinetobacter calcoaceticus-baumannii* has been implicated in multiple septic transfusion reaction cases suggesting a common source.<sup>21</sup>

### Adherence to plastics

Common bacterial contaminants of platelets such as *Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Serratia* spp. are able to adhere to platelets and to the inner surfaces of platelet containers where they form surface-attached bacterial aggregates (i.e., biofilms) during platelet storage; this results in reduced numbers of bacterial cells in suspension contributing to missed detection during platelet screening.<sup>17</sup> Biofilm-forming *Staphylococcus aureus* and *Staphylococcus epidermidis* have both been implicated in severe septic transfusion reactions.<sup>24,25</sup> Transfusion of biofilm-forming bacteria compounds risk to platelet recipients given their propensity to colonize biomedical devices, coupled with their resistance to antibiotic treatments and clearance by the immune system.<sup>17,26</sup>

### Final guidance to address risk of bacterial contamination of platelets in the United States

In recognition of the residual risk of bacterial contamination of platelets, in 2016 the FDA issued the first of a series of guidance documents offering a variety of approaches to contend with that risk.<sup>27</sup> The guidance document was ultimately finalized in December 2020, recommending implementation occur by October 2021.<sup>28</sup> Broadly, the mitigation strategies are categorized as

single- or two-step processes. Single-step approaches are confined to large volume delayed sampling and pathogen reduction. Under certain conditions, it may be permissible with certain approaches to extend the shelf life of platelets from five to seven days. The latter is very attractive to transfusing facilities as it minimizes the risk of out-dating and associated wastage. The conditions under which extension of shelf life becomes permissible include delayed timing of sampling of the platelet product for culture, increase in the sample volume (i.e.,  $\geq 8$  mL vs.  $\geq 16$  mL), the use of both aerobic and anaerobic bottles for culture (i.e., rather than aerobic bottles alone), and point of release testing. Pathogen-reduced platelets with seven-day storage are not yet FDA approved in the US but are widely used in Europe. Each approach has both advantages and disadvantages; institutions are best positioned to assess their individualized needs, balancing cost, logistical ease, efficiency, and integration into extant workflow.<sup>27</sup> All of the available approaches are nonetheless effective at addressing the residual risk of bacterial contamination of platelets.

### Strategies to reduce the risk of post-transfusion sepsis (Table 46.2)

Approaches to reduce the risk of post-transfusion sepsis can be categorized as follows: (a) reduction of bacterial contamination, (b) preventing exposure to high concentrations of bacteria, (c) improvement in bacterial detection, and (d) elimination of contaminating bacteria.

#### Reduction of bacterial contamination

##### Donor screening

Donors are routinely asked whether they feel well on the day of donation. Their vital signs are also tested (including temperature). Should the donor report a history of a recent infection, any antibiotic regimen must have been completed for donation to be permitted. The phlebotomy site is inspected to ensure that it is clean and without signs of inflammation or infection. Surgical wounds must be healed and dry. Donor screening also includes asking questions regarding general health, recent dental work, and signs of infection. Unfortunately, asking donors about symptoms suggestive of infections offers limited utility, given a lack of specificity for pathology that poses transfusion-associated risk.

##### Skin disinfection

This is an important step since most bacteria that are found in contaminated blood components are either resident or transient skin

flora; this underscores the need for optimal donor skin disinfection. Yet despite excellent technique, absolute sterility during venipuncture is not feasible given that organisms, which are harbored in sebaceous glands and hair follicles cannot be completely removed or killed and skin fragments, which are drawn up into the collection bag during the initial phase of donation, can also provide a source of infectious organisms.<sup>29,30</sup> Scarring or dimpling of the venipuncture site from prior donation has also been recognized as a risk factor for bacterial contamination because these areas frequently are difficult to disinfect.<sup>31</sup>

The effectiveness of skin preparation depends on the type and concentration of the disinfectant solution utilized, the number (generally one or two) and mode (e.g., scrub and swab) of applications, the dwell time during which the skin is exposed to the disinfectant, and the skill of the operator.<sup>32,33</sup> Blood centers have standard procedures that enforce optimal skin disinfection procedures. The AABB Technical Manual recommends a two-stage method involving disinfection of the venipuncture area with a 0.75% povidone-iodine scrub or a 10% povidone-iodine swab stick for a minimum of 30 seconds followed by the application of a 10% povidone-iodine preparation solution, which should stand for at least 30 seconds.<sup>34</sup> The skin of donors who are allergic to iodine is often cleansed with a chlorhexidine or isopropyl alcohol solution.<sup>35</sup> A one-step swab disinfection method (2% chlorhexidine and 70% isopropyl alcohol) is currently used in the US, the UK, Australia, China, and Canada, which has been shown to be as effective as two-step donor skin disinfection methods.<sup>36-40</sup>

##### First aliquot diversion

Diversion of the first 30–40 milliliters of whole blood during blood collection has been shown to reduce the amount of bacterial contamination from the skin, presumably by capturing skin fragments or a shower of bacteria released at the time of phlebotomy. Bruneau *et al.* collected the first and second 15 mL aliquots of 3385 whole blood collections and cultured these under aerobic and anaerobic conditions.<sup>41</sup> Seventy-three of them were positive in the first 15 mL and 21 in the second, including four species not detected in the initial 15 mL. The overall contamination rate was 2.2%, mainly with Gram-positive *Staphylococcus* spp. and *Bacillus* spp. The residual risk of contamination in the collection was 0.6%, demonstrating that diversion could significantly reduce the overall risk. A study from the Netherlands compared the bacterial contamination rates of whole blood collections with and without the removal of the first 10 mL. The diversion of the first 10 mL resulted in a significant decrease in bacterial contamination (18,263 collections with 0.39% contamination without diversion compared with 7115 collections with 0.21% contamination with diversion,  $p < 0.05$ ).<sup>42</sup> Diversion is most effective at interdicting contamination with skin flora. Similarly, investigators at the American Red Cross reported data comparing the rates of confirmed-positive bacterial cultures during one-arm collections, which incorporated inlet-line diversion, and two-arm collections, which did not, on the same apheresis equipment.<sup>14</sup> There was a 2.2-fold higher rate of skin contaminants with the two-arm procedure compared with the one-arm procedure and this difference was apparent only for skin contaminants. Of note, the majority of bacteria-related fatalities are associated with Gram-negative organisms; the latter are not impacted by diversion, i.e., diversion is intended to interdict skin flora that are introduced at time of collection.

**Table 46.2** Mitigation Strategies to Reduce the Incidence of Septic Transfusion Reactions

Time of Application	
<b>(a) Reduction of bacterial contamination</b>	
Donor questionnaire	Prior to blood collection
Donor skin disinfection	Prior to blood collection
Initial aliquot diversion	During blood collection
<b>(b) Preventing exposure to high concentrations of bacteria</b>	
Reduced PC shelf life	Transfuse early in the platelet shelf life
Point of issue testing	Within 24 hours of transfusion
Cold stored platelets	During platelet storage
<b>(c) Improvement in bacterial detection</b>	
PC screening with culture methods	Early in the platelet shelf life
PC screening with rapid testing methods	Late in the platelet shelf life, close to transfusion
<b>(d) Elimination of contaminating bacteria</b>	
Pathogen reduction methods	Early in the platelet shelf life

## Apheresis versus whole-blood-derived platelet concentrates

Therapeutic doses of platelets can be obtained from a single donor using apheresis technology or from whole blood donations. In the case of whole-blood-derived platelets, four to six platelets concentrate from whole blood donations (i.e., representing multiple donors) are pooled to make a therapeutic dose. Apheresis platelet units (i.e., single donor platelets) have been associated with lower rates of bacterial contamination as compared to pooled platelet concentrates due to reduced donor exposure resulting in lower frequency of bacterial contamination per transfusion episode.<sup>2,3,43,44</sup> This is not uniformly the case: Schrezenmeier *et al.* reported rates of bacterial contamination that did not differ significantly between apheresis and pooled buffy-coat platelet concentrates.<sup>45</sup> Interestingly, a comparison of the incidence of bacterial contamination in apheresis platelets collected in different devices showed that the rate of contamination and septic reactions was significantly higher with apheresis units collected with Amicus than with Trima indicating that apheresis collection technology affects the incidence of bacterial contamination in platelet components.<sup>46</sup>

## Bacterial detection

Bacterial contamination of platelet products is typically attributable to initially very low numbers of bacteria, some of which proliferate to high levels during storage. In vitro inoculation experiments confirm that the growth dynamics differ by bacterial species. In one study, bacterial growth characteristics were reported for 165 platelet units, each inoculated on the day of collection with 1–5 colony-forming units (CFU)/mL of one of the following organisms: *B. cereus*, *P. aeruginosa*, *K. pneumoniae*, *S. marcescens*, *S. aureus*, and *S. epidermidis*.<sup>47</sup> By Day 3 following inoculation, all the units that were contaminated with *B. cereus*, *P. aeruginosa*, *K. pneumoniae*, *S. marcescens*, or *S. aureus* had concentrations  $>10^2$  CFU/mL. By Day 4, all units with these organisms contained  $>10^5$  CFU/mL. Units contaminated with *S. epidermidis* exhibited slower and more varied growth. This study concluded that an assay capable of detecting  $10^2$  CFU/mL on Day 3 of storage would detect the vast majority of bacterially contaminated platelet units.

Based on these and similar findings from other studies, sensitive culture techniques that can detect 1–10 CFU/mL are used soon after platelet manufacture, while rapid, less sensitive detection methods are used closer to the time of transfusion. Examples of FDA approved screening systems with high sensitivity and may be used soon after manufacture includes the BACT/ALERT™ 3D and Virtuo Systems (BioMerieux Inc, Durham, NC) and the BACTEC™ System (BD Medical Device Company, Franklin Lakes, NJ). These can theoretically detect as few as a single viable bacterium in a 4–10 mL sample. In practice, they are validated to detect 1–10 CFU/mL of a range of transfusion relevant bacterial strains. In contrast, there are a number of detection systems that detect bacteria directly, such as the FDA-approved PGD™ assay (Verax Biomedical, Worcester, MA) and BacTx® Bacterial Detection Kit (Immunetics Inc.). There are other tests in use or development, including NAT assays, which typically have a lower analytical sensitivity than culture methods ( $\sim 10^2$ – $10^5$  CFU/mL), but may be performed rapidly (1–4 hours) and so are suitable for use close to the time of issue when bacterial concentrations are likely to be higher.

## Bacterial culture

The BACT/ALERT™ 3D and Virtuo systems use an automated liquid culture system that includes culture broth under aerobic or

anaerobic conditions. Each bottle contains a sensor that changes color as a consequence of increasing CO<sub>2</sub> produced by bacterial proliferation. The system monitors both the absolute color change and rate of change of the colorimetric sensor. The method reliably detects contamination of platelets with inocula of 10 CFU/mL and in many cases  $<5$  CFU/mL (e.g., *B. cereus*, *S. marcescens*, *C. perfringens*, *S. epidermidis*, *S. pyogenes*, *E. coli*, *K. pneumoniae*, *S. aureus*, and *viridans streptococci*) in 12–26 hours.<sup>48</sup>

There are several factors that may affect The BACT/ALERT system culture sensitivity including (a) the delay between collection and sampling, (b) the volume of product inoculated into the culture bottle, and (c) the evaluation of both aerobic and anaerobic culture conditions versus aerobic conditions alone.<sup>49</sup> Wagner and Robinette<sup>50</sup> used a model system of platelets contaminated in vitro with *S. epidermidis* or *E. coli* at 10, 1, or 0.1 CFU/mL and then sampled 0, 6, 24, or 48 hours later; 0.5, 1, or 2 mL were inoculated and incubated in 12 BACT/ALERT™ aerobic bottles for each data point. Their results clearly showed that higher initial concentrations, larger sample volumes, and longer delay before sampling were associated with a greater proportion of inoculated bottles signaling positive at any given time point. Their work was subsequently replicated for a wider range of organisms.<sup>48</sup> Various laboratories around the world have implemented the test in different ways (Table 46.3). False-negative screening BACT/ALERT results are either detected following the investigation of transfusion reactions or while performing quality control testing of expired platelet PC components.<sup>6</sup> False-negative results are associated with a low initial bacterial concentration and slow-growing or biofilm-forming organisms.<sup>3,16,51</sup>

The second culture system is the BACTEC automated culture system, which detects production of CO<sub>2</sub> by growing bacteria, which reacts with a fluorescent dye sensor at the bottom of the culture bottles.<sup>73</sup> Spiking studies have demonstrated the ability to detect Gram-positive organisms, including *B. cereus*, *S. aureus*, *S. epidermidis*, *S. agalactiae*, and Gram-negative species—including *E. cloacae*, *E. coli*, *K. oxytoca*, *Serratia marcescens*, and *P. aeruginosa*—at concentrations ranging from  $<1$  to 335 CFU/mL.<sup>74</sup> The system is routinely used in some European countries.<sup>1</sup> It has also been used in a study aimed at evaluating the incidence of bacterially contaminated PCs prior transfusion in a Ugandan Cancer Centre.<sup>75,76</sup> A study performed by Riedel *et al.* showed faster bacterial detection by the BACTEC system compared to the BacT/ALERT system.<sup>77</sup>

## Secondary culture

Primary bacterial culture of platelets has been in use in the United States and other high-income countries for over 15 years. It has been very effective at reducing the risk of contamination and associated septic transfusion reactions. But as described, risk has not been eliminated. Two root causes for cases of contamination escaping detection are the low inoculum at the time of sampling and insufficient time to allow growth, particularly for slow growing organisms. To contend with the residual risk of bacterial contamination, one approach that has been adopted at selected hospitals is secondary culture, whereby the unit of platelets is sampled at time of receipt at the transfusing institution.<sup>52</sup> This optimizes the probability of detection should there be contamination given the added 2–3-day interval between the donation and secondary sampling. In one institution, the investigators observed no cases of septic transfusion reactions in the three years following implementation.<sup>53</sup> By contrast, in the 13-month period prior to the implementation of secondary culture, there were a total of seven culture positive

**Table 46.3** International Approaches to Assuring Bacterial Safety of Platelet Products Utilizing Bacterial Culture (BACT/ALERT™) or Pathogen Reduction (INTERCEPT™) Technologies

Country	Storage Duration (days)	Bacterial culture					Ref.
		Pathogen Reduction	Aerobic (A)/Anaerobic (An)	Volume (mL)	Delay (hours)		
US	5	X	A/An	8–16	24–36	63	
Canada	7	–	A/An	16–20	36–48	17,56,64	
UK	7	–	A/An	16–20	≥36	9	
Australia	5	–	A/An	15–20	24	2	
Holland	7	–	A/An	15	16–22	65	
Hong Kong	5	X	A	10	–	66	
Switzerland	7	X*	–	–	–	67	
Germany	4	–	–	–	–	68	
France	7	X*	–	–	–	69	
Belgium	5	X*	–	–	–	–	
Japan	<3.5	–	–	–	–	–	66

\* Universal PR is mandated.

transfusion reactions, including one life-threatening event and one fatality.<sup>52</sup> Secondary culture has been included in the final FDA guidance document pertaining to bacterial control.<sup>28</sup> Secondary culture can be integrated into routine workflow using microbiology instrumentation that is commonly available in hospitals. The approach also allows for seven-day expiration if sampling is performed on Day 4 and both aerobic and anaerobic cultures are inoculated. The costs of secondary culture are also lower than those associated with other approaches that have been proposed to contend with residual risk.<sup>54,55</sup> Nonetheless, secondary bacterial culture has not been adopted widely. In part, this may be ascribed to the burden being placed on the transfusing facility where most might prefer the simplicity of pathogen reduction or high-volume delayed sampling that are undertaken by the collection center. Secondary bacterial culture with a five-day expiration, as outlined by the FDA, requires an incubation period to be defined by the transfusing institution. Of note, in published reports of use, no quarantine was applied following sampling such that when cultures turned positive, transfusion had already transpired in some cases, albeit without observed adverse clinical effect.<sup>53</sup>

### Large volume delayed sampling

In 2011, the National Health System Blood and Transplant (NHSBT) service in the UK adopted a large volume delayed sampling approach with platelet sampling performed at ≥36 hours post blood collection with inoculation of both aerobic and anaerobic culture bottles.<sup>9,17</sup> Using this approach, which remains in effect, platelet products are quarantined for at least six hours after sampling. Implementation of this testing algorithm has significantly reduced the incidence of septic transfusion reactions. A similar approach was adopted by Canadian Blood Services with extension of platelet storage from 5 to 7 days, demonstrating that the LVDS protocol has improved platelet safety. Héma-Québec has also implemented a more stringent LVDS protocol to screen platelets with sampling performed at 48 hours post blood collection and holding of units at least 12 hours post sampling.<sup>56</sup> Theoretical calculations suggest that doubling the sample volume may increase the proportion of contaminated products detected by as much as 25%, and Eder *et al.* found that BACT/ALERT™ culture was 1.54-fold (1.05–2.27) more likely to detect bacteria using an 8 mL sample versus a 4 mL sample.<sup>14,57</sup> Further theoretical calculations have been provided by Tomasulo and Wagner who advocate the use of a sample size that is a constant proportion of the product volume, in order to standardize the sensitivity of

the assay for single, double, and triple collections.<sup>58</sup> Despite a theoretical concern that larger volume products may be less safe if standard sampling volumes are applied, (i.e., a smaller proportion of the product is sampled), Eder *et al.* reported no difference in the septic transfusion reaction rates between single, double, and triple collections.<sup>59</sup> The downside of increased volume inoculation is the loss of product volume for transfusion and decreased splits rates for multiple component collections.

### Aerobic versus anaerobic culture conditions

Many centers inoculate both aerobic and anaerobic culture bottles in order to maximize culture sensitivity. Most clinically relevant bacteria that contaminate platelets grow under both conditions, although specific strains may grow faster in one condition than the other. This was best demonstrated for a single strain of *Streptococcus lugdenensis* implicated in a fatal transfusion reaction.<sup>60</sup> A small number of species are obligate aerobes (e.g., *Pseudomonas fluorescens*) or obligate anaerobes (e.g., *Clostridium* spp.). Utilizing both aerobic and anaerobic conditions enables the detection of a complete range of species, speeds up detection of some strains, and doubles the volume of product cultured, thereby increasing the sensitivity of the overall detection system.<sup>61,62</sup>

Even under optimal conditions that include both aerobic and anaerobic cultures, false-negative screening results continue to occur. Evaluation of outdated cultures suggests that only 25–40% of contaminated products are intercepted, thus accounting for reported septic reactions.<sup>70–72</sup> While culture screening has significantly reduced the risk of bacterial sepsis, especially for Gram-negative organisms, absolute protection from contamination has yet to be attained. Bacterial screening is also logistically burdensome, delaying product release into inventory. Further, there is product loss due to sampling especially when large volumes are used, and there is need for product recalls when culture screenings turn reactive after components are distributed to hospitals. Individual users have optimized the conditions of the culture assay to maximize analytical sensitivity while minimizing impact on logistics and blood availability, leading to the many different approaches as shown in Table 46.3.

### Point of care immunoassays

In September 2007, the FDA approved a rapid immunoassay for the detection of aerobic and anaerobic bacteria in leukocyte-reduced apheresis platelets as an adjunct quality control device. The Platelet PGD™ Test (Verax Biomedical, Worcester, MA) is a qualitative

immunoassay that differentiates between Gram-negative and Gram-positive bacteria through the detection of lipopolysaccharides (LPS) and lipoteichoic acid (LTA), respectively.<sup>10</sup> As approved, it is intended to be an adjunct safety test for leukocyte-reduced apheresis platelets; testing is required within 24 hours of transfusion, after the use of an FDA-cleared bacterial culture method at the time of manufacture. The system is also approved as a quality control test for pooled WBD platelets within four hours of transfusion. In this case, the platelets are not required to be prescreened using a culture method. The Platelet PGD™ Test can detect *B. cereus*, *C. perfringens*, *E. aerogenes*, *E. coli*, *K. pneumoniae*, *P. aeruginosa*, *S. aureus*, *S. epidermidis*, and *S. agalactiae* at a level of  $>10^5$  CFU/mL and *S. marcescens* at a level of  $8.6 \times 10^5$  CFU/mL. The test takes approximately 20–60 minutes to perform and is optimally undertaken after at least 72 hours of platelet storage. In a clinical study, the Platelet PGD™ test detected nine confirmed positive components of 27,620 apheresis platelets (1:3069) that had previously been screened and found to be negative for bacteria using the BACT/ALERT™ or eBDS™ culture systems. These were all Gram-positive organisms that were detected on Days 3 (four occurrences), 4 (two occurrences), and 5 (three occurrences) after collection. In the same study, two false-negative tests of 10,424 tests (1:5,212) were confirmed and the false-positive rate was 0.51%. An enhanced PGD test, the PGDprime test, was recently developed to simplify the workflow and improve specificity and sensitivity.<sup>78,79</sup>

### Nucleic acid testing detection techniques

Several amplification methods have been described for the detection of bacterial contamination, but none are currently available commercially. These comprise universal NAT assays that target genes with multiple copies (e.g., 16S ribosomal DNA or 23S ribosomal RNA) using real-time technology.<sup>80</sup> In theory, polymerase chain reaction (PCR) should be able to detect 1 CFU/mL of bacteria; however, the presence of bacterial DNA contaminating PCR reagents, and even in platelet products themselves, reduces the theoretical sensitivity of the assay to 20–1000 CFU/mL. Schmidt *et al.*<sup>81</sup> reported on the use of PCR to detect 16S ribosomal RNA from *S. aureus*, *E. coli*, *B. cereus*, and *K. pneumoniae* in pooled platelet concentrates. At an inoculum of 10 CFU/mL, the PCR testing detected all four bacteria at 12, 16, 20, and 24 hours after spiking. With a lower inoculum of 10 CFU/bag, the PCR testing detected 60% of *E. coli*, 80% of *B. cereus*, 90% of *K. pneumoniae*, and 100% of *S. aureus* 12 hours after spiking. Another study compared 16S ribosomal DNA PCR with the automated culture system BACT/ALERT.<sup>82</sup> A total of 2146 platelet concentrates were tested by both methods. When compared to the culture method, the PCR had a sensitivity and specificity of 100%. Recently, a Brazilian team reported an optimized RT-PCR method to detect bacterial contamination in platelets by targeting ribosomal 16S genes of common platelet contaminants, including *C. acnes*.<sup>83</sup> This method has enhanced specificity by treating the PCR Master Mix with ethidium monoazide and has a limit of detection of 10 genome equivalents. Given the complexities associated with such tests and the 2–6-hour delay to obtain results, nucleotide-based amplification has not been routinely applied to bacterial screening of platelets.

### Pathogen reduction

The ability to eliminate bacterial contaminants soon after collection as part of a global infection mitigation strategy offers a promising approach for preventing septic reactions completely. The FDA

recognizes that centers that implement approved pathogen reduction (PR) systems meet the requirements of the final FDA guidance for bacterial risk control.<sup>28</sup> Three pathogen reduction systems are described: (1) The INTERCEPT™ (Cerus Corp., Concord, CA) Blood System for Platelets uses amotosalen, a psoralen-based compound, plus ultraviolet-A (UVA) light to crosslink nucleic acids to prevent pathogen replication, (2) the Mirasol™ Pathogen Reduction Technology (PRT) system (TerumoBCT, Lakewood, CO) utilizes the vitamin riboflavin and ultraviolet plus visible light to achieve a similar effect by damaging nucleic acids and forming monoadducts.<sup>84,85</sup> These technologies are CE marked in Europe and are in various stages of implementation in individual European countries and are required for all platelets transfused in France, Switzerland, Belgium, Kuwait, and Iceland in 2021, while the Mirasol™ PRT system is used for all platelets in Luxembourg. At the time of writing, the INTERCEPT™ system is the only technology approved for use in the US. A third technology, (3) the THERAFLEX™ UV Platelets system (Macopharma, Tourcoing, France), is in development and uses UV-C light without any added photoactive compound to cause the formation of DNA pyrimidine dimers. This serves to block the elongation of nucleic acid transcripts and prevent replication.<sup>86,87</sup>

Efficacy in reducing the risk of bacterial contamination and sepsis is predicted by *in vitro* experiments that evaluate the ability to reduce the concentration of bacteria in artificially contaminated platelet products. In brief, components are inoculated with high concentrations of known bacterial species, and the titer is assessed directly before and after pathogen reduction treatment.<sup>84,88,89</sup> Technologies differ in their ability to inactivate various bacterial species, and all three technologies are less able to inactivate spores formed by *Bacillus* strains. PR is performed soon after platelet collection at a time when bacterial concentrations are low. PR treatments must render platelet components “functionally sterile” (defined as negative bacterial cultures at outdate). Since the components are stored for many days after treatment, even single residual viable bacteria may grow to sufficient concentrations to cause severe transfusion reactions. PR technology must be effective against Gram-negative bacteria (e.g., *Klebsiella* spp., *E. coli* etc.) that may grow rapidly after collection to overwhelm the capacity of PR systems.<sup>15</sup> The efficacy of PR is therefore contingent upon how soon after collection the PR process is performed.

Nussbaumer *et al.* inoculated double-dose apheresis platelet components with three different concentrations (1–10, 10–100, and 100–1000 CFU/mL) of each of seven different species of bacteria, performed INTERCEPT™ treatment 18–20 hours later, and compared the outcome to BACT/ALERT™ culture screening on Days 1, 2, and 5 after inoculation.<sup>90</sup> INTERCEPT™ was 100% effective at inactivating all species even when assessed five days after inoculation and treatment. Schmidt *et al.* confirmed these findings with apheresis platelets contaminated with eight different bacterial species and INTERCEPT™ treated 12 hours after contamination. A similar experiment was performed with whole-blood-derived (WBD) platelets with pathogen reduction performed 35 hours after collection and bacterial inoculation with either 100 CFU or 1000 CFU. All replicates were sterile at outdate with 100 CFU inoculation but with 1000 CFU inoculation, three of eight replicates of a strain of rapidly growing *K. pneumoniae*, and two of eight replicates of *B. cereus* exceeded the capacity of the PR system, allowing regrowth of bacteria during storage.<sup>91</sup> Similarly, Wagner *et al.* evaluated apheresis platelets inoculated with low concentrations (3–53 CFU/unit) of five transfusion relevant bacterial strains and treated with the INTERCEPT

System after either 24 or 30 hours of storage.<sup>92</sup> Platelets were stored for seven days and then cultured for sterility. All contaminated units treated with the INTERCEPT process 24 hours after inoculation were culture negative on all days tested. With inactivation performed 30 hours following inoculation, 1 of 15 units (1 of 3 replicates) was culture positive with *Klebsiella pneumoniae* by Day 5 of storage. These data support the use on the INTERCEPT Blood System for Platelets on the day of and the day after collection for both apheresis and WBD platelets.

Using the THERAFLEX UV Platelets system and pooled WBD platelet components spiked with each of 29 strains of bacteria, Gravemann *et al.* evaluated the ability to sterilize platelets contaminated with low levels of bacteria on the day of manufacture (~100 CFUs/unit).<sup>93</sup> They reported a mean  $\log_{10}$  reduction factor after UVC treatment ranging from 3.1 to 7.5, and that this varied between different strains of the same species. All platelets ( $n = 12/\text{species}$ ) spiked with up to 200 CFUs/bag remained sterile until the end of storage when treated six hours after spiking. UVC treatment eight hours after spiking resulted in single breakthrough contaminations with the fast-growing species *Escherichia coli* and *S. pyogenes*. These data support the use of the THERAFLEX UV Platelets system within six hours of platelet component collection.

Goodrich *et al.* inoculated multiple platelet samples with each of 20 different bacterial strains and performed PR using the Mirasol PRT system two hours later.<sup>89</sup> They found that for components contaminated with <20 CFU/mL, PR was 98% effective at inducing functional sterility and substantially more effective than bacterial culture screening, whereas for components contaminated with 20–10<sup>3</sup> CFU/mL, PR was 91% effective and as effective as culture screening. Subsequently, Kwon *et al.* demonstrated that platelets inoculated with low titers (45–147 CFU/bag) of *S. aureus* and *B. subtilis* and immediately treated with the Mirasol PRT system led to regrowth to clinically significant concentrations by Day 5 of storage, suggesting that cases of breakthrough contamination could be detected even when treatment was performed immediately after contamination.<sup>94</sup>

Finally, McDonald *et al.* investigated the inactivation capabilities of the Mirasol PRT and the INTERCEPT Blood System for Platelets, by evaluating the absence of viable bacteria after seven days for terminal sterility.<sup>95</sup> Bacterial count was determined post-spiking, immediately prior to treatment (two hours after spiking), immediately after treatment, and at the end of shelf life (Day 7). Using sterility at the end of platelet shelf life as the outcome, the inactivation capabilities of INTERCEPT and Mirasol PRT treatment were assessed. With INTERCEPT treatment, the inactivation capacity was measured as  $\geq 10^5 \log_{10}$  CFU/mL for *S. aureus*, *S. epidermidis*, *K. pneumoniae*, *S. bovis*, *E. coli*, *S. pneumoniae*, *S. mitis*, *L. monocytogenes*, and *S. dysgalactiae*. For *S. marcescens* and *P. aeruginosa*, the inactivation capacity was 10<sup>3</sup>–10<sup>5</sup>  $\log_{10}$  CFU/mL, and for *B. cereus* (sporulating strain), the inactivation was <10<sup>2</sup>  $\log_{10}$  CFU/mL. For the Mirasol PRT system, the inactivation of *S. pneumoniae* was 10<sup>3</sup>  $\log_{10}$  CFU/mL, and for all other strains tested, it was <10<sup>2</sup>  $\log_{10}$  CFU/mL.

The authors conclude that the inactivation capability of INTERCEPT was greater than that of Mirasol PRT. PR offers a possible alternative to bacterial screening if treatment is performed at an appropriate time dependent on the inactivation capabilities of the system. Mirasol PRT treatment needs to be performed promptly after manufacture to achieve maximum benefit, while INTERCEPT treatment could be applied much longer after donation.

Taken together, these data suggest that while PR systems are promising technologies for preventing septic transfusion reactions, the available technologies differ in their ability to inactivate clinically relevant strains to sterility. Technologies with less robust inactivation should be performed close to the time of collection in order to be as effective as available culture screening techniques.<sup>96</sup>

Blood collectors need to validate each PR technology and how it is implemented, as local practices such as buffy-coat PC preparation and the timing of leukoreduction may influence outcomes.<sup>96</sup> Alabdullatif *et al.* inoculated whole blood (WB) units with one of six *K. pneumoniae* strains, which are generally known to be fast growing, at a concentration of 3–38 CFU/unit, and processed these using the platelet-rich plasma (PRP) method 18 hours after collection.<sup>97</sup> Each spiked PC was pooled with four unspiked units and split into three components, one control, and the other two that were treated with the Mirasol PRT system 26 and 32 hours after collection, respectively. Culture at 5 and 7 days after treatment revealed that Mirasol treatment after 26 hours of WB collection resulted in complete inactivation of all *K. pneumoniae* strains. However, treatment 32 hours post-WB collection resulted in the breakthrough of one clinical isolate in two of the three replicates with ~7.8  $\log_{10}$  CFU/unit detected on Day 5 of PC storage. Although other bacterial strains were not tested and the outcomes may be variable, for *K. pneumoniae* it seems that Mirasol PRT can be performed up to 26 hours after collection under these specific conditions of non-leukoreduced WB storage which are thought to suppress bacterial growth.

Clinical data on the effectiveness of PR system in routine use have begun to be published. Hemovigilance programs in Belgian, Swiss, and French had not documented any septic reactions attributable to transfusion of INTERCEPT platelets with 609,290 platelets transfused (<1.6 per million) up to 2016;<sup>98</sup> further, no subsequent cases have been reported under these hemovigilance programs with over 1.5 million platelets transfused. This rate is significantly lower than those reported with nonpathogen-reduced platelets in the same settings. In the US, two confirmed septic reactions, including one fatality, were reported with PR-treated platelet components.<sup>19,99</sup> In both cases, there is strong evidence that the PR process would have effectively inactivated the implicated bacterial strains if they were present at the time of treatment and that contamination occurred after the PR process was performed. Environmental bacterial strains were implicated and in the fatal case, a defect in the storage container was demonstrated suggesting that bag damage provided a portal for bacterial entry. These reports highlight the need for proper platelet storage bag handling techniques and the storage and transport of platelets in clean environments as sterile platelets can be contaminated after PR treatment through “holes in the bag.”

Pathogen reduction systems are effective at reducing the risk of bacterial sepsis and enjoy the added advantages of providing a level of protection against emerging pathogens and the residual risks of known pathogens, as well as reducing the risk of transfusion associated graft versus host disease. They allow earlier release of platelets into hospital inventories as they do not require the delay before release that is inherent to the use of delayed large volume sampling strategies.<sup>100</sup> Both culture and PR incorporate processing losses of 5–10% of each platelet collection. All three PR technologies lead to lower platelet count increments than conventional platelets; however, multiple studies with the PR system available in the US demonstrate noninferiority with respect to the ability to prevent and treat bleeding.

## Bacterial contamination of red blood cells

Sepsis associated with the transfusion of bacterially contaminated Red Blood Cell (RBC) components is very rare and appears to be declining in incidence. In part, this is ascribed to the storage conditions of RBCs that are unfavorable to bacterial growth. Specifically, RBC concentrates (RBCCs) are stored at 1–6 °C in North America for a maximum of 42 days and at 2–6 °C for up to 35 days in Europe. Most bacteria have poor viability under these storage conditions. However, some psychrophilic (i.e., that grow optimally at refrigeration temperatures) pathogenic organisms can proliferate in RBCCs reaching clinically significant levels. RBCCs are not routinely screened for bacterial contamination; therefore, the incidence of bacterial contamination in RBC units is estimated based on reports of septic transfusion reactions or analysis of data from quality control sterility testing of expired units.

RBC transfusion associated sepsis may be severe. From 2014 to 2018, the US Food and Drug Administration (FDA) reported seven fatalities caused by RBCC contaminated with bacteria.<sup>6</sup> Implicated organisms included *P. fluorescens*, *Pseudomonas veronii*, *Enterococcus faecium*, and the obligate intracellular bacterium *Anaplasma phagocytophilum*. Since 2010, there have been no cases of transfusion-transmitted infections caused by bacterially contaminated RBCCs reported to the United Kingdom's Serious Hazards of Transfusion (SHOT) program.<sup>9</sup> Funk *et al.* reported one fatality in 2018 caused by transfusion of a RBCC contaminated with *E. coli* in Germany.<sup>101</sup>

The major contaminants of RBCCs are Gram-negative bacteria of the Enterobacteriace family, with *Yersinia enterocolitica* being historically the most commonly isolated species.<sup>102–104</sup> Isolation of this species has decreased in recent years, which has been ascribed to the implementation of leukocyte reduction in many high-income countries.<sup>105</sup> However, the risk of *Y. enterocolitica* has not been eliminated. In 2015, Frati *et al.* published a case report of a fatal septic reaction in Italy caused by the transfusion of a RBC unit contaminated with *Y. enterocolitica*.<sup>106</sup> *Serratia* spp. are also Gram-negative bacilli that are ubiquitous in the environment and grow well under RBC storage conditions. In particular, *Serratia liquefaciens* has been implicated in RBC transfusion-associated sepsis, with a high mortality rate.<sup>107,108</sup> In 2016, Héma-Québec reported a fatal RBC transfusion case involving another environmental contaminant, *Aeromonas veronii*.<sup>109</sup> *K. pneumoniae* is a bacterium also often implicated in severe septic transfusion events involving contaminated RBCC.<sup>15,110</sup> An interesting case study was published by Woodring and Farrel involving the transfusion of an RBC unit contaminated with *Pseudomonas poae*. Although Gram-negative bacilli were visualized by Gram staining of the transfused unit, blood cultures of the patient and cultures of the RBC unit yielded negative results given that this organism fails to grow at 37 °C.<sup>111</sup> Other species of *Pseudomonas* have also been implicated as RBC contaminants. For example, *Pseudomonas putida* was identified in a septic reaction in Australia,<sup>2</sup> and *P. fluorescens* was implicated in a transfusion reaction that was reported to the NHSN Hemovigilance Module.<sup>7</sup>

Septic reactions caused by contaminated RBCC are mostly associated with Gram-negative bacteria due to infused endotoxin (lipopolysaccharide of the cell wall), which can provoke an uncontrolled immune response and septic shock. Most severe reactions occur following the transfusion of older RBCC (i.e., that have been stored for more than 21 days) as these are more likely to have a high endotoxin load.

Although infrequent, Gram-positive bacteria, including *S. aureus*, *S. epidermidis*, *S. pneumoniae*, and *Streptococcus mitis*, have also been implicated in septic transfusion reactions associated with

contaminated RBCC.<sup>2,7,112,113</sup> Damgaard *et al.* performed a study in which blood samples that had been collected from healthy blood donors were separated into RBC and plasma; approximately 35% of the samples showed evidence of bacterial contamination in the RBC component with *Cutibacterium acnes* being most commonly identified.<sup>114</sup> Similarly, a spiking study performed by Taha *et al.* demonstrated that bacteria present in whole blood migrate preferentially to cellular fractions (RBC and platelets) during manufacturing of buffy-coat platelet pools.<sup>115</sup> These two studies raise the question as to whether screening of RBCC for bacterial contamination should be considered. Approximately 40% of RBC units associated with bacterially contaminated whole-blood-derived platelets test positive. The most frequent organism isolated in companion RBC units of whole-blood-derived platelets is the aerotolerant anaerobe *C. acnes*. Although *C. acnes* does not replicate during RBCC storage, there is evidence that transfusion reactions caused by this organism are delayed and may go unrecognized and underreported.<sup>116</sup>

## Clinical presentation

The clinical sequelae following transfusion of bacterially contaminated blood products are highly variable, spanning asymptomatic infection, low grade fever (which may be indistinguishable from a nonhemolytic transfusion reaction) acute sepsis, hypotension, and death.<sup>11</sup> In general, the clinical manifestations are less severe following platelet transfusion than those following transfusion of bacterially contaminated RBCs.<sup>117</sup>

Sepsis caused by the transfusion of contaminated platelets remains vastly under recognized and underreported. Indeed, Jacobs *et al.* found that during periods at their institution where active culture screening was in place, contaminated platelet components and sepsis were 32.0- and 10.6-fold more likely to be documented than during a period when detection relied solely on clinician recognition and reporting (i.e., passive surveillance).<sup>118</sup> The severity of reactions was greatest with components containing  $\geq 10^5$  CFU/mL and with higher bacterial virulence. At lower concentrations and with less virulent organisms, patients frequently did not display symptoms or presentation of symptoms is delayed. Similarly, patients who are on antibiotic therapy or who are neutropenic may not exhibit classical signs of sepsis (e.g., fever), and even when they do, those signs are often ascribed to other infectious causes or underlying disease.<sup>11</sup>

Patients may not react immediately after transfusion of contaminated platelets: in one well-documented outbreak of *Salmonella choleraesuis*, seven patients were linked to one repeat donor with occult, chronic osteomyelitis. The time to the onset of illness ranged from 5 to 12 days (mean 8.6 days). In all cases, the platelet units had been stored for less than one day.<sup>119</sup> Similarly, in 2006 the CDC reported a multistate outbreak of *Pseudomonas fluorescens* septicemia. All cases could be traced to contaminated heparin flushes.<sup>120</sup> A total of 28 patients had delayed onset of *P. fluorescens* infections, ranging from 84 to 421 days after their last potential exposure. As reported by Hong *et al.*, signs of septic transfusion reactions are not typically identified in the first 4–6 hours following transfusion; by contrast, active surveillance has shown that septic events can be detected up to 24 hours after the transfusion events.<sup>121</sup> A report of the isolation of the Gram-negative strict aerobe *Bordetella holmesii* during platelet culture screening revealed that this organism does not proliferate yet still remains viable during PC storage. It was shown that the contaminating organisms may be neutralized by bacteriostatic factors present in the platelet unit impeding detection.<sup>122</sup>

During transfusion, any change in clinical condition that prompts suspicion of sepsis, even in the absence of fever, should lead to

halting of the transfusion, patient support and investigation for possible transfusion of a contaminated component.<sup>123</sup> Similarly, patients who develop fever  $\geq 38^\circ\text{C}$  ( $<100.4^\circ\text{F}$ ), rigors, hypotension, shock, tachycardia, dyspnea, or nausea/vomiting within 24 hours of a platelet transfusion merit investigation given the potential for transfusion associated sepsis.

### Bacterial contamination of plasma, cryoprecipitate, and derivatives

Cell-free products such as plasma and cryoprecipitate are stored in the frozen state and thus are rarely associated with significant contamination. *Burkholderia cepacia* and *P. aeruginosa* have been cultured from cryoprecipitate and plasma thawed in contaminated waterbaths.<sup>124,125</sup> The increasing use of thawed plasma and liquid, never-frozen plasma<sup>126</sup> warrants greater vigilance for septic reactions with these components, given allowable storage at 1–6 °C for up to 5 days and 26 days, respectively.<sup>127</sup> From 2014 to 2018, the FDA documented one fatality that was attributable to plasma contaminated with coagulase-negative *Staphylococcus*.<sup>6</sup> Thawed plasma contaminated with *Bacillus* spp. was implicated in a transfusion reaction reported to the NHSN Hemovigilance Module.<sup>7</sup> Spiking studies have shown that other bacteria such as *S. liquefaciens* and *P. fluorescens* are also to grow in thawed cryoprecipitate.<sup>128</sup>

Products derived from blood components may also be contaminated with bacteria. Human serum albumin is a good culture medium and preserves the viability of contaminants. A heating step (60 °C for 10 hours) is performed during the manufacture of albumin to inactivate certain viruses rather than to ensure bacterial sterility.<sup>129</sup> The latter would require autoclaving (superheating under pressure), which would cause albumin to denature. On occasion, specific lots of albumin product have been found to be contaminated with bacteria, typically *Pseudomonas* spp.<sup>130</sup> These lots have resulted in endotoxic shock, transient bacteremia, and febrile reactions in recipients. Two patients in different hospitals developed *Enterobacter cloacae* septicemia following infusion of contaminated albumin.<sup>130,131</sup> Cultures of unopened product grew *Stenotrophomonas multophila* and *Enterococcus gallinarum* in addition to *E. cloacae*. This prompted a worldwide recall of selected lots of 5%, and 25% albumin. Cracks in the glass bottles were suspected to be responsible for the contamination. In short, deficiencies in manufacturing processes of blood derivative can pose risk of bacterial contamination with serious ramifications.

### Transfusion-transmitted syphilis

*Treponema pallidum*, the causative agent of syphilis, is a thin-walled, motile, spiral Gram-negative spirochete that cannot be visualized with Gram's stain. Historically, *T. pallidum*, a fastidious organism, was not able to be grown on bacteriologic media or in cell culture, instead relying on inoculation of rabbits and other animals for study.<sup>132</sup> Although *T. pallidum* is a bacterium, it is often regarded as a distinct entity, that is different from other transfusion-transmitted bacteria. Only 25% of patients with primary syphilis have a reactive serologic test for syphilis, and the test does not become routinely positive until the fourth week after the onset of symptoms; therefore, donors infected with *T. pallidum* may be asymptomatic with negative serology during periods of spirochetemia.<sup>133–135</sup> The organism does not survive prolonged storage at 4 °C, remaining viable for 1–5 days at these cold temperatures.<sup>136,137</sup> Nonetheless, infection is conceivable following transfusion of a fresh RBC unit from a donor who is still seronegative at the time of donation. While platelets

provide a more hospitable environment for *T. pallidum* given their storage at 20–24 °C, *T. pallidum* does not thrive in modern platelet storage bags owing to the associated high oxygen tension.

On occasion, the merits of screening for syphilis have been questioned, at least in high-income countries.<sup>138</sup> For one, transfusion-transmitted syphilis is exceedingly rare. Since 1969, only three cases of transfusion-transmitted syphilis have been published in the US.<sup>139–141</sup> Factors that likely contribute to the low incidence include (1) a low prevalence of syphilis among blood donors; (2) donor questioning that targets high-risk behavior for syphilis and other transfusion transmissible infections; (3) universal laboratory screening for syphilis; (4) refrigerated storage, which results in the death of spirochetes; (5) the high proportion of transfusion recipients who are on antibiotics at the time of platelet transfusion (i.e., many of which are bactericidal to *T. pallidum*); and (6) potential exclusion for another positive infectious marker such as HIV, HCV, or HBV.<sup>142,143</sup>

Advocacy for elimination of syphilis testing surrounds the low risk of transfusion-transmitted syphilis, conferring high cost—albeit low yield—to extant screening, coupled with the potential for false-positive test results that require unnecessary counseling and investigation. The counter argument is that donor syphilis testing can uncover individuals at risk of other sexually transmitted diseases (i.e., it serves as a surrogate marker for high-risk behavior) and, therefore, should be retained. There is also an argument for a public health benefit given surveillance of large numbers of individuals (donors). Both counterpoints are debatable: first, coinfections with other transfusion-transmissible infections have been reported but are rare. Second, while donor screening does provide surveillance, this is not the primary intent of blood donor screening and could even incentivize test seeking behavior. However, syphilis, even in high-income settings, is prevalent in selected high-risk groups.<sup>143</sup> Importantly, the argument does not hold true in low-income countries where high prevalence of syphilis, suboptimal donor selection, and transfusion of freshly collected blood, with often absent post-transfusion surveillance, argues for continued testing for syphilis.<sup>144</sup>

### International comparison

There is wide variation in the way different countries approach the problem of bacterial contamination of platelets. An international survey published in 2007 of 12 countries revealed multiple approaches; routine culture screening was performed in a minority.<sup>98</sup> A similar survey in 2014 revealed that all but a few countries had implemented BACT/ALERT™ culture screening in some form. Almost all countries recognize the danger of contaminated platelets and have instituted routine screening of donors for risk factors, standardized skin preparation techniques, and diversion for the initial blood drawn during phlebotomy. With only these interventions, France reported a continued high risk of sepsis (1:40,000 transfusions) and fatality (1:194,000 transfusions). In order to manage this risk, PR was implemented in France. Similarly, universal use of PR was implemented in Switzerland in 2011 and in Belgium in 2015. In contrast, The NHTSB in England adopted universal large volume delayed culture screening in 2011 and reported their five-year experience in 2017 documenting that the protocol was an effective risk reduction measure and increased the safety of the blood supply.<sup>16</sup> From February 2011 to September 2015, a total of 1,239,029 PCs were screened. False-negative cultures, all with *S. aureus*, occurred on four occasions; three were visually detected before transfusion and one confirmed transmission resulted in patient morbidity. The NHTSB screening protocol effectively reduced the number of clinically adverse transfusion transmissions

by 90%. Subsequently, Canada implemented a similar protocol and the US FDA has recognized large volume delayed culture as an effective intervention for use in the US.

In Germany, a pilot study, where 52,243 platelet components were screened with both BACT/ALERT™ and eBDS™ cultures before release, documented a false-negative result for one collection that leads to a fatality and severe reaction in the recipients of split units.<sup>45</sup> Culture screening was not implemented, and subsequently in 2009 the Blood Working Party (Arbeitskreis Blut: National Advisory Board of the Federal Ministry of Health) mandated a shortened shelf life of four days.<sup>66,145</sup> Permission to extend shelf life to five days has been granted based on bacterial screening on Day 3 or 4 after collection. Japan likewise restricts the shelf life of platelets and does not perform bacterial screening. Recently, shelf life was extended from 72 hours to midnight on the third day effectively allowing up to 3.5 days ( $\leq 85$  hours from collection) shelf life, in order to facilitate logistics of supplying hospitals. Seventeen septic reactions were reported from 2008 to 2018 with platelets two and three days old, including one fatality caused by *E. coli*.<sup>146</sup>

To date, attention on bacterial contamination of blood products has largely been confined to the US and other HIC. Blood transfusion safety remains a major challenge in low- and middle-income countries (LMICs). Deficiencies span the complete blood safety continuum from national oversight through donor recruitment, collection to testing, and transfusion and post-transfusion surveillance. Structural barriers that impede blood transfusion safety include competing health-care priorities, funding, education, and infrastructure. Collectively, this has manifested as increased infectious risk, often in locations where the incidence and prevalence of the major transfusion transmissible viruses (e.g., HIV, HBV, and HCV) are high. Where there has been investment in blood safety, initiatives have largely targeted prevention of HIV, HBV, and HCV.<sup>147</sup> By contrast, bacterial contamination has been neglected despite environmental (i.e., heat and humidity in tropical countries) and technical (e.g., inattention to aseptic technique, suboptimal quality systems, and hemovigilance) conditions that favor contamination.<sup>144,148,149</sup> There are comparatively few studies that have evaluated rates of contamination of blood products in LMICs.<sup>76</sup> One review of related studies in Africa suggested much higher rates of contamination (i.e., up to 17.5% for whole blood or packed red cells; up to 17.9% for platelets) than have been reported in HICs.<sup>149</sup> In a survey of members of the African Society of Blood Transfusion, the collated responses suggested low uptake of strategies to contend with contamination that are otherwise routine in HICs, such as bacterial culture and pathogen reduction.<sup>149</sup> While not unique to this topic, it highlights the ever growing divide between high and low resource settings, with little sign of change.<sup>150</sup> Technological advances such as nucleic acid testing and pathogen reduction, which have arguably had modest (i.e., incremental) effect on blood safety in HICs, could prove transformative in low resource settings. Nonetheless, a myriad of challenges (notably related to funding and infrastructure) still preclude their wider adoption.<sup>151</sup>

## Conclusion

Bacterial contamination of blood components is the most common cause of transfusion-transmitted infectious disease. Septic transfusion reactions related to RBC transfusions have declined rapidly in the last decade, possibly related to the more universal application of leukoreduced blood products. Most cases of post-transfusion sepsis

involve platelets that must be stored at room temperature under conditions conducive to bacterial proliferation. With the introduction of improved skin disinfection, diversion techniques, and bacteria detection of platelets using culture techniques, the rate of clinically significant septic reaction has decreased; however, a substantial risk of septic transfusion reactions remains. Many blood centers have moved to universal use of PR, and clinical evidence suggests that this further reduced the risk of sepsis, but recent reports identify a previously under recognized risk of contamination after culture testing or PR treatment through breaches in the integrity of the storage bag. An alternative solution is to use cold stored platelets; however, these products are not yet approved for use and are restricted to the treatment of active bleeding.

In the US, the implementation of the FDA Guidance in October 2021 will further improve patient safety; however, no technology can claim the elimination of all risks. Clinicians must continue to be vigilant for evidence of bacterial contamination and septic reactions, and respond rapidly to support patients when these events occur.

## Disclaimer

Dr. Bloch is a member of the US Food and Drug Administration (FDA) Blood Products Advisory Committee. Any views or opinions expressed in this chapter are those of the authors and are based on their own scientific expertise and professional judgment; they do not necessarily represent the views of the Blood Products Advisory Committee or the formal position of the FDA and also do not bind or otherwise obligate or commit either the Advisory Committee or the FDA to the views expressed.

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## CHAPTER 47

# Hemolytic transfusion reactions

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A hemolytic transfusion reaction (HTR) is the accelerated clearance or destruction of red blood cells associated with a transfusion event. HTRs can be due to immunologic incompatibility or to non-immune factors. The majority of HTRs are a result of red blood cell (RBC) transfusion, but they may also derive from the transfusion of plasma-containing blood components or plasma-derived products, or as a result of immune incompatibility in a hematologic or solid organ transplant.

Harmonized definitions for HTRs are available from expert organizations, and their use is encouraged in hemovigilance practice.<sup>1,2</sup> They are classified as acute (AHTR) or delayed (DHTR) hemolytic reactions, based on whether they occur within or after 24 hours of the implicated transfusion. Hyperhemolysis refers to a life-threatening DHTR presentation that is encountered most commonly in sickle cell disease (SCD) patients,<sup>3</sup> but has also been reported in myelofibrosis,<sup>4,5</sup> thalassemia,<sup>6</sup> and other disorders.<sup>7,8</sup> Otherwise known as “bystander hemolysis,” this is an immune phenomenon characterized by accelerated destruction of both autologous erythrocytes and transfused blood cells. It can be accompanied by reticulocytopenia and may not be associated with alloantibody formation.

Transfusion may also stimulate the production of alloantibodies without clinical evidence of hemolysis. This phenomenon has been termed delayed serologic transfusion reaction (DSTR) and is fundamentally distinct from DHTR.<sup>2</sup> DSTR is clinically benign and is often identified during routing blood bank testing. Although there is no evidence of hemolysis, a DSTR is essentially an alloimmunization event and thus predisposes a patient to subsequent HTRs. Earlier reports of hemolytic reactions have not necessarily differentiated clearly between serologic and true hemolytic reactions. One national hemovigilance system stopped collecting DSTR data in 2016.<sup>9</sup>

### Incidence

National hemovigilance programs have become important tools to understand the epidemiology of hemolytic transfusion reactions, identify their root causes, and design and monitor the efficacy of preventive strategies. The extent and scope of hemovigilance surveillance systems varies across the globe. In the

USA, the FDA mandate is limited to reporting fatality events,<sup>10,11</sup> while the Serious Hazards of Transfusion (SHOT) program in the UK encompasses the much more frequent, and highly informative, near-miss incidents.<sup>9</sup> Other programs that provide periodic, public reports, and valuable benchmark data include the Canadian Transfusion Transmitted Injuries Surveillance System (TTIIS)<sup>12</sup> and the Australian Haemovigilance Report.<sup>13</sup> The International Haemovigilance Network (IHN) released a summary of adverse reactions using data submitted by 25 countries throughout 2006–2012.<sup>14</sup> Interpretation of this epidemiologic data must take into account historical changes in imputability and case definitions, as well as variable participation by local health organizations and a pervasive degree of underreporting.

In its 2019 publication, the SHOT group describes 49 reported HTRs within a background of 2,306,983 total transfused blood components that year, yielding a final estimate of one HTR per 47,800 transfusions.<sup>15</sup> Twenty-two (45%) of these reported hemolytic reactions were classified as acute, 23 (45%) as delayed, and 4 as a hyperhemolysis event. No HTR deaths were registered that year, but 11 cases were linked to resulting major morbidity.<sup>15</sup> The number of deaths attributed to hemolytic reactions has oscillated between 0 and 4 per year in the UK since 2010. Statistics from the USA exhibit a similar trend, with a historical reduction of HTR fatalities and a residual low, yet persistent incidence during the last decade (5–9 HTR-related deaths yearly reported FY10 through FY18).<sup>10</sup> Data from IHN indicates that AHTRs accounted for 6.9% of the 349 transfusion-associated deaths reported by the 25 participating nations during 2006–2012.<sup>14</sup> In the same dataset, DHTRs were reported as the fourth most frequent classification (4.3%) within the total 92,850 reported transfusion reactions internationally, but they represented 16% of the reported serious events. DSTRs were reported more frequently (12.1% of all reactions and 20.8% of serious reactions), but DSTR case and severity definitions were inconsistent across participating groups.<sup>14</sup>

Hemolytic reactions secondary to ABO-incompatible transfusions are considered fully preventable sentinel events.<sup>16</sup> A 20-year retrospective review of ABO-incompatible transfusion fatalities reported to the FDA confirmed a significant and sustained decrease in their rate after 2008,<sup>17</sup> indicating a growing focus on

patient safety and improved transfusion procedures, but also a remaining gap for practice improvement. Four ABO-incompatible RBC transfusions, with no associated fatalities, were recorded in the UK in 2019; their historical incidence over the preceding 23 years also shows a marked decreasing trend.<sup>15</sup> The SHOT group, however, also captures ABO-incompatible near-miss events, which totaled 329 during the same year and thus provide powerful insight into the systemic deficiencies that can potentially lead to these serious events.

## Causes of HTRs

Causes of HTRs and differential diagnoses are summarized in Table 47.1. Surveillance data indicate that hemolytic transfusion reactions frequently result from inadvertent administration of incompatible blood components or from failure to detect a potential incompatibility. Incompatible components may also be transfused when clinical urgency dictates emergency release of blood products before completion of pretransfusion testing, although the literature indicates that acute HTRs develop in less than one per 1000 patients in this setting.<sup>18</sup> In 2019, one nonfatal HTR was reported due to emergency transfusion of antigen-positive blood in the UK.<sup>15</sup>

Among 80 ABO-incompatible related fatalities reported to the FDA during 2000–2019, 72.5% of the source errors were traced to clinical service processes, and the remaining fraction was attributed to errors in the transfusion service or laboratory.<sup>17</sup> The latter can be broadly divided into two categories: wrong components issued at the blood bank and laboratory testing errors linked to manual processes (including wrong tube tested, mislabeling, and incorrect typing and clerical errors). The most recent (2019) SHOT document describes 329 incidents related to the transfusion of an incorrect blood component, but their distribution by location exhibits the inverse pattern: 39.8% were attributed to errors in the clinical arena and 60.2% to the blood bank laboratory.<sup>15</sup> Notably, the five ABO-incompatible RBC or FFP transfusion events that provided data for a patient identification method indicated that it was performed manually.<sup>15</sup> Furthermore, SHOT describes an additional 1314 near-miss incidents, which account for 38.7% of the total reports and which could have potentially resulted in erroneous blood group typing or transfusion of an incorrect blood component.<sup>15</sup> Wrong blood in tube (WBIT) incidents accounted for 85.7% of the wrong blood component near-miss events and could be traced back to incorrect patient identification or failure to follow sample labeling protocols.<sup>15</sup>

In the United States, antibodies responsible for HTR fatalities were most commonly of the ABO group during FY14–FY18, followed by two cases attributed to anti-c and two cases attributed to anti-Fy<sup>a</sup> during that period.<sup>10</sup> Other reported specificities include single instances of anti-Jk<sup>a</sup>, anti-Jk<sup>b</sup>, anti-C, anti-U, anti-Fy<sup>a</sup>, anti-e, and anti-Wr<sup>a</sup>.<sup>10</sup> In the UK, three of four AHTRs in 2019 were caused by anti-Wr<sup>a</sup>, and the remaining case was associated with weak anti-E; all four cases were undetected in the initial antibody screen.<sup>15</sup> DHTRs were mostly associated with evanescent antibodies that were not detectable in the pretransfusion sample, including one incident where the patient had informed the clinical service of antibodies formed during a prior pregnancy. Anti-Jk<sup>a</sup> was the most common specificity (36.5%) reported among 104 DHTRs in the UK during 2015–2019, followed by an antibody mixture including anti-Jk<sup>a</sup> or anti-Jk<sup>b</sup> (13.5%), anti-c (10.6%),

anti-Fy<sup>a</sup> (9.6%), and an unspecified mixture of specificities that did not include Jk<sup>a</sup> or Jk<sup>b</sup> (9.6%).

Transfusion of ABO-incompatible platelet products is not infrequent due to inventory constraints,<sup>19–21</sup> and the hemolysis of the recipient's RBCs has been reported due to passive transfer of ABO antibodies in this scenario.<sup>22–26</sup> Such hemolytic reactions have been traditionally attributed to the transfusion of apheresis platelets carrying high titers of anti-A/A,B,<sup>27</sup> but similar titers were documented in pooled products,<sup>28</sup> and although less frequent, a fatality has been reported due to passive transfer of anti-B.<sup>23</sup> Many centers screen group O platelets for high titers; however, the tittering method and the titer threshold vary widely across institutions.<sup>29</sup> Dilution in platelet additive solution (PAS) reduces ABO antibody titers in platelet components,<sup>30</sup> but one fatality has been reported in spite of this mitigation strategy.<sup>23</sup> The use of group A plasma and group O whole blood during emergency resuscitation in trauma patients has been documented as a safe strategy, where the benefits are reported to outweigh the risks of hemolysis.<sup>26,31–33</sup> A growing number of studies suggest that the ABO titer alone is not predictive of the risk of hemolysis,<sup>23,34–37</sup> highlighting the need for further research in this area.

Acute hemolysis can be caused by infusion of an ABO-incompatible hematopoietic progenitor cell (HPC) graft.<sup>38</sup> In addition, delayed engraftment and pure red cell aplasia can result from major ABO-incompatible HPC transplantation (HPCT).<sup>39</sup> An important complication to monitor after minor ABO-incompatible HPCTs is passenger lymphocyte syndrome (PLS), which can be observed as brisk hemolysis 1–3 weeks after infusion of the graft due to viable, immunocompetent donor lymphocytes that produce isoantibodies directed to the recipient's RBC antigens.<sup>40,41</sup> PLS has also been documented after solid organ transplant, including lung and heart, and liver and kidney.<sup>42</sup>

A prospective observational study published in 2021 followed 78 nongroup O patients that received a total of 99 high-dose intravenous immunoglobulin (IVIG) doses.<sup>43</sup> Hemolysis was reported after 32 IVIG infusion events, with 19 (19%) of 99 graded severity 2 or higher.<sup>43</sup> Risk factors included blood group AB, first-time administration of IVIG, and the absence of concomitant immunosuppressive treatment.<sup>43</sup> This reported hemolysis rate following IVIG is higher than the 1.6% frequency previously reported in a 2008 retrospective study of 1000 patients.<sup>44</sup> An analysis of IVIG hemolysis cases published during 2003–2012 identified that hemolysis correlates with high IVIG doses,<sup>45</sup> and a subsequent study documented that heterozygosity, with the presence of the O allele, mitigates this risk.<sup>46</sup> No association was identified between the risk of IVIG hemolysis and recipient secretor (*FUT2*) status, or recipient *FCGR2/3* genomic locus variations.<sup>46</sup> An incidence of 15% was described among 419 pediatric patients with Kawasaki disease that received IVIG, with an increased risk with high-dose (4 g/kg) administration.<sup>47</sup> The rates of IVIG-associated hemolysis in Kawasaki disease patients range from 0.36%<sup>48</sup> to 16%<sup>49</sup> in the literature and may be related to different IVIG preparations.<sup>50</sup> Hemolysis has also been documented after infusion of intravenous anti-D in D+ patients with immune thrombocytopenic purpura.<sup>51–53</sup>

Nonimmune causes of hemolysis associated with transfusion, underlying hemolytic conditions that can be exacerbated post-transfusion, and diagnostic entities that may confound the laboratory and clinical picture should also be considered in the differential diagnosis. These are discussed below (see the Clinical manifestations and diagnosis section) and summarized in Table 47.1.

**Table 47.1** Summary of Hemolytic Transfusion Reactions (HTRs) Causes, Targeted Prevention Strategies, and Differential Diagnoses

Causes of Hemolytic Transfusion Reactions and Differential Diagnosis	
<b>Hemolysis Induced by Alloantibody to Red Cell Antigen</b>	<b>Preventive Strategy</b>
Accidental <ul style="list-style-type: none"> <li>Wrong blood in tube (misidentification and poor labeling practices)</li> <li>Patient or product misidentification at bedside</li> <li>Wrong component issued at blood bank</li> <li>Incomplete or inappropriate pretransfusion testing</li> <li>Errors associated with manual processes</li> <li>Emergency release of un-crossmatched blood</li> </ul>	Robust hemovigilance and safety culture Root-cause analysis encompassing near-miss events Centralized alloimmunization registries Electronic identification systems Prophylactic antigen matching for hemoglobinopathies Patient participation and education
Passive transfer of ABO alloantibodies <ul style="list-style-type: none"> <li>Platelet mismatch due to limited inventory or prioritization of HLA matching</li> </ul>	Titer screening, limiting volume of incompatible plasma, platelet volume reduction, and PAS
Group A plasma in emergency resuscitation of trauma patients Immunoglobulin products: IVIG and RhIg	Report to appropriate agencies and quarantine IVIG lots with high-titer ABO antibodies
ABO-incompatible HSCT <ul style="list-style-type: none"> <li>Acute hemolysis (ABO-incompatible HSCT)</li> </ul>	Red cell or plasma volume reduction and proper selection of peri-transplant blood products
Passenger lymphocyte syndrome (minor incompatibility HSCT or solid organ transplant) Delayed engraftment and pure red cell aplasia (major ABO-incompatible HSCT)	
<b>Coexisting Conditions That May Confound Clinical and Laboratory Presentation</b>	
Autoimmune hemolytic anemia G6PD deficiency Sickle cell disease Drug-induced hemolysis Sepsis Active bleeding Mechanical thrombectomy Hematoma resorption	
<b>Nonimmune Hemolysis Associated with Transfusion</b>	
Incompatible fluids coadministered with the transfusion Thermal damage Mechanical factors Improper deglycerolization G6PD-deficient red blood cell donor	

## Pathophysiology

Early transfusion reactions described in the 1600s developed following the infusion of xenogeneic blood products from sheep or calves into humans. As expected, repeated transfusions uniformly resulted in patient deaths, interdicting the use of transfusions for several years. Almost three centuries later, Karl Landsteiner's discovery of ABO blood groups allowed for the accurate prediction of immunological incompatibility among human-to-human transfusions,<sup>54</sup> paving the way for blood product transfusions in routine clinical practice. However, despite these and several other advances in the field, transfusion reactions continue to occur in clinically significant numbers as highlighted in the previous sections. It is thus critical to understand and further study the pathophysiology of hemolytic transfusion reactions.

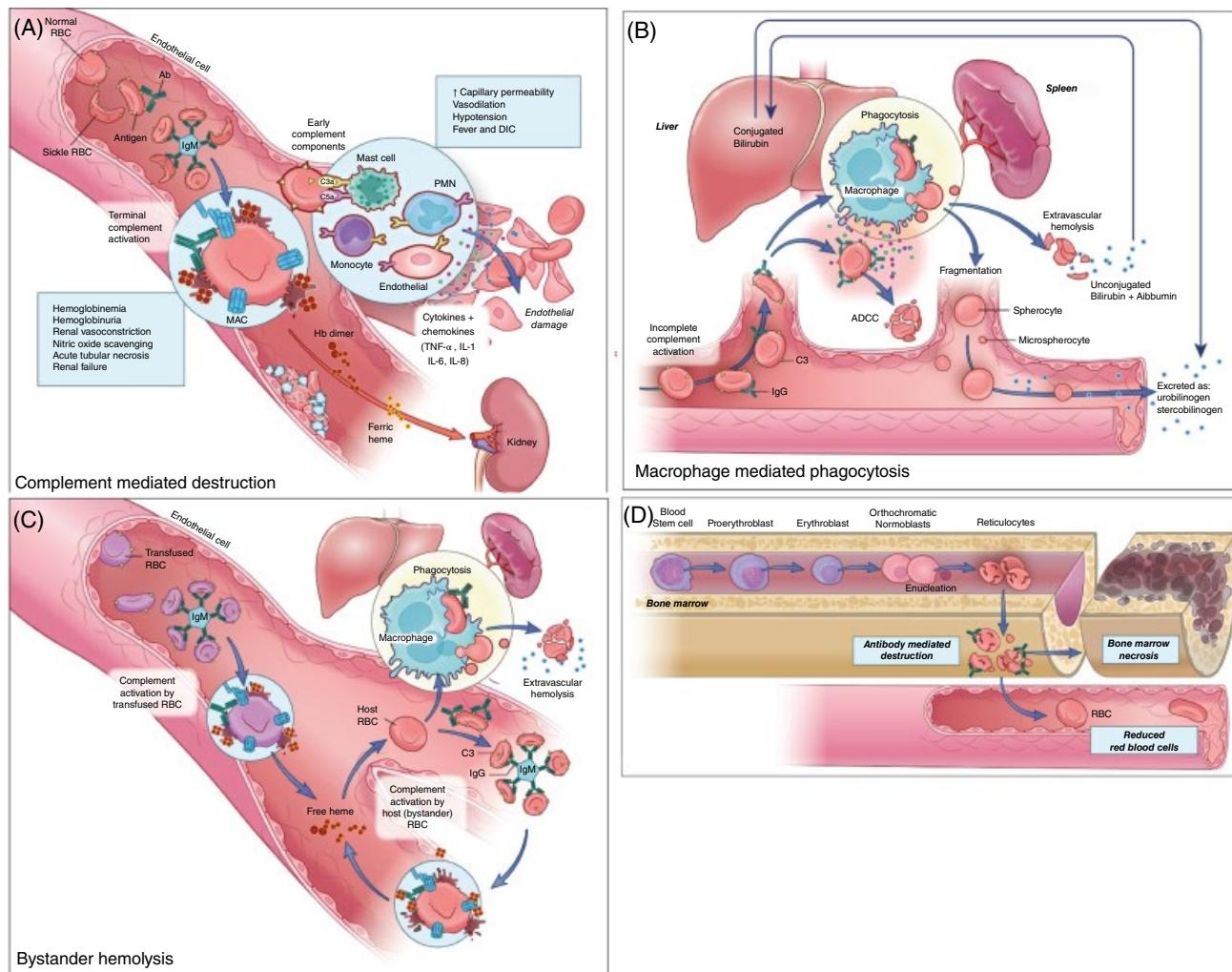
The pathophysiology of hemolytic transfusion reactions is dependent on the type of antigens and antibodies involved, as well as host factors. Broadly, mechanisms of immune hemolysis can be categorized based on the location of hemolysis (intravascular or extravascular) or by timing of onset after transfusion (acute if <24 hours or delayed if 24 hours to 28 days after cessation of transfusion).<sup>2</sup> However, they often present as overlapping phenomena in clinical scenarios. Based on our current knowledge, at least four mechanisms may be important in acute and delayed hemolysis, as well as in hyperhemolytic reactions: the complement system, macrophage mediated mechanisms, bystander hemolysis, and reticulocyte suppression or destruction.

## Role of complement

The complement system is composed of about 50 serum and membrane proteins with tightly regulated proteolytic activation cascades that culminate in the production of effector molecules with multiple biological functions. It represents a major component of the innate immune system, and it provides a powerful and effective mechanism to protect the host from pathogens. It was first described in 1899 by Paul Ehrlich as a heat-labile component of serum that "complemented" the effects of antibodies in bactericidal activity as well as red blood cell lysis.<sup>55</sup>

Activation of the complement system is thought to be the key player in a majority of acute hemolytic transfusion reactions. Acute hemolytic reactions are typically characterized by the interaction of preexisting IgM antibodies in the host with incompatible blood group antigens from the donor (transfusate). Naturally occurring ABO antibodies and reactions following ABO-incompatible transfusions have been implicated in a majority of the fatal cases.<sup>10,56</sup> Circulating IgM, when bound to foreign blood group antigens, activates the complement system, initiating a cascade of events described below and illustrated in Figure 47.1A. More recently, the role of complement mediated cell destruction is also increasingly recognized in the context of delayed hemolysis and hyperhemolytic transfusion reactions.<sup>57</sup>

In the context of transfusion mediated immune hemolysis, the classical and the alternative complement pathways are relevant. The classical pathway is initiated by antibody–antigen immune complexes



**Figure 47.1** Pathophysiologic mechanisms of hemolytic transfusion reactions (HTR). Acute intravascular HTRs are typically characterized by complement-mediated destruction (A). Extravascular macrophage-mediated phagocytosis (B) is the hallmark of delayed HTRs. In addition to mechanisms (A) and (B), bystander hemolysis (C) of host RBCs due to complement activation by transfused RBCs, and reticulocyte suppression (D) and rarely bone marrow necrosis have been described in the context of hyperhemolysis, particularly notable in sickle cell disease.

via C1q recognition of Fc domains in conformationally altered immunoglobulin (Ig) M or clustered IgG. In contrast with the classical pathway, the alternative pathway is constitutively active and provides a primed and rapid response mechanism for the deposition of complement on foreign surfaces. This pathway accounts for about 80% of generated complement activation products (even if the classical pathway is initially activated), and as such it is a major driver of inflammation. Both pathways converge at the cleavage and activation of C3 with the subsequent generation of various biological effector molecules. Specifically, the formation of excessive terminal membrane attack complexes consisting of components C5 through C9 creates multiple functional pores in the transfused red-cell membranes, initiating intravascular osmotic lysis.<sup>55</sup> Consequently, free hemoglobin (Hb) is released into the intravascular space, which results in end-organ damage, including acute tubular necrosis and renal failure (Figure 47.1A).

In addition, complement components are also potent anaphylotoxins. Complement components C5a and C3a have proinflammatory effects, and the activation of less than 1% of C3 or C5 in plasma will cause cellular responses. These anaphylotoxins not only trigger

the inflammatory response but also directly alter the cellular functions of parenchymal cells. Their receptors are present on a wide variety of cells including monocytes, macrophages, neutrophils, platelets, endothelium, and smooth muscle (Figure 47.1A). The engagement of these complement components on this variety of cells can result in distinct immunological outcomes. Effects on myeloid cells results in chemokinesis, degranulation, and stimulation of the respiratory burst. Further, macrophages are stimulated to express proinflammatory cytokines. Tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) appears in plasma within two hours and has potent proinflammatory effects, including fever, leukocyte activation, procoagulant activity, and increased expression of a large number of genes related to the inflammatory response. TNF- $\alpha$  in the blood as a result of ABO incompatibility causes endothelial cells to express leukocyte adhesion molecules, chemokines, and procoagulant activity. Chemokines (and the chemokines CXCL8 (interleukin-8 [IL-8]) and CCL2 (monocyte chemoattractant protein-1 [MCP-1])) appear later than TNF- $\alpha$  and may be a secondary phenomenon. These chemokines have effects on granulocytes, lymphocytes, and macrophages expressing CXC and CC receptors.<sup>58</sup>

The resulting endothelial damage and increased capillary permeability contribute to the development of disseminated intravascular coagulation and multiorgan failure.

Incomplete complement activation also destroys incompatible red cells through C3b opsonization and monocyte-and-macrophage-induced erythrophagocytosis in the liver and spleen (extravascular hemolysis). As a potential pathophysiologic process in delayed hemolysis, complement-coated red cells are phagocytosed in stages. This gradual removal of red-cell membrane and surface area results in the formation of spherocytes and microspherocytes. This extravascular destruction process, with minimal release of free hemoglobin in the plasma, may also be mediated by immunoglobulins that are recruited by B-cell growth and differentiation factors (interleukin-1 $\beta$  and interleukin-6). Reactions to Rh antibodies and to other non-ABO antigens may be manifested in this manner.<sup>59,60</sup>

Most clinically significant hemolytic transfusion reactions are due to ABO incompatibility. However, antibodies to other blood group systems, such as Rh, Kell, Duffy, and Kidd, can be implicated in significant HTRs and may be fatal. Antigen density varies significantly for different RBC antigens, and its abundance influences the severity of the reaction. For example, ABO antigens are present at approximately 200,000–800,000 per cell, while Kell antigens are present at approximately 3000–6000 per cell, a 100-fold difference.<sup>61,62</sup> It is postulated that lower antigen density reduces antibody-induced clustering rendering C1q engagement and complement activation less efficient.<sup>63</sup>

Recent studies have elucidated the role of a chronically activated complementemia in the context of sickle cell disease.<sup>57</sup> Chronic hemolysis is a hallmark of sickle cell disease wherein increased free plasma hemoglobin, cell-free heme, and heme-loaded microvesicles directly activate complement. Further an overwhelmed and ineffective haptoglobin and hemopexin scavenging system may aid and abet this process.<sup>64</sup> Furthermore, sickle erythrocytes themselves appear to activate the alternative complement pathway by exposure of phosphatidylethanolamine and phosphatidylserine on these erythrocytes, amplifying hemolysis.<sup>65</sup> As in conditions like atypical hemolytic uremic syndrome, the already inflamed endothelium in patients with SCD, particularly following triggers such as pain crisis, acute chest syndrome, and/or infection, can drive terminal complement activation leading to end-organ damage very rapidly.<sup>57,66</sup>

### Role of macrophages

The role of macrophages in hemolysis (Figure 47.1B) has been studied since the 1950s.<sup>67</sup> Immune incompatible RBCs may become coated with IgG (IgG1, IgG2 or IgG3), IgA, or complement (C3b, iC3b), and these proteins react with receptors specific for these proteins present on macrophages (Fc receptors, CR1, CR3, and CR4) in the spleen and liver (Kupffer cells). Studies using chromium labeled RBCs starting in the 1980s have shown that macrophages may shorten RBC survival by at least three mechanisms. The macrophage surface expresses receptors for the Fc region of the immunoglobulin molecules, which enables engulfment and ingestion of the opsonized RBCs as a whole. Often, however, phagocytosis is incomplete and results in the formation of spherocytes. This has been explained in part by the removal of more membranes than volume. In addition, ectoenzymes on the macrophage surface cause microperforations of the RBC membrane, increasing its permeability and thereby promoting the transition

from a biconcave to a spherical shape of the cell.<sup>68</sup> Spherocytes are prone to further destruction during subsequent passages through the spleen. As a third mechanism, investigators have proposed that part or all the destruction may occur external to the macrophage by antibody-dependent cellular cytotoxicity (ADCC), wherein the macrophage secretes toxic molecules aiding the ADCC process. Some RBCs may escape immediate destruction but have a shortened lifespan because of macrophage-enzyme-induced membrane defects.<sup>60,69</sup> In the laboratory, the monocyte monolayer assay has been used to measure adherence and/or phagocytosis, but not ADCC. Chromium-labeled (<sup>51</sup>Cr) ADCC assays have provided mechanistic insights on the role of macrophages in ADCC.

Interactions between sensitized RBCs and macrophages may be influenced by multiple factors—Characteristics of antibody (class, subclass, specificity, thermal amplitude, complement-activating efficiency, and affinity and amount of galactose on the Fc carbohydrate), quantity of RBC bound, type of complement on RBCs, activity of the macrophage system, and/or CD47/inhibitor balance.<sup>70,71</sup> Although macrophages are undoubtedly the most important cell line involved, it is still unclear how important the role of other cells (e.g., lymphocytes [cytotoxic T-cells; natural killer, NK, cells]; dendritic cells; and granulocytes) can be in individual patients. All these cells can be shown to interact with sensitized RBCs (e.g., direct lysis) in vitro, when conditions are optimal, but are thought by most investigators to generally play a minor role.<sup>60,69</sup>

### Bystander hemolysis

Bystander hemolysis has been defined as the destruction of antigen-negative red cells during immune hemolysis; it plays an important role in DHTRs in SCD and is a hallmark of hyperhemolysis.<sup>72,73</sup> In their retrospective analysis, King *et al.* evaluated five patients with sickle cell disease (SCD) who underwent exchange transfusion and subsequently experienced a DHTR.<sup>72</sup> Serial samples were examined for complete blood counts, the percentage of hemoglobin A and S, and the percentage of reticulocytes. Some of these patients had a decrease in HbS red cells in addition to the transfused HbA cells suggesting the destruction of autologous red cells. This was evident in one patient despite a high/normal reticulocyte index providing additional evidence for bystander hemolysis. While the mechanism for bystander hemolysis is unclear, it may likely arise from autoimmunization following transfusion and red cell destruction.<sup>72</sup> Young and colleagues described two patients with autoimmune hemolytic anemia (AIHA) that was suspected to have been associated with previous blood transfusions. In a retrospective study, they found that 5% of 2618 patients with a positive DAT had demonstrable RBC autoantibodies, and 34% of these had a history of the positive DAT developing after transfusions and subsequently developed alloantibodies.<sup>72,74–76</sup> Several inciting events have been proposed. T lymphocytes from multiple transfused patients develop cytotoxicity against autologous cells; this was not necessarily associated with tissue damage. Other studies have demonstrated increases in preexisting endogenous antibody levels following transfusions, as well as alterations in self-reactive IgM and IgG antibody repertoires in plasma that are independent of a specific immune response to RBC antigens.<sup>70</sup>

### Reticulocyte suppression/destruction

Paradoxically, in severe delayed hemolytic transfusion reactions as well as with hyperhemolysis seen hemoglobinopathies, severe retic-

ulocytopenia has been reported following transfusions.<sup>77,78</sup> Studies have hypothesized that suppression or erythropoiesis and rarely bone marrow necrosis may contribute to this phenomenon.<sup>79,80</sup> Yet others have identified that both mature sickle cells and sickle reticulocytes adhere more readily to macrophages, and that hyperactive macrophages perpetrate a peripheral consumption rather than suppression of erythropoiesis, as evidenced by bone marrow erythroid hyperplasia in some of these cases.<sup>81</sup>

### Clinical manifestations and diagnosis

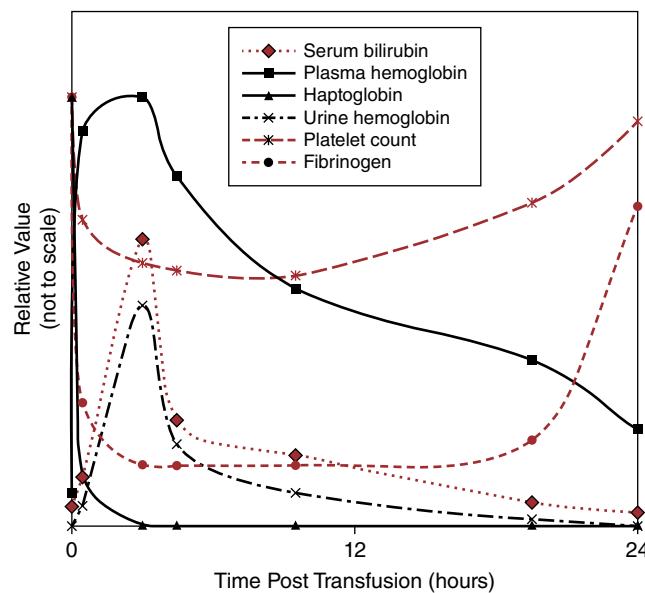
In an acute, immune-mediated hemolytic reaction, signs and symptoms present within 24 hours of the implicated transfusion event and may include fever and/or chills, flushing, nausea, vomiting, tachycardia, dyspnea, pain at the infusion site, abdomen/chest/flank/back pain, a subjective sensation classically described as “impending doom,” and red or dark urine signaling hemoglobinuria. These are not only broad and highly variable in their presentation, but also overlap with other etiologies, although fever is most commonly reported presentation. A high index of suspicion is warranted for prompt cessation of the transfusion and active investigation. The cause of pain is unclear, but may be related to spasm of coronary vessels, vascular occlusion of agglutinated cells, or direct bradykinin stimulation of nociceptive nerves in perivascular tissue.<sup>82,83</sup> In the anesthetized patient, hypotension refractory to fluid replacement, oliguria, red urine, and abnormal bleeding may be the only warning signs.

It has been traditionally believed that the severity of an AHTR is related to the total volume and infusion rate of incompatible blood, but even small volumes (10 mL) can produce rapid hemolysis and symptoms.<sup>37</sup> An analysis of 80 fatal ABO-incompatible transfusions reported to the FDA during 2000–2019 failed to detect a correlation between the number of transfused incompatible units and the time interval to death.<sup>17</sup> When an AHTR is suspected, the transfusion must be immediately stopped, and a bedside clerical check should be performed to verify the blood product label and patient identification band. Intravenous access should be maintained for supportive therapy. This should be followed by prompt reporting; a patient post-transfusion blood sample, along with the blood bag and remaining product if available, should be sent to the blood bank for evaluation. Initial laboratory testing typically includes a clerical check, visual inspection of the post-transfusion sample for hemolysis, a direct antiglobulin test (DAT), and verification of ABO and Rh typing. Ancillary tests that may be ordered by the physician include a post-transfusion CBC, LDH, urinalysis, serum haptoglobin, bilirubin, coagulation studies, blood smear, and hemoglobin electrophoresis. Table 47.2 summarizes the investigation of suspected transfusion reaction events, and Figure 47.2 illustrates a timeline of laboratory values in intravascular HTR.

Visual inspection of the postreaction plasma in an adult can detect as little as 10 mL of hemolyzed red cells, equivalent to free hemoglobin in the range of 20–50 mg/dL.<sup>86</sup> It should be remembered that free serum hemoglobin may also be present in nonimmune causes of hemolysis, hemoglobinopathies, severe burns, infusion of hemoglobin-based oxygen-carrying solutions, and after percutaneous intravascular thrombectomy.<sup>87</sup> If visual inspection is positive, a second sample is frequently requested to exclude mechanical factors due to an inappropriate blood draw. False-negative test results can be due to an excessive delay in the collection of the specimen, by which time the free hemoglobin has already been cleared

**Table 47.2** Investigation of Hemolytic Transfusion Reactions

Bedside	Blood Bank Laboratory
Stop transfusion Verify patient/product identifier Report incident	Visual check for hemolysis DAT ABO/Rh verification
Maintain IV access Draw post-transfusion sample and send to blood bank along with blood bag	Antibody screen in pre- and post-transfusion sample Eluate (include testing with A1 and B cells for non-group O recipients)
Additional baseline laboratory tests as indicated by physician (CBC, LDH, urinalysis, haptoglobin, bilirubin, BUN, creatinine, coagulation studies, blood smear, and Hb electrophoresis)	Antigen typing of units
Interconsultation with renal and intensive care specialist and management of complications as necessary	Repeat crossmatch with pre- and post-transfusion samples
	Investigation of nonimmune causes (Gram stain and culture, investigation of transfusion technique and blood storage conditions, check blood bag, and tubing and segments for hemolysis)



**Figure 47.2** Time course of hemolytic and coagulation parameters in intravascular HTR. Source: Based on Duvall *et al.* (1974).<sup>85</sup>

from the circulation. A low level of hemolysis may be challenging to detect visually in an icteric specimen.

A newly positive or stronger DAT supports an immune hemolytic etiology, but the strength of the testing does not correlate with the severity of the reaction. A false-negative DAT may occur if antibody-coated red cells have been already cleared from the circulation, or if the number of bound IgG molecules is below the sensitivity of the assay. Manual DAT using the spin antiglobulin tube method requires a minimum level of 100–150 bound anti-D IgG molecules for detection.<sup>88,89</sup> In a study that compared the relative sensitivity of DAT performed with monospecific IgG antiglobulin technique, flow cytometry, and antibody elution, the DAT could

detect 10% antibody-coated cells.<sup>90</sup> Antibody could be detected in the eluate of the samples with as little as 1% antibody-coated red cells present; flow cytometry, however, was consistently the most sensitive method with a detection limit of approximately 1%.<sup>90</sup> This underscores the need to perform an eluate when the clinical suspicion of hemolysis is high, a sensitive tool that can identify antibodies that are not detectable in plasma. In addition to the conventional reagent screening cells, the eluate should be tested with A and B cells in nongroup O patients that have received plasma containing products or immunoglobulin concentrates. Additional laboratory tests include repeating the antibody screen and confirming antigen typing and crossmatch of the implicated unit using pre- and post-transfusion samples.

Manifestations of DHTR appear more than 24 hours after the implicated transfusion, typically within the next two weeks but up to four weeks later, due to a delayed, anamnestic antibody response. Previous alloimmunization occurs from transfusion, pregnancy, or HSCT, with subsequent evanescence of the corresponding alloantibody titers to levels that are undetectable by common laboratory techniques. The most common signs of a DHTR are fever and/or chills, in addition to an unexpected hemoglobin drop (or failure to sustain a post-transfusion hemoglobin increment) due to the extravascular lysis of transfused cells. Hemoglobin degradation leads to an increase in plasma bilirubin levels and may manifest as jaundice 5–10 days after the triggering transfusion.<sup>83</sup> Figure 47.3 illustrates a time course of laboratory parameters in delayed, extravascular hemolysis. The degree of hyperbilirubinemia depends upon the patient's liver function and rate of red cell destruction. Spherocytosis may be observed in the blood smear. Laboratory testing will characteristically reveal a newly positive DAT. Eluate testing is important since it may be the only mean to identify the responsible alloantibody, which is often undetectable in plasma.<sup>15</sup> Once identified, segments from units previously transfused to the patient should be antigen-typed; this will provide an estimate of the burden of incompatible transfused erythrocytes with shortened survival.

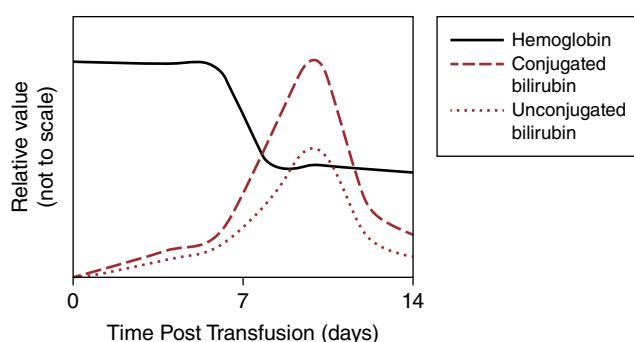
In patients with SCD, the clinical presentation of a DHTR may mimic a vaso-occlusive crisis, posing a risk of misdiagnosis.<sup>92–94</sup> In a retrospective five-year review of 637 adult SCD patients treated at a tertiary care center, at least one DHTR was documented in 7.7% of the 220 patients that received transfusions under an Rh and Kell-matched protocol.<sup>93</sup> Overall, a DHTR was implicated in 1.1% of the total 2158 documented transfusions in this study.<sup>93</sup> Most importantly, 47.8% of DHTR events were not diagnosed as such at the

time of presentation, most frequently mistaken for an SCD pain crisis.<sup>93</sup> The most common reported presenting symptom in this dataset was pain, followed by fever and hemoglobinuria.<sup>93</sup> A larger, 12-year retrospective analysis described 99 DHTRs in 69 adult SCD patients and reported hemoglobinuria as the most common presenting sign in 94% of cases, followed by pain in 89% and fever in 64%.<sup>95</sup> Only 7% DHTRs in this study were diagnosed at initial presentation in the emergency room.<sup>95</sup> Clinical signs of DHTR were reported to appear  $10.1 \pm 5.4$  days<sup>93</sup> and  $9.5 \pm 5$  days<sup>95</sup> post-transfusion in each of these studies. A significant fraction of these DTHR cases—56%<sup>93</sup> and 38%<sup>95</sup>—did not document the formation of new antibodies; in the remaining cases, new alloantibodies, autoantibodies, or nonspecific new reactivity were identified. No association was reported between the presence and specificity of the detected antibodies and the DHTR clinical severity.<sup>95</sup>

DHTRs are not only more frequent in the SCD population, but also more concerning due to the propensity to evolve into hyperhemolysis.<sup>3</sup> Awareness of the risk and variable manifestations of DHTR in these patients is critical to establish an early diagnosis and initiate appropriate interventions. A thorough transfusion history should always be obtained in SCD patients presenting with vaso-occlusive crisis, followed by the appropriate laboratory investigation. This includes the traditional hemolysis markers (CBC, LDH, serum bilirubin, urinalysis), hemoglobin electrophoresis, repeat DAT, antibody screen, and eluate. Comparison with a pretransfusion sample, and if available, a sample obtained shortly after transfusion, can provide valuable diagnostic information. The hallmark of hyperhemolysis is a hemoglobin nadir that is below the pretransfusion value. A predictive scoring system, based on a patient's previous DHTR episodes and their alloimmunization and transfusion burden history, is available for clinicians to estimate the risk of DHTR.<sup>96</sup> A second strategy employs a nomogram of expected HbA decay after transfusion, using the relative HbA change in relation to the post-transfusion elapsed time to calculate the risk of selective hemolysis.<sup>97</sup> The latter method requires two separate HbA percentage values, one taken as a baseline shortly (within one week) after transfusion, in addition to the value at the time that DHTR is suspected.

Drug-mediated immune hemolysis may present with a positive DAT and should also be considered in the differential diagnosis.<sup>98</sup> Suspicion of drug-induced immune hemolytic anemia (DIIHA) necessitates a detailed clinical history that establishes a temporal association between drug administration and the start of hemolysis. The list of drugs that have been implicated in DIIHA is vast and growing; the most frequent culprit reported by a research lab in California during the period of 2008–2013 was piperacillin, followed by ceftriaxone and cefotetan.<sup>99</sup> Other drugs frequently reported in the literature, in association with drug-dependent antibodies, include catechin, cephalothin, diclofenac, oxaliplatin, penicillin G, phenacetin, quinidine, rifampin, and tolmetin, among others.<sup>99</sup> In these cases, DIIHA classically presents in the laboratory as a positive DAT (IgG and/or C3), with a positive autocontrol and a negative eluate. Confirmatory testing in a reference laboratory involves the use of the soluble causative drug and/or manufacture of drug-treated reagent RBCs. Other medications, such as methyldopa, fludarabine, interferon, and mefenamic acid, have been associated with drug-independent antibodies and are therefore indistinguishable from warm autoantibodies by serologic methods.

Nonimmune hemolysis can have a similar clinical presentation to HTR (Table 47.1). Lysis of red cells can be caused by physical factors, such as overheating in a blood warmer or accidental freezing.<sup>100</sup>



**Figure 47.3** Time course of hemolytic parameters in extravascular hemolysis. Source: Cummins *et al.* (1997).<sup>91</sup> Reproduced with permission of Sage.

Excessive heat damages the RBC membrane, and this can cause spontaneous lysis of RBCs and result in intravascular hemolysis. The cells that have not already been lysed are cleared from the circulation by the spleen (extravascular hemolysis). Freezing injury occurs when red blood cells are exposed to below-freezing temperatures in the absence of a cryoprotective agent such as glycerol or dimethyl sulfoxide (DMSO). This can lead to a dehydration injury if the freezing is slow, or ice crystal formation if the freezing is rapid, both resulting in intravascular destruction. Hemolysis can also be caused by inadequate removal of glycerol from frozen red cells,<sup>101</sup> by attempting to force blood through a filter or small-bore needle,<sup>102</sup> or from defective pumps or blood administration sets. Infusion of blood in the same tubing with hypotonic solutions can lead to entry of free water into the RBCs, with subsequent cellular swelling and intravascular lysis. Intravenous DMSO infusion has been reported to mimic HTR.<sup>103</sup> Resorption of a hematoma can have manifestations very similar to extravascular HTR as well, including hyperbilirubinemia, elevated LDH, and depressed haptoglobin levels.<sup>83</sup> In addition, the presence in the serum of fibrin degradation products from the hematoma may be mistaken with DIC, one of the known complications of HTRs.<sup>86</sup> Transfusion of blood from donors with glucose-6-phosphate dehydrogenase (G6PD) deficiency can lead to hemoglobinemia and hyperbilirubinemia in the recipient<sup>104</sup> and may be particularly marked in the setting of concomitant oxidative medications.

Establishing the diagnosis of HTR may be particularly difficult in patients with liver disease, autoimmune hemolytic anemia (AIHA), sepsis, or active bleeding, due to overlapping features and laboratory abnormalities. Characteristically, in both bleeding and AIHA there is proportionate loss of both autologous and transfused red cells. One indication of HTR in these settings is the persistence of transfused red cells that lack the implicated antigen, but the absence of transfused cells bearing the antigen.

## Complications

Although the half-life of IgG is approximately 3–4 weeks, a positive direct antiglobulin test (DAT) by IgG may persist for longer periods than the transfused cells, and there may be evidence of complement deposition on autologous red cells, with a positive DAT with C3 persisting for weeks to months.<sup>105</sup> Autoantibodies are frequently found concomitantly with alloantibodies, with a reported range of 15–53%.<sup>106–108</sup> A retrospective review of cases with coexistent auto and alloantibodies reported that 75% were associated with a red cell transfusion.<sup>106</sup> Autoantibodies are also a common specificity developed by SCD patients with DHTR and/or hyperhemolysis.<sup>93,95</sup>

Hypotension occurs in some cases of intravascular HTR but is rare in extravascular reactions. Complement activation, with subsequent release of vasoactive peptides, is likely to be the most important determining factor (Figure 47.1A). As discussed previously, the anaphylatoxins C3a and C5a are released during immune hemolysis. Additionally, consumption of C1-esterase inhibitor contributes to activation of the kinin pathway, leading to generation of bradykinin.<sup>109</sup> The proinflammatory tumor necrosis factor (TNF) produced by phagocytes during HTR may also contribute to hypotension and shock.

Intravascular hemolysis also modulates blood pressure and local blood flow through alterations in the metabolism of the potent physiologic vasodilator nitric oxide (NO). NO can combine with heme and thiol groups of hemoglobin.<sup>110</sup> In oxyhemoglobin, NO

causes reduction of ferrous iron ( $\text{Fe}^{2+}$ ) to form ferric methemoglobin [ $\text{Hb}(\text{Fe}^{3+})$ ]. NO combines with deoxyhemoglobin to form  $\text{Hb}(\text{Fe}^{2+})\text{NO}$  but does not cause reduction. Scavenging of NO by free hemoglobin through these pathways results in vasoconstriction and hypertension. NO can also combine with cysteine on the beta-globin chain to form S-nitrosohemoglobin (SNO-Hb). This process is reversible, so SNO-Hb can act as a NO donor with resultant vasodilation. Free hemoglobin reacts with NO much more rapidly than does intraerythrocytic hemoglobin.<sup>111</sup> The effects of intravascular hemolysis may be very similar to the infusion of stroma-free hemoglobin (i.e., hemoglobin-based oxygen carriers [HBOCs]). Indeed, prominent hypertensive effects limited early trials of hemoglobin solutions as a blood substitute.<sup>112</sup>

Disseminated intravascular coagulation (DIC) is a rare complication of intravascular HTRs and is reported even less frequently in extravascular hemolysis. The production of proinflammatory cytokines, triggered by potent complement activation, is likely to be a major factor in this complication (Figure 47.1A). It can be challenging to distinguish DIC from other causes of coagulopathy, particularly in massive transfusion or liver disease. Uncontrolled bleeding caused by DIC may be the initial manifestation of an acute HTR in the intraoperative setting. Unfortunately, if HTR is not recognized early in this situation, more incompatible blood may be transfused in an attempt to keep up with blood loss. In one report, 9 of 35 patients experiencing HTR while under anesthesia received 4–6 additional units because of excessive bleeding.<sup>113</sup>

Impairment of renal function is seen in both intravascular and extravascular HTR, although it is more common in the former. The degree of renal function abnormality varies from an asymptomatic elevation of serum blood urea nitrogen (BUN) and creatinine to complete anuria. Both hypotension and intravascular coagulation contribute to renal impairment. Thrombus formation in renal arterioles caused by disseminated intravascular coagulation (DIC) may cause cortical infarcts. Free hemoglobin contributes to renal injury, causing so-called pigment nephropathy. Experimental evidence indicates that hemoglobin is toxic to renal tubular epithelium cells.<sup>114</sup>

Recent epidemiologic data on the mortality of HTRs were discussed previously. Mortality due to AHTRs is historically believed to depend on the volume of incompatible red cells transfused.<sup>83</sup> A review of 41 HTRs causing acute renal failure indicated that no deaths occurred among patients receiving less than 500 mL of incompatible blood; there was 25% mortality in the group receiving 500–1000 mL and 44% mortality among those receiving greater than 1000 mL of incompatible blood. However, the transfusion of even small amounts of incompatible blood is not necessarily safe. A 20-year retrospective review of ABO-incompatible transfusion fatalities reported to the FDA failed to identify a significant correlation between the number of RBC units transfused (dichotomized at  $\leq 1$  unit or  $> 1$  unit) and the reported time interval of days until death.<sup>17</sup>

A rapid hemoglobin drop due to HTR could itself exacerbate any preexisting SCD complications in this patient group.<sup>115</sup> Development of acute chest syndrome as a complication of DHTR was documented in 50% of cases, hepatic dysfunction in 25.3%, followed by pulmonary hypertension (16.2%) and renal failure (10.1%). ICU admissions were required in 41.4% of cases, with a mean ICU stay of  $6.2 \pm 4$  days. Mortality as a complication of DHTR in SCD patients was reported as 6%, with a mean interval of  $10 \pm 2$  days between the implicated transfusion and the patient's demise.<sup>95</sup>

**Table 47.3** Therapeutic Options in HTR

Therapeutic Intervention	Indication	Typical Dose or Goal
Hydration	Prevention of renal impairment	Maintain urine output to 0.5–1 mL/kg
Supplemental diurects	Prevention of renal impairment	Furosemide 40 mg IV bolus and then 10–40/mg. Monitor for hypotension
Alkalization of urine	Prevention of renal impairment	$\text{NaH}_2\text{CO}_3$ 130 mmol/L in 5% dextrose at 200 mL/hour; maintain urinary pH > 6.5, discontinue if it exceeds 7.5
Blood product transfusion	Treatment of DIC complications	Maintain platelet > 20,000/mcL, INR > 2.0, fibrinogen > 100 mg/dL
Glucocorticoids	Prevention of hemolysis in incompatible transfusion	Hydrocortisone 100 mg before transfusion and 24 hours later
IVIG	Prevention of hemolysis in incompatible transfusion	1.2–2.0 g/kg over 2–3 days

## Management

An AHTR is considered a medical emergency. The first important intervention is interrupting any ongoing transfusion and avoiding further infusion of incompatible blood, followed by initiation of the establishment's transfusion reaction response and investigation procedure. Clear protocols must be in place at each institution describing the interdisciplinary steps in this process. Intravenous access should be maintained, and baseline laboratory samples should be collected as instructed by the physician. Therapy is primarily supportive, following current practices for the management of shock, DIC, and renal failure, as required by the patient's clinical evolution.<sup>83</sup> Renal consultation is advised to assess the need for dialysis, and admission to the intensive care unit may be required. Vigorous hydration is recommended to maintain a minimum urine output; supplemental diuretics and forced alkaline diuresis may also be helpful (Table 47.3). Renal-sparing pressor support with dopamine may be required if hypotension develops, and any electrolyte abnormalities should be corrected. DIC should be addressed with blood product support to maintain minimal platelet, INR, and fibrinogen values.

Pretreatment with glucocorticoids and IVIG has been employed with some success when transfusion is clinically warranted and compatible RBC units are unavailable.<sup>116</sup> The monocyte monolayer assay has been useful in predicting the clinical significance of an alloantibody, and thus the probability of a hemolytic transfusion reaction, upon infusion of incompatible blood; but this assay requires specialized laboratory resources and skills.<sup>117</sup> A regimen of rituximab and methylprednisolone has been employed in heavily alloimmunized patients undergoing transplantation with incompatible grafts. Appropriate selection of blood components, which should be donor and recipient compatible, is essential in the peritransplant period of an ABO-incompatible HSCT. For minor incompatible grafts, awareness of the risk of PLS by the clinical team, watchful monitoring of hemoglobin levels 5–14 days post-transplantation, and prompt transfusion support with donor and recipient-compatible blood products are recommended.

DHTRs are often asymptomatic or clinically mild, with the exception of SCD patients who can present as a vaso-occlusive crisis or develop hyperhemolysis. Early diagnosis of hyperhemolysis is critical for prompt initiation of the appropriate management strategy. This typically includes avoiding further transfusions, except for severe cases with signs of end-organ hypoperfusion. Documented management strategies for DHTR in SCD include watchful and supportive, use of erythropoietin stimulating agents, and immunomodulatory drugs (IVIG, corticosteroids, rituximab, bortezomib, eculizumab, and tocilizumab).<sup>118–122</sup> Literature is often limited to case reports and case series; further studies are needed to better understand the underlying pathophysiology and to prospectively evaluate different treatment strategies and their clinical benefit. In

DHTRs that present without detectable antibodies, testing should be repeated three months after the event to capture any new, developing antibodies and establish the appropriate future transfusion strategy for each patient.

## Prevention

Many core blood bank procedures are ultimately directed toward the prevention of HTRs. Proper typing of donor units, pretransfusion testing, antibody identification, and crossmatching are critical elements that are discussed in Chapter 28.

A robust hemovigilance system is essential to document HTRs, dissect their underlying causes, and implement the appropriate corrective and preventive actions at a system-wide level. National hemovigilance surveillance systems, often coupled with open, periodic data sharing, have proven invaluable for the global transfusion community in its quest for continuous quality improvement and maximum patient safety.<sup>123</sup> The World Health Organization encourages the implementation of such a surveillance system at a national scale and provides a detailed guide document for this purpose.<sup>124</sup> Participation in the International Haemovigilance Network (IHN) has progressively increased, with 41 countries listed as members at the time of this writing.<sup>125</sup>

Lessons extracted from recent hemovigilance reports include the need for simple and clear standard operating procedures in the laboratory, rigorous competency assessment practices, addressing staff shortages, investigating incidents and near-misses systematically with a learning approach, and establishing a just patient safety culture.<sup>15</sup> The 2019 SHOT report recommends that all staff receive holistic training, including a focus on human factors. This refers to the study of human elements such as teamwork, workspaces, tasks, equipment, and culture within the functions of a clinical setting. Adequate staffing is critical to ensure safe patient care; four of the six ABO-incompatible transfusions reported in 2019 in the UK occurred after 8:00 pm or before 8:00 am,<sup>15</sup> when there is likely to be minimal or only one laboratory staff member. Elements of a strong safety culture include a climate where staff feels motivated and safe to report incidents and where this behavior is encouraged and even rewarded, a willingness to adapt to changing circumstances and demands, a growth mindset with a desire to learn from incidents and near-misses, and an open environment where practices and protocols are regularly questioned.<sup>9</sup> The concept of Safety-II was set forth to describe a proactive approach that recognizes existing health-care resilience and successful events, capitalizing on the lessons therein.<sup>126</sup>

A health system that views patients as active participants in their care, rather than only as passive recipients, supports safe health-care practices. In transfusion medicine, this includes asking patients for a history of alloimmunization or transfusion reactions, and ensuring that this information is documented and acted upon.<sup>15</sup> Establishment

of universally available alloimmunization registries has been advocated to ensure appropriate blood product selection for patients that are transfused at multiple sites or that have a history of evanescent alloantibodies, which pose risk factors for HTRs.<sup>127–129</sup> A review of fatalities due to ABO-incompatible RBC transfusions, reported to the FDA over a 20-year period, confirms the importance of verification practices for the ABO blood group and the value of electronic identification systems.<sup>17,130,131</sup> Rigorous labeling and patient identification protocols, including a final bedside check, remain critical elements for transfusion safety and prevention of HTRs.<sup>17,132</sup>

Full crossmatch procedures and selection of donor units lacking antigens corresponding to a patient's known clinically significant alloantibodies are essential for the prevention of HTRs. The concept of prophylactic red cell antigen matching has been advocated for hemoglobinopathy patients as a strategy to prevent alloimmunization in this frequently transfused group that is also known to have higher alloimmunization risk.<sup>127,133,134</sup> The rationale for prophylactic antigen matching is to provide these patients with units that are matched for RBC antigens beyond ABO and RhD, in order to prevent future alloimmunization and therefore reduce the risk of ensuing difficulty in finding compatible units, transfusion delays, and HTRs.

Guidelines from the American Society of Hematology and the International Collaboration for Transfusion Medicine (ICTM) recommend prophylactic matching for Rh (C/c and E/e) and K antigens in patients with SCD;<sup>127,134</sup> ICTM extends these recommendations to β-thalassemia patients as well. Extended matching to include Jk<sup>a</sup>/Jk<sup>b</sup>, Fy<sup>a</sup>/Fy<sup>b</sup>, and S/s is mentioned in these two guideline documents as a strategy to further reduce the possibility of alloimmunization in these patients. Provision of extended-matched units may be hindered by a limited inventory of compatible donor units,<sup>135,136</sup> and care should be taken to avoid transfusion delays that would have an adverse clinical impact.<sup>134</sup> Although extended matching has been shown to result in a lower incidence of red cell antigen alloimmunization compared to ABO/RhD matching alone,<sup>137</sup> no studies are yet available to address the impact of prophylactic antigen matching in clinical outcomes. Thus, the optimal degree of antigen matching in this patient population is still uncertain, and further trials are needed to clarify the best strategy and evaluate its clinical value.

Guidelines suggest obtaining an extended red cell antigen profile for hemoglobinopathy patients at the earliest opportunity.<sup>127,134</sup> Genotyping assays are advantageous over serologic phenotyping for recently transfused patients, and they can provide information for antigens for which no commercial antisera is available.<sup>138</sup> In addition, molecular platforms can precisely identify genomic variations associated with weak or partial antigen expression, and they can interrogate the silencing promoter variant that is frequently encountered upstream of the Fy<sup>b</sup>-encoding gene in individuals of African descent.<sup>138,139</sup> A comparison of hemagglutination and molecular RBC antigen typing in 494 SCD patients at a pediatric hospital reported higher accuracy and a larger information yield from genotyping assays.<sup>140</sup> Although genotyping cost and availability are important considerations, declining sequencing price and development of new technologies augur a feasible solution in the future.<sup>141–143</sup> RH genotype matching for provision of blood products in SCD patients has been explored and would likely require concerted efforts to increase the ethnic diversity of the donor pool.<sup>135,144</sup>

Transfusion services should have a policy addressing passive transfer of ABO-alloantibodies in plasma-containing products. Strategies currently employed to mitigate this risk include screening for platelet units that exceed a titer threshold that has been deemed

acceptable by the transfusion service,<sup>145,146</sup> limiting the quantity of incompatible plasma products transfused per patient,<sup>21</sup> volume reduction of ABO-incompatible platelet products,<sup>21,147</sup> and dilution with platelet additive solution.<sup>30</sup> The optimal ABO-titer threshold and its clinical significance remain undefined;<sup>29,34</sup> thus, these strategies might evolve as future research resolves with more detail the patient and immune determinants of hemolysis in this scenario.

Hemolysis in the setting of major and/or minor ABO-incompatible HSCT can be tempered by graft red cell or plasma volume reduction, respectively.<sup>148,149</sup> Reduction of graft-directed ABO antibodies has also been documented through therapeutic plasma exchange or through deliberate transfusion of incompatible, donor-type RBCs prior to transplant.<sup>150,151</sup> Establishment of transfusion policies for blood product selection in the peri-transplant period, considering ABO and donor/recipient alloimmunization to other red cell antigens, is also important.<sup>38</sup>

## Summary

Although the incidence of HTRs has significantly reduced during the last decade, the prevention of these adverse transfusion reactions continues to be a major focus in transfusion medicine. Establishment of robust hemovigilance programs and systematic protocols to identify and investigate all suspected HTRs remain as central practices. Advances in the understanding of the pathophysiology of HTRs and hyperhemolysis continue to provide insights to help guide the management of patients undergoing these reactions.

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## CHAPTER 48

# Nonhemolytic transfusion reactions

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This chapter reviews a variety of acute, nonhemolytic, and noninfectious transfusion reactions, the most common of which are febrile, nonhemolytic transfusion reactions (FNHTRs) and allergic reactions. Other acute, nonhemolytic reactions are reported less frequently and include transfusion-related acute lung injury (TRALI) and anaphylactic or anaphylactoid reactions. Additional acute adverse effects can occur in massive transfusion because of the large volume of blood components transfused over a short period. The complications of massive transfusion include dilutional coagulopathy, hypothermia, citrate toxicity, and electrolyte disturbances, among others. Some patients cannot tolerate the acute increase in intravascular blood volume caused by transfusion and experience the complications of transfusion-associated circulatory overload (TACO). Acute reactions can be caused by the toxicity of chemicals that leach into blood components from blood storage containers or filters or by chemicals added to improve storage conditions, such as dimethyl sulfoxide (DMSO). Other reactions are caused by endogenous mediators generated in the blood during filtration, processing, or storage, such as bradykinin-mediated hypotensive reactions. It is important that these complications of transfusion be recognized by patient care teams and blood bank personnel, and that appropriate treatments and preventive measures be instituted for patient safety and well-being.

## Febrile nonhemolytic transfusion reactions

### Description

An FNHTR is commonly defined as an increase in body temperature of 1 °C or more that occurs during or within several hours of transfusion and is unrelated to hemolysis, sepsis, or other known causes of fever. The use of a 1 °C increase in body temperature as a threshold for defining an FNHTR avoids undue concern over small fluctuations in body temperature unrelated to transfusion which do not justify discontinuation of transfusion and follow-up investigation. Many FNHTRs begin with the patient feeling uneasy and experiencing chills. In mild reactions, the signs and symptoms do not progress. Chills with or without an increase in body temperature can be classified as an FNHTR if other possible causes of chills

are unlikely and the time course of the reaction correlates with the transfusion. In the most severe reactions, patients may experience rigors (severe shaking chills) or a fever elevation of 2 °C or more over baseline. Although signs and symptoms usually are limited to chills and fever, some patients may also rarely experience more severe symptoms such as headache, nausea, and/or vomiting.

The fever of FNHTR usually persists no more than 8–12 hours after the start of transfusion. If fever persists 18–24 hours or longer, it is unlikely to be transfusion related. Generally, FNHTRs are self-limited and have no sequelae. However, elderly patients, patients with compromised cardiovascular status, or critically ill patients are at risk of cardiorespiratory complications associated with FNHTR. Because fever increases oxygen demand and consumption by an estimated 13% for every 1 °C over 37 °C and shivering increases oxygen demand approximately 300%, FNHTRs can aggravate pre-existing cardiac, pulmonary, and cerebrovascular insufficiency. Therefore, prompt recognition and antipyretic management of FNHTRs can be very beneficial.

An FNHTR almost always is associated with the transfusion of cellular blood components, such as red cells, platelets, and granulocyte preparations, and less commonly with noncellular components, such as plasma and cryoprecipitate. The incidence of FNHTR varies widely, and median rates have been reported as higher for platelets (4.6%) than for red cells (0.33%).<sup>1</sup>

The reaction risk of blood components, however, varies according to numerous factors, such as the method of preparation of the blood component (e.g., leukocyte reduction, storage time, medications, patient and donor characteristics, monitoring practices, and many others). These factors vary among different geographic regions and medical centers. In addition, rates based on reactions reported to blood banks are lower than those based on systematic surveillance of responses to all transfusions. One study demonstrated that the rate of reported FNHTR was low for prestorage-leukocyte reduced pooled platelets compared to poststorage-leukocyte reduced pooled platelets.<sup>2</sup> Longer platelet storage times are also associated with higher rates of FNHTR.<sup>3–5</sup> Reactions are also more frequent among certain recipients, such as multitransfused patients or multiparous women who have developed leukocyte or platelet alloantibodies.

## Etiology

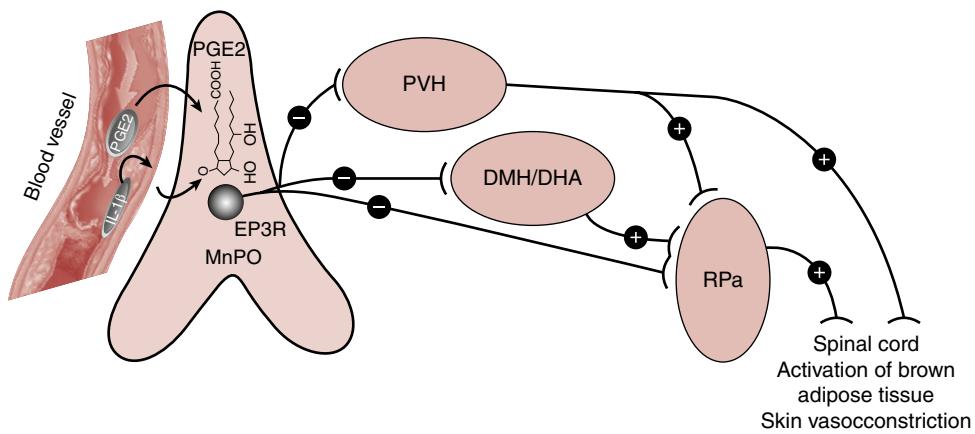
An FNHTR appears to be part of the systemic inflammatory response syndrome (SIRS) provoked in transfusion recipients by the immune challenge of transfusing foreign cells or infusing soluble inflammatory mediators present in stored blood components. The term *systemic inflammatory response syndrome* was coined to describe the constellation of observed body responses to various insults, such as infection, trauma, burns, and ischemia. It is defined as the presence of two or more of the following: body temperature more than 38 °C or less than 36 °C; heart rate more than 90 beats/minute; tachypnea (respiratory rate >20 breaths/minute or  $\text{PaCO}_2$  less than 32 mmHg); and white cell count more than 12,000/ $\mu\text{L}$  or less than  $4 \times 10^9/\text{L}$ , or more than 10% immature neutrophils (band forms). Although a mild FNHTR may not completely fulfill these criteria, FNHTR is nevertheless an inflammatory response.

Exogenous pyrogens such as lipopolysaccharide (LPS) and pyrogenic cytokines initiate a series of responses leading to hyperthermia. These responses include rapid muscle contractions that cause shivering, rigors, and an increase in heat generation. Heat conservation is achieved through cutaneous vasoconstriction, which also contributes to the sensation of a chill. Perceived chills lead to behavioral changes that can further increase body temperature. For example, the patient may cover up, and the result is inhibition of heat dissipation.

An FNHTR appears to have two possible underlying causes: (1) the more “classical” pathway of infused antigens, such as leukocytes, that stimulate the *in vivo* generation of cytokines in the recipient; and (2) the infusion of pyrogenic cytokines or other inflammatory response mediators (e.g., activated complement proteins, LPS, or neutrophil-priming lipids) that accumulate in the plasma portion of cellular blood components during storage.<sup>5–6</sup>

Other causes include immune recognition of donor leukocytes by recipient antileukocyte antibodies, passive transfer of donor inflammatory cytokines, and immune destruction of incompatible donor platelets by recipient antibodies.<sup>7</sup> Another cause of fever is the infusion of blood components contaminated with bacteria or bacterial products, will produce a febrile

response, but it is not usually categorized as an FNHTR but rather as a bacterial septic reaction, if recognized. The common pathway by which these different stimuli induce post-transfusion fever has been attributed to an increase in circulating pyrogenic cytokines in the recipient, such as interleukin-1 $\beta$  (IL1 $\beta$ ), IL6, and tumor necrosis factor  $\alpha$  (TNF $\alpha$ ). Pyrogenic cytokines induce fever by mediating upregulation of the thermostatic set point for body temperature in the thermoregulatory center of the hypothalamus. This mechanism is supported by the association of febrile reactions with a specific cytokine polymorphism *IL1RN*\*2.2 genotype.<sup>8</sup> Severe nonhemolytic transfusion reaction have also being linked to inflammatory cytokines and chemokines generated by mononuclear cells in donor blood by HLA Class II antibody containing plasma unit.<sup>9</sup> Recently, research has led investigators to a more complex model of fever generation, building on a model where cytokines principally induce fever to one where central nervous system stimulation by prostaglandin E2 (PGE2) is pivotal. Alternative hypotheses have resulted from the finding that a febrile response to LPS occurs even with blockade of either IL1 or TNF $\alpha$  and that the presence of circulating cytokines lags behind the development of fever.<sup>10</sup> Research has found that LPS-induced C5a production via complement activation results in rapid peripheral PGE2 production.<sup>11</sup> In addition, LPS binds to toll-like receptor 4 and induces cytokine production, leading to a two-phase rapid and delayed febrile response. Hyperthermic stimuli compete with hypothermic stimuli to achieve a central thermal balance point that may elevate or decrease based upon physiologic stimuli.<sup>12</sup> Central to the febrile response is the presence of EP3 prostaglandin receptors that bind PGE2 in the hypothalamus (Figure 48.1).<sup>13</sup> These newer models of the fever response provide possible explanations for why FNHTRs continue to occur despite prestorage leukocyte reduction that minimizes cytokine accumulation in storage. Clinical evidence also supports the hypothesis that febrile reactions can be caused by noncytokine hyperthermic stimuli present in cellular transfusion products.<sup>14</sup> More research is needed for the development of more targeted antipyretic medications that may eventually lead to the extinction of FNHTRs.



**Figure 48.1** A model for neuronal action of the EP3 receptors (EP3Rs) in the fever response after systemic immune challenge. In response to lipopolysaccharides or cytokines, prostaglandin E2 (PGE2) is generated by macrophages of the liver and lung and endothelial cells lining venules in the brain. PGE2 is transported or diffuses across the blood-brain barrier and acts on EP3Rs in the median preoptic area (MnPO). These are hypothesized to be GABAergic neurons that inhibit thermogenic systems in the hypothalamic paraventricular nucleus (PVH), the dorsomedial nucleus/dorsal area (DMH/DHA), and the raphe pallidus nucleus in the medulla (RPa). These thermogenic nuclei activate sympathetic preganglionic neurons in the spinal cord, resulting in cutaneous vasoconstriction and activation of brown adipose tissue that ultimately raise body temperature.

Source: Based on Lazarus *et al.* (2007).<sup>13</sup>

### **Alloimmunization to leukocytes or platelets**

Transfusion recipients at greatest risk of an FNHTR are those with leukocyte or platelet antibodies who receive transfusions with blood components containing large numbers of passenger leukocytes or platelets.<sup>15–16</sup> Less frequently, donor antibodies to leukocytes, present in the plasma portion of blood components, are associated with FNHTRs. The implicated antibodies most often have HLA specificity, although they also may be platelet- or granulocyte-specific. A minimum of approximately  $1 \times 10^7$  leukocytes per unit of red blood cells (RBCs) appears necessary to cause an FNHTR, although this number varies among individuals.<sup>17–18</sup> The role of donor leukocytes in FNHTR is supported by the finding that decreasing the leukocyte content of blood components below this threshold reduces the incidence of FNHTRs.

A variety of mechanisms are possible by which antibody-leukocyte or antibody-platelet interactions cause fever. For example, donor monocytes may be activated and secrete pyrogenic cytokines when recipient antibodies bind to them. An alternative explanation is that immune complex formation between recipient antibodies and donor leukocytes or platelets leads to the generation of activated complement components, such as C5a, which stimulate the production of PGE2.

### **Storage-generated cytokines**

Antibodies to leukocytes or platelets do not appear to account for all FNHTRs, particularly those caused by platelet transfusions. For example, some patients with no history of transfusion or pregnancy experience an FNHTR to their first transfusion of platelets.<sup>19</sup> It is unlikely that these reactions are mediated by recipient leukocyte or platelet antibodies because these recipients have no previous exposure to foreign cells. In addition, the rate of FNHTRs to platelet transfusion increases with increasing blood bank storage time of the transfused platelet concentrate.<sup>3–5</sup> This indicates that time-dependent change occurs in the platelet concentrate during storage that has a role in stimulating an FNHTR in some patients. Furthermore, febrile reactions still occur with the use of prestorage leukocyte reduction. In some cases, this is the result of inappropriate filter use or filter failure. However, this observation also supports the possibility that a substance or substances in the plasma portion of blood components not removed by filtration may be responsible for mediating at least some FNHTRs. The discovery that proinflammatory cytokines accumulate in the plasma portion of platelet concentrates may account for many of these findings.

A variety of leukocyte-derived, proinflammatory cytokines, including IL1 $\beta$ , IL6, IL8, tumor necrosis factor- $\alpha$  (TNF $\alpha$ ), macrophage inflammatory protein-1 $\alpha$  (MIP1 $\alpha$ ), and growth-related oncogene- $\alpha$  (GRO $\alpha$ ), are generated and accumulate in the plasma portion of platelet concentrates during storage.<sup>20–22</sup> Extracellular levels of these cytokines generally increase with increasing component storage time and are roughly proportional to the passenger leukocyte content of the blood component bag. Prestorage or early storage leukocyte reduction (within 1–2 days of collection) prevents or greatly reduces the generation of these cytokines. Because they have pyrogenic activity, many of these cytokines (if present in high enough concentration) can induce febrile responses in transfusion recipients. Elevated levels of IL1 $\beta$ , IL6, and TNF $\alpha$  in the plasma portion of platelet concentrates correlate positively with the occurrence of an FNHTR. Some studies have shown that IL6 levels in the plasma portion of platelet concentrates correlate best with the occurrence of FNHTR. In one study, chills, fever, or both occurred more frequently after infusion of the plasma portion of the platelet

concentrates than after infusion of the cellular portion containing platelets and leukocytes.<sup>20</sup> The plasma portions that caused an FNHTR contained higher levels of IL1 $\beta$  and IL6 than did those that did not cause chills or fever. These data support the role of the plasma portion of platelet concentrates as a source of inflammatory mediators and as a possible stimulus of FNHTR in some transfusion recipients. Although levels of a variety of proinflammatory cytokines in the plasma portion of platelet concentrates correlate with the occurrence of FNHTRs, it is unknown which, if any, of these actually mediate FNHTRs.

Soluble CD40L (sCD40L) in the supernatant of stored platelets can induce cytokines, chemokines, and lipid mediators by activating CD40 bearing cells. Increased levels of sCD40L in transfused blood are associated with milder transfusion reactions such as fever and rigors.<sup>23</sup>

Platelet-derived cytokines, such as CD40L (CD154), CCL5 (RANTES), transforming growth factor  $\beta$ 1 (TGF- $\beta$ 1), CXCL4 (platelet factor 4; PF4), CXCL8 (IL8), and MIP1 $\alpha$ , are present in the plasma portion of platelet concentrates and apheresis platelets. All platelet-derived cytokines accumulate during platelet storage, and CD40L has been associated with clinical FNHTRs.<sup>24</sup> These cytokines are not known to be directly pyrogenic, but they stimulate the synthesis of proinflammatory mediator, IL1 $\beta$ , IL6, IL8, and TNF $\alpha$ . Because RANTES can activate basophils and mast cells and stimulate histamine release, it may play a role in mediating some allergic reactions (see the “Allergic reactions” section).<sup>25</sup>

Some proinflammatory cytokines, such as IL1 $\beta$  and IL8, have been detected in the supernatant portion of stored RBCs, although at much lower levels than in platelet concentrates.<sup>26</sup> Because the cold storage temperature of RBC units, 1–6 °C, has an inhibitory effect on cellular metabolism, the capacity of passenger leukocytes in RBC units to synthesize and secrete cytokines is less than those in platelet concentrates. As a result, the levels of cytokines in RBC units appear to be too low to mediate significant physiologic reactions.

The stimulus for cytokine generation during storage of blood components remains unknown. Measurements of cytokine messenger RNA levels and total cytokine levels (intracellular plus extracellular) in platelet concentrates indicate that accumulation of leukocyte-derived cytokines is caused in part by new synthesis and secretion. The stimulus for synthesis and secretion of leukocyte-derived cytokines may be, for example, contact activation of monocytes after these cells interact with the plastic of the storage containers or tubing.<sup>27</sup> Other possibilities include the direct stimulatory effects of C5a on PGE2 or the stimulatory effects of activated complement components on monocytes or other leukocytes in the blood component bag. The presence of platelet derived cytokines (e.g., CD40L, RANTES, and TGF- $\beta$ 1) in the plasma portion of platelet concentrates likely results from their release from preexisting stores because the biosynthetic activity of platelets is limited.

### **Bacterial contamination of blood components**

An FNHTR may result from infusion of a blood component contaminated with bacteria or bacterial products such as LPS. Unless a Gram's stain and bacterial cultures are performed, mild septic transfusion reactions characterized by only fever and chills are likely to be classified clinically as FNHTR, especially if the patient is concurrently receiving antibiotic therapy for their clinical condition.<sup>28–29</sup>

Transfusion reactions caused by contaminating bacteria, whether mild or severe, are manifestations of the SIRS, described earlier.

Proinflammatory cytokines (such as IL1 $\beta$ , IL6, IL8, and TNF $\alpha$ ) are implicated in the pathogenesis of SIRS associated with sepsis.<sup>30</sup> If bacterial contamination of blood components has occurred, the greatest source of cytokines is likely the transfusion recipient's cells stimulated by the infused bacteria or bacterial products. However, cytokine production by leukocytes in the component bag during storage stimulated by bacteria or bacterial products may also contribute to the reaction.

### Diagnosis

As a routine part of the transfusion procedure, the vital signs of transfusion recipients (pulse, temperature, and respiratory rate and oxygen percent saturation) should be measured immediately before transfusion and at intervals during and soon after transfusion. The patient should be watched closely, particularly in the first 30–60 minutes of transfusion, for the onset of chills, shivering, or rigors, which often precede a fever.

A transfusion reaction is a possibility if chills, fever (1 °C or more over pretransfusion temperature), or both develop any time during transfusion or up to several hours after the transfusion has ended. A febrile response to transfusion, however, is not specific for an FNHTR. For example, a fever may be the early manifestation of a more serious acute hemolytic, septic, or TRALI transfusion reaction. When a patient has a febrile reaction to transfusion, an evaluation to rule out hemolysis and possibly bacterial contamination should be undertaken promptly. Nursing staff should stop the transfusion immediately and notify the physician caring for the patient. They should verify that the identity of the transfusion recipient, based on the patient's identification bracelet and verbal confirmation with the recipient, if possible, matches that of the intended recipient, as indicated on the blood component tag. All containers and transfusion sets should be sent to the blood bank laboratory along with a post-transfusion blood specimen and a report that summarizes the clinical reaction. The clinical team should also have verbal communication with the blood bank staff to ensure that the postreaction blood specimen, component bag, and infusion set are received by the blood bank as soon as possible.

Investigation of a febrile reaction in the blood bank generally begins with a recheck of the records for clerical errors. The post-transfusion serum/plasma must be visually evaluated for hemolysis and should be compared with the pretransfusion specimen. A direct antiglobulin test must be performed on the post-transfusion blood specimen and ideally on a pretransfusion specimen for comparison. The ABO grouping of the patient sample and the donor unit should be repeated. When suggested by the preliminary serologic results, the crossmatch may be repeated for RBC transfusions to confirm patient–donor compatibility. The results of these tests confirm or exclude a hemolytic transfusion reaction as the basis for the fever. When a septic reaction is highly suspected, for example if the patient arrives with a high fever (2 °C or more) or accompanying hypotension, the bag contents should be examined by means of a Gram's stain and culture for bacterial contamination. Blood cultures should also be obtained from the transfusion recipient's post-transfusion blood specimen to correlate bacteremia with the same organism that may be detected in the blood component bag. Most blood banks do not test for HLA-specific, platelet-specific, or granulocyte-specific antibodies in the recipient's serum as possible causes of an FNHTR. Identification of these antibodies and pyrogenic cytokines is reserved for specialized laboratories and does not play a role in the immediate evaluation of most reactions.

The patient is examined by the primary team and the blood bank physician to determine whether associated symptoms or circumstances can explain the fever, such as drug reactions, sepsis, or other inflammatory conditions unrelated to transfusion. The time course of the development and resolution of fever should be examined in relation to the transfusion. In cases in which the transfusion recipient has a fever at the start of transfusion or is experiencing intermittent spiking fevers, a post-transfusion increase in body temperature can be difficult to interpret. In such cases, *possible* FNHTR may be the most definitive diagnosis that can be made. An FNHTR is a diagnosis of exclusion, arrived at by means of eliminating the possibility of immune hemolysis, bacterial contamination of the blood component, TRALI, or other causes of fever unrelated to transfusion.

### Treatment

The transfusion should be stopped immediately. The intravenous line should be kept open with normal saline solution to provide ready access for the possible infusion of crystalloid and intravenous medication in case the fever is a sign of a more serious hemolytic or septic reaction. Most patients, however, should be reassured that febrile transfusion reactions are usually harmless and that the fever typically responds to antipyretic therapy. The antipyretic agent of choice is acetaminophen (adults, 325–650 mg orally; children, 10–15 mg/kg orally or rectally). Aspirin and nonsteroidal anti-inflammatory drugs are contraindicated in the treatment of many transfusion recipients, such as those receiving platelets. Unless the patient has signs of an allergic reaction, such as urticaria, erythema, or pruritus, antihistamines are not indicated in the management of FNHTR. However, it is not recommended for physicians to prescribe both an antipyretic and an antihistamine in combination for mild reactions.

Patients occasionally develop rigors (severe shaking chills) after a transfusion and meperidine had been a mainstay therapy for many years. Because shivering can increase oxygen demand significantly, it is important to control the shaking chills, particularly for patients with cardiac or respiratory insufficiency. When administered to adults at doses of 25–50 mg intravenously, meperidine remains a very effective treatment for rigors. Meperidine is effective in rapidly arresting rigors through mechanisms not clearly understood. Unfortunately, meperidine has fallen out of favor with some hospitals because of unacceptable central nervous system toxicities and other downsides. Use of meperidine is generally contraindicated in the care of patients with renal failure because of accumulation of the proconvulsant metabolite normeperidine. Use of meperidine is also contraindicated in the care of patients who have taken monoamine oxidase inhibitors within the previous 14 days because of the risk of serotonin syndrome (excess serotonin activity). Toxicities secondary to metabolite accumulation, short half-life, and higher equianalgesic dose compared to morphine have decreased interest but were used in 36% of cases.<sup>31</sup> The advantages of meperidine include rapid and effective results, sedation and analgesia, and the avoidance of long-acting muscle relaxants or the acute hypotensive effect of chlorpromazine.<sup>32–33</sup>

On the basis of anecdotal evidence, morphine may be slightly less efficacious for the treatment of rigors, but its safety profile is more acceptable when given as a onetime dose of 2–4 mg intravenously.

After symptoms of an acute febrile reaction have been treated and the patient has been stabilized, any unused portion of the blood component should be returned to the blood bank and not transfused, even if blood bank testing rapidly rules out hemolysis. A new

device that has been FDA approved for the detection of bacteria in both whole-blood-derived platelets and apheresis PLT products is the Pan Genera Detection (PGD) test, which can detect  $10^3$ – $10^5$  colony-forming units per mL and may be more sensitive than the Gram stain.<sup>34</sup> The use of Gram's stain helps detect heavily contaminated units (with detection limits not less than  $10^6$  organisms per mL), and lower levels of contamination may be missed. If the febrile reaction is caused by bacterial contamination of the component bag, restarting transfusion of the same component can cause a severe and even fatal septic transfusion reaction as more bacteria or bacterial products are infused. For this reason, a new blood component unit should be used if transfusion is still needed after the patient's condition has been stabilized. Hemolysis of either donor or recipient red cells is usually not significant because of the small amount of red cells and plasma in platelet preparations. The transfusion should generally not be restarted at all as a precaution to allow other possible signs or symptoms of a serious reaction to develop. High transfusion-related fevers, such as a  $2\text{ }^\circ\text{C}$  increment or more, are more likely to be associated with septic reactions and should preclude restarting the transfusion. However, lesser fevers do not rule out bacterial contamination of the blood component. If the transfusion is restarted, the patient should be made as comfortable as possible with appropriate antipyretic therapy, as described earlier. The transfusion should proceed slowly and the patient should be observed closely for further signs of a reaction or further temperature elevation throughout the transfusion, which should be stopped if symptoms recur. Restarting transfusion of a blood component that has caused an FNHTR should not be routine.

## **Prevention**

### **Premedication**

Premedication with acetaminophen but not diphenhydramine should be considered for patients with a history of FNHTR. Patients who have no history of FNHTR do not need premedication. Despite a number of studies showing no benefit to premedication in preventing transfusion reactions, the practice remains common in many institutions.<sup>35</sup> A prospective, randomized, double-blind controlled trial of acetaminophen and diphenhydramine pretransfusion medication versus placebo showed that pretransfusion medication may decrease the risk of FNHTR to leukoreduced blood products.<sup>36</sup> Premedication with the glucocorticoid hydrocortisone sodium succinate (adults, 100 mg intravenously) may be useful in the care of reaction prone patients when an antipyretic agent alone is ineffective. Glucocorticoids have anti-inflammatory effects that may help prevent or reduce the severity of FNHTRs. For example, they inhibit the enzyme phospholipase A<sub>2</sub>, thereby blocking production of arachidonic acid and its metabolites such as PGE2, a key mediator of fever. Glucocorticoids also inhibit synthesis of pyrogenic cytokines, such as IL1 and IL6. A variety of glucocorticoids other than hydrocortisone are available. However, hydrocortisone has the advantage of being a short-acting glucocorticoid (biologic half-life, 8–12 hours), and it induces a shorter period of immunosuppression than do many other glucocorticoid preparations. Because glucocorticoids generally act through changes in gene expression, hydrocortisone should be administered at least 4–6 hours before transfusion so that its anti-inflammatory action has time to take effect.

### **Rate of infusion**

Slowing the speed of infusion of a blood component can possibly prevent or decrease the severity of FNHTR. The rate of increase in body temperature in FNHTR caused by leukocyte alloimmunization

appears to be directly related to the rate of infusion of leukocytes in the blood components.<sup>18</sup> A slower rate of infusion is of theoretic advantage in decreasing the severity of reactions caused by bacterial contamination or storage-generated cytokines. Slower infusion avoids a sudden bolus of bacterial toxins or cytokines that may provoke an immediate and possibly massive inflammatory response.

### **Leukocyte reduction**

The prophylactic transfusion of leukocyte-reduced components in the treatment of patients receiving repeated transfusions is effective in avoiding alloimmunization to leukocytes, which is one of the major causes of FNHTR. Leukocyte-reduced blood components should ideally be transfused to such patients beginning with the first transfusion. Leukocyte reduction is effective in the care of patients already alloimmunized to leukocytes because FNHTRs in these patients are directly related to the number and rate of infusion of passenger leukocytes. The threshold number of white cells associated with the development of an FNHTR generally ranges from  $0.25 \times 10^9$  to  $2.5 \times 10^9$ .<sup>18</sup> The removal of approximately 90% of leukocytes ( $1 \times 10^{10}$ ), which usually leaves less than  $5 \times 10^8$  white cells per RBC unit, is sufficient to prevent most FNHTRs.<sup>17,37</sup> For that reason, leukocyte reduction for the purpose of preventing FNHTRs often is defined as decreasing the passenger leukocytes to less than  $5 \times 10^6$  per transfusion. Leukocyte reduction of blood components can be performed either at the time of component preparation (prestorage leukocyte reduction) or immediately before transfusion (poststorage leukocyte reduction). Poststorage leukocyte reduction by means of filtration can be performed in the blood bank before distribution of the component for transfusion or during administration of blood components. The latter is often called *bedside leukocyte reduction*.

Leukocyte-reduced RBC units have in the past been prepared by various poststorage techniques, including simple centrifugation with buffy coat removal, saline washing, and deglycerolization of frozen RBCs.<sup>38–39</sup> Saline-washed and frozen deglycerolized RBCs are rendered leukocyte reduced because approximately 1–2 log<sub>10</sub> leukocytes are removed by repeated centrifugation and washing steps on automated cell washers. Filtration of RBCs units through microaggregate filters designed to remove microaggregate debris more than 40  $\mu\text{m}$  in diameter after an extra centrifugation step or after centrifugation and cooling (spin, cool, and filter) has also been shown to reduce leukocytes in RBC units sufficiently to reduce the incidence of FNHTRs.<sup>40–43</sup>

High-efficiency leukocyte reduction filters for red cells and platelets have been developed that are capable of removing both microaggregate debris and nonaggregated leukocytes.<sup>44</sup> These leukocyte reduction filters can remove three or more log (99.9% or more) leukocytes, thereby decreasing the leukocyte content to approximately  $1 \times 10^6/\text{unit}$  or less. Despite their efficacy in leukocyte reduction, the use of bedside leukocyte reduction filters has had variable and sometimes disappointing results in reducing the incidence of FNHTRs to platelet concentrates.<sup>45</sup> This may be the result of causes of FNHTR other than leukocyte antibodies in the transfusion recipient. For example, storage-generated, extracellular cytokines in the component bag that either are not removed or are inadequately removed by means of poststorage filtration are now believed to mediate some reactions. As a result, the practice of prestorage leukocyte reduction is increasingly replacing poststorage leukocyte reduction. Prestorage leukocyte reduction not only removes leukocytes but also removes leukocytes before they have a chance to release cytokines that can accumulate extracellularly in blood

component bags during storage. Prestorage leukocyte reduction has also yielded conflicting results on the efficacy of leukocyte reduction to mitigate FNHTRs.<sup>46</sup>

Prestorage leukocyte reduction of platelet concentrates or RBC units is achieved by the use of blood component containers with in-line leukocyte reduction filters in a closed system between the primary collection bag and satellite containers. Prestorage leukocyte-reduced platelets can also be prepared with some automated apheresis instruments equipped with centrifugation chambers designed to minimize leukocyte collection (so-called *process leukocyte reduction*). Because some data indicate that only approximately 15% of patients who experience an FNHTR will have a similar reaction to the next transfusion, some blood banks provide a leukocyte-reduced component (either prestorage or bedside leukocyte reduced) only when a patient has had two or more documented febrile reactions.<sup>47</sup> This practice is cost-effective but has the disadvantage of subjecting some patients to two uncomfortable reactions before a preventive measure is taken. Prestorage leukocyte reduction by means of filtration is a more efficient and cost-effective way to eliminate extracellular leukocyte-derived cytokines while reducing passenger leukocytes. Moreover, in evaluations of plasma removal from platelet concentrates to reduce the risk of FNHTR, this technique is still associated with FNHTR in a relatively large percentage of recipients. Neither leukocyte reduction nor poststorage plasma removal has been effective in eliminating all FNHTRs to platelet transfusions.

## Allergic reactions

### Description

An allergic reaction can be classified as an immediate hypersensitivity response consisting of transient localized or generalized urticaria, erythema, and pruritus. More serious allergic reactions can be complicated by hypotension and angioedema of the face and larynx. Allergic reactions can be categorized as those that have only cutaneous manifestations and usually are mild, resolving soon after administration of antihistamines. If other organ systems—cardiovascular, respiratory, or gastrointestinal—are involved beyond mild hypotension, particularly if the reaction is serious enough to necessitate treatment beyond antihistamines, the reaction would be considered *anaphylactic* or *anaphylactoid* (see the “Anaphylactic and anaphylactoid reactions” section). Allergic and anaphylactic reactions, however, are part of a continuum. Allergic reactions occur during or soon after transfusion of plasma-containing blood components. Atopic individuals—those with other known allergies—appear at greater risk of allergic reactions. A large retrospective review of reported transfusion reactions noted that 17% of all reactions in a nine-year period were allergic and 1% of reactions were very severe.<sup>48</sup> Other papers report allergic reaction rates of approximately 0.19% for red cells and 0.53% for platelet transfusions.<sup>49,50</sup> Hemovigilance studies have demonstrated a lower rate of allergic transfusion reaction associated with red cells in the US 0.05%.<sup>46</sup> Studies have also shown that ABO-compatible platelet transfusions have the lowest rate of transfusion reactions. Transfusion of ABO-antigen-incompatible platelets had the highest rate of allergic transfusion reactions and resulted in a transfusion reaction rate 1.5–2 times that of ABO-compatible transfusions.<sup>51</sup>

### Etiology

Allergic reactions are mediated by recipient immunoglobulin E (IgE) or non-IgE antibodies to proteins or other allergenic soluble substances in the donor plasma. The result of the hypersensitivity reaction is secretion of histamine from mast cells and basophils, which mediates cutaneous reactions by increasing vascular permeability.

Although the source of histamine in allergic reactions is believed in many cases to be the transfusion recipient's mast cells and basophils, it has been hypothesized that histamine generated by leukocytes in stored cellular blood components may play a role. Several studies have shown that histamine accumulates in the plasma portion of platelet concentrates and RBC units with increasing storage time. However, histamine is not synthesized during storage but rather it leaks into the extracellular plasma or may be due to calcium ion ( $\text{Ca}^{2+}$ ) influx-inducing activity toward mast cell in patients prior to transfusion.<sup>52–54</sup> These data are consistent with the observation that allergic transfusion reactions are also more common with increasing storage time of blood components.<sup>55</sup> Several of the chemokines that accumulate in the plasma portion of platelet concentrates during blood bank storage, such as IL8, RANTES, and MIP1 $\alpha$ , can recruit and activate basophils and stimulate histamine release. Therefore, it is theoretically possible that the infusion of storage generated donor cytokines during transfusion may contribute to the onset of allergic reactions among transfusion recipients. Consistent with this hypothesis, the biologic activity of RANTES is present at higher levels in apheresis platelets that cause allergic reactions.<sup>54</sup> One study measured the concentration of allergic agonists such as C5a, brain-derived neurotrophic factor (BDNF), and CCL5 (RANTES) in apheresis platelets showed that high levels of these agonists were associated with allergic transfusion reactions. Therefore, levels of acute inflammatory mediators and growth or chemotactic factors of basophils and mast cells do not appear to be associated with allergic transfusions reactions according to the study. However, the study only evaluated 20 platelet transfusions with associated allergic transfusion reactions.<sup>55</sup>

Allergic (and anaphylactic) reactions have been reported after infusion of antibodies in donor plasma, such as penicillin antibody infused into recipients receiving penicillin or related antibiotics, and after infusion of drugs in donor plasma, such as penicillin infused into recipients already sensitized to penicillin.

### Diagnosis

Urticaria is readily diagnosed clinically by the presence of the cutaneous wheal-and-flare reaction. Because allergic symptoms are usually mild and are not characteristic of hemolytic transfusion reactions, serologic blood bank investigations to rule out hemolysis usually are unrevealing. Isolated, mild urticarial reactions not accompanied by other signs and symptoms necessitate minimal diagnostic evaluation. If the reaction is severe, has atypical manifestations, or is accompanied by fever (uncharacteristic of allergic reactions), a more elaborate laboratory evaluation to rule out a hemolytic or septic transfusion reaction is indicated. In the diagnosis of an allergic reaction as transfusion related, it is important to rule out, if possible, urticarial drug reactions that may be circumstantially attributed to transfusions. Careful attention to the timing of onset of urticaria relative to the transfusion may help avoid this confusion. Administration of medications should generally be discouraged in the peritransfusion period to avoid such confusion.

Even mild allergic reactions should be reported to the blood bank. Monitoring allergic reactions and correlating reactions with any newly implemented changes in blood component collection, processing, storage, or filtration are important in detecting new and unexpected causes of reactions. In the care of patients with repeated allergic reactions, notification of the blood bank allows the blood bank medical director to consult on measures to manage or prevent such reactions in the future.

### Treatment

The patient can be treated with a first-generation, H<sub>1</sub>-blocking antihistamine (adults, 25–50 mg diphenhydramine intravenously or orally). If the sedating side effects of first-generation antihistamines must be avoided, newer, less sedating antihistamines are available for oral administration (adults, cetirizine 10 mg orally, loratadine 10 mg orally, or fexofenadine 60 mg orally); however, parenteral antihistamines are preferred in the management of acute reactions because of their more rapid bioavailability. An H<sub>2</sub> blocker, such as cimetidine (adults, 300 mg intravenously) or ranitidine (adults, 50 mg intravenously), may be added to the H<sub>1</sub> blocker to speed resolution of the reaction. However, as of 1 April 2020, the FDA has requested the immediate market withdrawal of all prescription and OTC ranitidine products due to the presence of a contaminant, *N*-nitrosodimethylamine (NDMA), found in some ranitidine products. NDMA is considered a probable human carcinogen, and sustained higher levels of exposure may increase the risk of cancer.<sup>50</sup>

Combining H<sub>1</sub> and H<sub>2</sub> antagonists has given better results in treating patients with allergic reactions in nontransfusion settings than has use of an H<sub>1</sub> antagonist alone.<sup>56–57</sup> For reactions characterized by only localized urticaria, such as a few hives, the transfusion can be temporarily discontinued while an antihistamine is administered. The transfusion can be resumed in approximately 30 minutes if the urticaria has cleared and if no further symptoms occur. For patients with generalized urticaria or a more serious allergic reaction accompanied by facial or laryngeal edema or hypotension, the transfusion should be discontinued and the infusion set with any untransfused blood returned to the blood bank. If laryngeal edema causes breathing difficulties or if hypotension is severe, epinephrine (adult dose, 0.2–0.5 mL of 1:1000 solution [0.2–0.5 mg] subcutaneously) can be administered.

### Prevention

Transfusion recipients are often given routine premedication with an antihistamine such as diphenhydramine in an effort to prevent or reduce the severity of allergic transfusion reactions, even when they have had no previous reactions. The value of this approach is uncertain because few patients have allergic reactions. At least two randomized double-blind placebo-controlled studies of premedication using diphenhydramine and acetaminophen have failed to show a benefit of premedication to reduce reactions.<sup>58–59</sup> When premedication is restricted to patients who have had two or more previous allergic reactions, overall reaction rates do not increase. Accordingly, premedication with an antihistamine should probably be reserved for recipients who have had a previous allergic reaction. For patients with repeated reactions not eliminated by premedication with an H<sub>1</sub> blocker alone, a combination of H<sub>1</sub> and H<sub>2</sub> blockers has been shown more effective.<sup>56–57</sup>

Should premedication not prevent repeated allergic transfusion reactions, another option is to reduce the plasma content of transfused blood components. This can be achieved in RBC and plate-

let preparations with automated saline “washing.”<sup>60–61</sup> However, washing or plasma removal steps generally should be reserved for patients with two or more serious allergic reactions (e.g., those that include angioedema or hypotension) that are not prevented with premedication with both H<sub>1</sub> and H<sub>2</sub> blockers because cell washing is time-consuming and can delay transfusion. Patients with two or more severe allergic reactions can undergo testing for IgA deficiency to rule out a relative deficiency of IgA because this has been reported to cause both a severe allergic and anaphylactic reactions.<sup>62</sup>

## Anaphylactic and anaphylactoid reactions

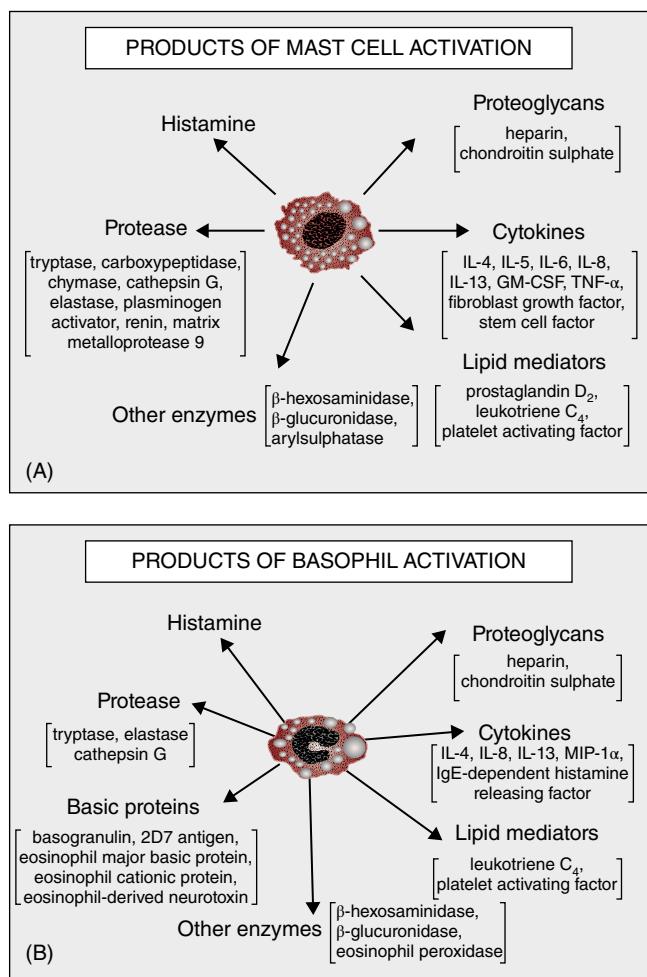
### Description

Anaphylactic reactions are serious and potentially life-threatening immediate hypersensitivity reactions to allergens in the plasma of transfused blood components.<sup>63</sup> These reactions can have a rapid onset beginning as early as seconds to minutes after the start of the transfusion and can occur with small transfused volumes. Anaphylactic reactions are differentiated from other allergic (urticarial) transfusion reactions by their systemic nature and severity. These reactions generally affect multiple organ systems as evidenced by cutaneous, respiratory, cardiovascular, and gastrointestinal effects. The symptom complex often includes the rapid onset of laryngeal edema and bronchospasm with stridor, wheezing, coughing, and respiratory distress. Other symptoms include generalized urticaria, erythema, tachycardia, hypotension, nausea, vomiting, diarrhea, and cramping abdominal or pelvic pain. Severe reactions can proceed rapidly to shock, syncope, respiratory failure, and death. Fatal anaphylactic reactions are less common than are fatal hemolytic or septic reactions.

### Etiology

Anaphylactic reactions occur when an allergen present in plasma is transfused to a patient who through previous sensitization has an IgE directed against that allergen.<sup>64</sup> Immunoglobulin E is bound by means of Fc receptors to mast cells and basophils. The binding of allergen to cell-bound IgE results in crosslinking of IgE and Fc receptors. This crosslinking activates the mast cells and basophils to secrete preformed mediators, such as histamine, as well as newly synthesized mediators, such as leukotrienes, prostaglandins, cytokines, and platelet-activating factor (PAF) (Figure 48.2).<sup>65</sup> PAF induces downstream production of nitric oxide (NO) through inducible and possibly constitutively expressed NO synthase.<sup>66</sup> As a potent vasodilator, NO is believed to be the principal compound causing hypotensive and cardiovascular collapse during anaphylaxis, although the exact mechanism remains under debate.

Anaphylactoid reactions are acute hypersensitivity reactions that are clinically identical to anaphylaxis but are not mediated by IgE antibodies or IgE involvement cannot be established. For example, immune complexes involving antibodies other than IgE may result in complement fixation and generation of the anaphylatoxins C3a, C4a, and C5a, which activate basophils and mast cells. Some cytokines secreted by monocytes as part of the inflammatory cascade initiated by non-IgE immune complex formation can also directly activate basophils and mast cells and initiate anaphylactoid reactions. Moreover, IgG4 subclass antibodies can bind to Fc receptors of mast cells and basophils and, in a manner analogous to that of IgE, mediate cellular activation and degranulation after binding



**Figure 48.2** (A) Mast cell with its activation products. (B) Basophil with its activation products. Note that currently only two products of mast cell activation (histamine and total tryptase) and one product of basophil activation (histamine) can be measured in clinical laboratories as markers of acute anaphylaxis events. Used with permission from Simons *et al.* (2007).<sup>66</sup> IL: interleukin; GM-CSF: granulocyte macrophage colony-stimulating factor; TNF $\alpha$ : tumor necrosis factor alpha; MIP: macrophage inflammatory protein.

of allergen. The term *anaphylactoid* is sometimes used to describe mild or clinically atypical anaphylactic reactions. However, *anaphylactoid* is better used to differentiate the mechanism of the reaction, not its clinical severity or presentation.

The best documented anaphylactoid reactions have resulted from the transfusion of donor plasma containing IgA- to IgA-deficient recipients who have produced a class-specific IgG anti-IgA antibody that reacts with all IgA subclasses. A recent case report of neutropenia and monocytopenia in a recurrent anaphylactoid reaction after red blood cell transfusions showed an atypical signs and symptoms of anaphylactoid reaction with the patient only experiencing rigors, fever, and hypertension in addition to neutropenia and monocytopenia. The patient was later found to be IgA deficient (<0.05 mg/dL) with IgG anti-IgA in serum.<sup>67</sup>

Less commonly, patients with normal total IgA levels have a subclass-specific IgA deficiency and may make an anti-IgA of restricted specificity. Although IgA deficiency is relatively common (approximately 1 case among 700 persons), anaphylactoid reactions

occur only among some IgA-deficient transfusion recipients because not all make anti-IgA. Anaphylactic or anaphylactoid reactions have been documented among patients with deficiencies of other plasma proteins, such as complement, von Willebrand factor, and haptoglobin.<sup>68–69</sup> In an analogous manner, these patients produce an antibody to the missing factor that reacts with transfused, plasma-containing blood components. Angiotensin-converting enzyme (ACE) inhibitors are drugs commonly prescribed to treat hypertension. Patients taken ACE inhibitors have been reported to have anaphylactoid reactions during online extracorporeal apheresis such as plasma exchange.<sup>70</sup> In most anaphylactic or anaphylactoid reactions, however, the allergen is never identified, nor is evidence obtained to differentiate anaphylactic from anaphylactoid mechanisms.

### Diagnosis

Anaphylactic and anaphylactoid reactions are diagnosed from clinical signs and symptoms.<sup>71</sup> The cutaneous signs and symptoms and the often rapid onset of the reaction help differentiate anaphylactic reactions from acute hemolytic and septic transfusion reactions. Serum  $\beta$ -tryptase levels may be measured to confirm an anaphylactic reaction because it is a marker for mast cell degranulation. However, no laboratory measurement is available in time to meaningfully affect recognition and management of an anaphylactic reaction.

Recipient IgA levels should be measured in a pretransfusion blood specimen to determine if the recipient is IgA deficient. Although the results of tests for IgA deficiency do not affect diagnosis or management of the reaction at hand, it is important for avoiding future reactions.<sup>63</sup> Testing should be performed on a specimen drawn before transfusion because IgA deficiency can be masked by any IgA provided by the transfusion. Recipient anti-IgA can also be measured, especially for rare cases in which the anti-IgA is subtype-specific and total IgA levels are within the reference range. Although IgA is the most commonly known allergen in anaphylactoid reactions, in many anaphylactic and anaphylactoid reactions, the offending allergen is not IgA and is never identified.

### Treatment

Anaphylactic and anaphylactoid reactions are managed identically.<sup>71</sup> Severe reactions are true medical emergencies and should be managed by experienced critical care staff, if possible. The patient should be placed in an intensive care unit as soon as it is practical without jeopardizing emergency care. Once anaphylaxis is evident clinically, 1:1000 epinephrine solution (1 mg/mL) should be administered subcutaneously in a dose of 0.2–0.5 mL for adults (0.01 mL/kg of body weight for children). The dose may be repeated every 15–30 minutes as needed. Intravenous crystalloid or colloid solution should be administered as needed to support the patient's blood pressure. For example, 500 mL to 1 L of normal saline solution can be administered in the first 15–30 minutes. Further infusion should be titrated to blood pressure. If the systolic blood pressure is less than 60 mmHg, intravenous epinephrine in a dose of 1–5 mL of a 1:10,000 solution (0.1 mg/mL) for adults and 0.1 mL/kg for children is administered over 2–5 minutes by means of intravenous push. An epinephrine drip (1–4  $\mu$ g/minute) may be started, and administration of other pressors, such as dopamine, can be considered. Blood pressure, pulse, and urine output should be monitored. It may be necessary to monitor the effectiveness of fluid replacement and pressor infusion through measurement of central venous pressure.

Respiratory distress is managed with supplemental oxygen. The patient's upper airway may have to be secured with endotracheal intubation if obstruction from laryngeal edema is imminent. Stridor is a sign of laryngeal edema. Endotracheal intubation and mechanical assistance with ventilation are indicated if the PaCO<sub>2</sub> increases to more than 65 mmHg. When intubation is difficult or impossible because of laryngeal obstruction, cricothyrotomy or tracheostomy is an option. Wheezing caused by obstruction of small bronchi and bronchioles by increased mucus production and smooth muscle contraction can be managed with nebulized albuterol or metaproterenol and intravenous aminophylline.

Urticaria, angioedema, or gastrointestinal distress is managed with an antihistamine (adults, 50 mg diphenhydramine intravenously; children, 1–2 mg/kg intravenously). H<sub>2</sub>-blocking antihistamines may be added as an adjunct to H<sub>1</sub> blockers. Glucocorticoids, such as hydrocortisone, 200 mg given intravenously every six hours, are also administered because they reduce late-phase inflammatory responses. Glucocorticoids, however, are not expected to be of benefit in the initial management of anaphylaxis because of their delayed onset of action.

### **Prevention**

Patients with IgA deficiency who have already had an anaphylactic reaction or who are known to have anti-IgA should receive transfusion of RBC and platelet preparations that have been saline-washed with an automated cell washer.<sup>59,62</sup> If plasma transfusion is necessary, only IgA-deficient donors should be used. Patients who have anaphylactic reactions to any other known plasma allergen should also be treated with transfusion of saline-washed RBCs or platelet preparations. Because anaphylactic reactions can be induced by very small amounts of allergen, washing must be extensive. Washing and saline replacement by means of automated cell washers have been shown generally successful in removing IgA sufficiently to prevent recurrences of anaphylactoid reactions.

If a patient has had one anaphylactic reaction of unknown causation, the next transfusion need not necessarily be performed with washed RBCs or platelets because the reaction might have been donor specific. The next transfusion may be administered slowly with vigilance after premedication with both H<sub>1</sub> and H<sub>2</sub> blockers and a glucocorticoid. The patient care team should be prepared to respond to an anaphylactic reaction. The patient should ideally be in a critical care unit with monitoring at the time of transfusion and with a critical care physician and nurses in attendance. Some blood banks with the capability of automated cell washing, nevertheless, may choose to provide saline-washed RBCs and platelet concentrates for future transfusions after a single anaphylactic reaction as a precautionary measure, particularly if the patient is not expected to receive many more transfusions.

### **Complications of massive and rapid transfusion**

Massive transfusion is defined as the replacement of one blood volume within a 24-hour period. For practical purposes for adults of average size, this is roughly equivalent to 10 units of RBCs with any accompanying crystalloid, colloid, platelet, or plasma infusions. An infusion of greater than four units of RBCs in an hour and ongoing use anticipated could also be regarded as a massive transfusion. The possible complications include citrate toxicity, electrolyte imbalance (hyperkalemia from transfusion of older RBCs and hypocalcemia from citrate toxicity), circulatory overload, and hypothermia.

Recipients of massive transfusions are at increased risk of hemolytic transfusion reactions (including ABO incompatibility), FNHTR, and allergic reactions because of the number of units they receive. Reactions can be more severe with massive transfusion because rapid infusion means the implicated unit often has been completely administered before the onset of symptoms. The large number of units transfused in a short time complicates the investigation of transfusion reactions because each transfused component must be investigated. Due to the resurgent interest in transfusing whole blood for hemorrhagic shock, low-titer group O whole blood (LTOWB) is being used at many trauma centers, and there is increasing evidence both in the military and civilian populations that the early intervention with blood products in patients with traumatic bleeding saves lives.<sup>72–74</sup> The AABB Standard 5.15.1 in the 31st edition states “recipient shall receive ABO group-compatible Red Blood Cell (RBC) components, ABO group-specific Whole Blood, or low-titer group O Whole Blood (for non-group -O recipients or for recipients whose ABO group is unknown).” This new standard permits the use of LTOWB in a manner that is similar to how group O un-crossmatched RBCs are provided to a recipient of unknown ABO group in an emergency.<sup>75</sup> Transfusion of LTOWB to nongroup O recipients has the potential for hemolysis because of the presence of anti-A and anti-B in the plasma of the whole blood. The safety of using group A plasma and LTOWB in trauma resuscitation had been previously demonstrated in the STAT study and other studies.<sup>76–78</sup>

The nonhemolytic lethal triad of severe trauma consists of hypothermia, acidosis, and coagulopathy. “Damage control” resuscitation methods have been developed to directly address the coagulopathy of trauma. The coagulopathy of trauma develops because of severe injury and is already present when a patient presents for emergency medical care. Trauma coagulopathy is not caused by the resuscitation efforts of emergency medical interventions as traditionally understood.<sup>79</sup> Advances in trauma research have shown that early application of damage control resuscitation can greatly improve survival through an application of a 1:1:1 plasma-red-cell-platelet ratio from patient presentation.<sup>80</sup> In well-coordinated trauma centers, massive transfusion protocols now provide six units of plasma, six units of RBCs, six whole-blood-derived platelet concentrates (or one apheresis platelet) or four units of plasma, four units of RBCs, and one unit of apheresis platelets. Aggressive damage control transfusion has been associated with a number of risks including hyperkalemia. Despite these risks, retrospective studies have shown that the application of early, aggressive transfusion support improves overall survival in this severely injured population that would otherwise have very poor prognosis.

Rapid transfusion can also occur during therapeutic apheresis and red cell exchange apheresis (erythrocytapheresis). During apheresis procedures, as many as 10–20 units of fresh frozen plasma or 4–8 units of RBCs can be transfused over 1.5–2 hours. Although any acute transfusion reaction can occur during apheresis-associated transfusion, citrate toxicity in particular is a common but usually mild complication.

### **Citrate toxicity**

Rapid blood transfusion can cause a transient decrease in the level of ionized calcium because of the calcium-chelating properties of the citrate anticoagulant in stored blood components.<sup>81</sup> The clinical presentation of citrate-induced symptoms is also termed *citrate toxicity*. Citrate toxicity can occur whenever large volumes of plasma

that contain citrate are transfused, such as during massive transfusion, plasma exchange, or other apheresis procedures.<sup>82–84</sup> Citrate infusion can induce hypocalcemia, hypomagnesemia, and other electrolyte imbalances, and these imbalances are associated with clinical symptoms. Apheresis procedures can produce a unique clinical paradox of urinary calcium excretion in the setting of hypocalcemia.<sup>83,85</sup> Hypocalcemia is a recognized complication of liver transplantation, in which large amounts of plasma are transfused. However, the precise mechanism of hypocalcemia is not well understood and may not be caused entirely by citrated plasma.<sup>86–87</sup>

Citrate ordinarily is rapidly metabolized to bicarbonate in mitochondria-rich tissue, such as liver, skeletal muscle, and kidney.<sup>81</sup> In the routine transfusion of blood components, patients with normal liver function usually tolerate the citrate infusion without significant complications. However, patients with liver or renal failure or parathyroid dysfunction are at greater risk of citrate toxicity when they receive rapid transfusions of plasma or plasma-containing blood components. Citrate anticoagulates blood by binding divalent cations such as calcium, thus hypocalcemia is a primary symptom. Other divalent cations such as magnesium and zinc can also be bound by citrate, but the contribution of hypomagnesemia to clinical symptoms is less pronounced.<sup>88</sup> During apheresis, citrate is administered as acid–citrate–dextrose formula A (ACD-A) in constant proportion to the whole blood flow rate. Healthy plateletpheresis donors receive relatively large doses of citrate, and many experience mild symptoms of the citrate effect, but the symptoms usually do not progress because of the short duration of the procedure. Donors of peripheral blood stem cells (PBSCs), however, receive smaller doses of citrate per unit of time but usually experience more severe citrate toxicity because of the longer duration of the procedure. Paresthesia caused by transient hypocalcemia is common in apheresis. It typically occurs after the initial infusion of the priming solution (if citrate is used) or later as apheresis progresses. Apheresis practitioners should be aware that peripheral paresthesia caused by hypocalcemia can be masked in patients with a preexisting neuropathy as a result of chemotherapy (vincristine) or as part of a neurologic condition.

Citrate toxicity is recognized clinically because of the signs and symptoms of hypocalcemia. It can be confirmed by measuring the plasma-ionized calcium level in the patient. Symptoms of hypocalcemia include peripheral and perioral paresthesia (Chvostek and Trousseau signs can occasionally be elucidated), muscle spasm, cramping, nausea, vomiting, cardiac arrhythmia, bradycardia, hypotension, and, if severe, tetany. An electrocardiogram (ECG) can show prolongation of the QT interval with hypocalcemia, but the relation is not linear with the ionized calcium level, and ECG findings are an unreliable guide to calcium therapy.

Mild citrate toxicity during transfusion or apheresis is managed or prevented in part by means of slowing the rate of transfusion or reinfusion. When slowing the infusion rate is impossible or ineffective and the patient has signs and symptoms of hypocalcemia, calcium supplementation is indicated. The best guide to determining a need for calcium supplementation is the measurement of the patient's ionized calcium levels, if results can be obtained rapidly. Calcium replacement during apheresis should generally be given when a patient has symptoms, when the patient's clinical condition may exacerbate citrate effects, or when prolonged large-volume leukapheresis is expected to cause citrate toxicity. Infusion of calcium itself, however, is associated with the development of ventricular arrhythmia and even cardiac arrest. Therefore, intravenous calcium replacement for the management or prophylaxis

of apheresis-induced citrate toxicity should be administered only by experienced apheresis staff. Under no circumstances should calcium be added directly to a unit of blood because it causes clots to form in the bag.

Citrate toxicity during apheresis is related to the citrate concentration of the reinfused blood or colloid solution, the infusion rate, the blood volume of the patient, and the total time over which the citrate is infused.<sup>89</sup> It is difficult to establish a definitive safe rate of citrate infusion because of the large number of variables involved. However, citrate dosages of up to 1 mg/kg/minute given during plateletpheresis are usually well tolerated during a 60–90 minute collection. A safe rate of calcium replacement for controlling citrate toxicity during more prolonged (several hours) PBSC apheresis is 0.5–0.6 mg of calcium ion for every 1.0 mL of infused ACD-A.<sup>90–91</sup> These dosages have been successful for prophylaxis against citrate toxicity during large-volume leukapheresis. To avoid excessive volume during PBSC apheresis, the administration of a concentrated calcium solution (calcium chloride or calcium gluconate) is appropriate. Care must be taken to coordinate the calcium infusion with whole blood flow during the apheresis procedure to avoid the potential for catheter thrombosis. Calcium administration should be halted soon after interruptions in whole blood flow.

### **Electrolyte disorders**

Because of inhibition of sodium–potassium–adenosine triphosphatase in red cell membranes by the cold storage temperature of RBC units, extracellular potassium accumulates with increasing blood bank storage times. Extracellular potassium increases at the rate of approximately 1 mEq/day during the first three weeks of RBC storage in citrate–phosphate–dextrose–adenine-1 (CPDA-1).<sup>92</sup> Potassium levels in additive solution-1 (Adsol) units are markedly higher on Day 7 of storage (17 mmol/L) than on Day 0 (1.6 mmol/L). The increase is even greater by Day 42 (46 mmol/L).<sup>93</sup> Total extracellular volume in additive solution is less than half of that in CPDA-1 blood; this must be taken into account when the amount of transfused extracellular potassium is considered.

Hyperkalemia resulting from massive transfusion of older RBC units with an elevated amount of extracellular potassium can cause significant cardiac complications and possibly death in some patients.<sup>94</sup> Acidosis can contribute to hyperkalemia, and severely injured patients presenting with a potassium level greater than 4 mmol/L are at increased risk. Other patients at risk of hyperkalemic complications are neonates and those with renal failure. The diagnosis of hyperkalemia is made by means of measurement of potassium in the serum and observation of ECG changes, which include peaked T waves, prolongation of the PR interval, and ventricular arrhythmia.<sup>95</sup> In neonatal transfusions, hyperkalemia can be avoided by use of fresh RBC units (less than seven days old) or older units that have been saline-washed to remove the extracellular fraction containing the potassium.<sup>96–97</sup> However, the transfusion of older RBC units does not place neonates at risk of hyperkalemia if small-volume transfusions (10–15 mL/kg) are given slowly.<sup>98</sup> Hypokalemia can also develop during massive transfusion or large-volume apheresis.<sup>83,99</sup>

As the anticoagulant citrate in blood components is metabolized to bicarbonate, the blood can become alkalotic, producing hypokalemia. The degree of hypokalemia may be sufficient to necessitate infusion of potassium if symptoms develop. However, the use of newer RBC additive solutions such as Adsol has helped to decrease the effect of hypokalemia. RBC units are plasma-reduced before the addition of additive solution, which itself

contains no additional citrate. Therefore, most of the citrated plasma is removed from additive solution RBC units during production. Animal studies have shown fewer physiologic aberrations during massive transfusion with Adsol RBCs than with CPDA-1 units.<sup>100</sup> The complications of hypokalemia, therefore, are more likely when large numbers of units of plasma rather than RBC units are transfused. Post-transfusion hypokalemia can also be due to potassium uptake by transfused old RBCs.

### Hypothermia

Hypothermia, defined as a core body temperature of less than 35 °C, may be caused by rapid infusion of large quantities of cold (1–10 °C) blood or RBC units. Hypothermia during massive transfusion has been shown to induce cardiac arrhythmia and arrest.<sup>101</sup> Hypothermia is a known independent risk factor for early coagulopathy, multiple organ failure (MOF) development, and mortality.<sup>102</sup> Even smaller quantities of cold blood can be cardiotoxic if transfused into central venous lines because the newly infused cold blood can reach the heart before sufficient warming has occurred. Data published in the early 1960s showed that massive transfusion at a rate of approximately one unit every 5–10 minutes was sufficient to lower the temperature of an esophageal probe behind the right atrium to nearly 30 °C.<sup>101</sup> The resulting decrease in sinoatrial node temperature was associated with the development of ventricular fibrillation.

For most routine transfusions given at a standard rate of administration, blood does not have to be warmed.<sup>103</sup> The patient may experience minor chills, but this is easily remedied by warming the patient, as with extra blankets. Transfusion of cold RBC units through central venous lines, however, should be avoided. Indications for warming blood include rapid transfusion, which are generally considered to be more than 50 mL/kg/hour for an adult and more than 15 mL/kg/hour for a child, and exchange transfusion for infants. Because blood warming during certain massive transfusions sometimes delays infusion and impedes resuscitation, it is not always practical. Warming blood for transfusion in the treatment of patients with cold agglutinin disease has a theoretic basis but is debatable because supportive outcome data are lacking.

If blood has to be warmed, an approved warming device should be used and the temperature must be kept below a point where hemolysis occurs.<sup>103</sup> Care must be taken when warming up blood because warming to a temperature above 42 °C may cause hemolysis.<sup>104</sup> Infusion of thermally injured cells can induce disseminated intravascular coagulation and shock. Heating blood with a device other than an approved blood warming device, such as a commercial microwave oven, is unacceptable. Blood that has been warmed but not used should not be reissued for another patient because of the increased risk of bacterial proliferation at warmer temperatures.

The maximum flow rate that can be achieved with commercially available blood warmers is 850 mL/minute; however, most can provide a rate of only 150 mL/minute. Most recent research has focused on comparisons between commercial warming devices, and the comparison studies usually evaluate warming capability in the rapid transfusion setting.<sup>105</sup> Most of the available federally approved blood warming devices safely warm blood and other intravenous fluids across a range of flow rates. However, although little recent data exist correlating the clinical benefits of warming blood, many emergency centers and trauma services use such devices routinely without incident.

An alternative to using mechanical blood warmers that circumvents such flow limitations is rapid admixture with warm or hot

saline solution immediately before transfusion.<sup>103,106</sup> This technique immediately warms a unit of RBCs, yet does not cause significant hemolysis. However, it necessitates that warmed saline solution be available at all times in trauma care and requires attention to technique to avoid the direct infusion of hot saline solution into the patient.

### Reactions attributed to microaggregate debris

Microaggregate debris ranges in diameter from 20 to 120 µm and consists of nonviable platelets, white cells, and strands of fibrin that form in blood during storage.<sup>107–108</sup> Because of their size, microaggregates are not removed from transfused blood with the standard 170–260-µm screen filters. A variety of adverse events have been attributed to the presence of microaggregate debris after large-volume and massive transfusion.

Studies in the 1960s showed that patients undergoing open heart surgery with cardiopulmonary bypass experienced post-perfusion syndrome during the postoperative period. This symptom complex consisted of cerebral and renal dysfunction attributed in part to occlusion of end-organ capillaries with microaggregate debris. Cotton wool (Swank) microaggregate blood filters capable of retaining particles or debris with a size of 40 µm or more appeared to eliminate many of these reactions. Microaggregate blood filters (MBFs) with 10–40 µm pore size are able to eliminate larger-diameter, retained, blood component microaggregate materials and nonblood, particulate matter that is potentially harmful to the recipients. In other words, MBFs ensure the patient protection from microaggregate materials, clots, and particulate debris that can cause pulmonary embolization.<sup>109</sup>

During the Vietnam War, some soldiers who underwent massive transfusion experienced respiratory distress syndrome (shock lung). At autopsy, the cause was presumed to be the material found in soldiers' lungs that was positive on a periodic acid-Schiff (PAS) test. Because microaggregate debris stains PAS-positive, this was taken at the time as evidence of the pathologic nature of microaggregate debris.

During the 1970s and 1980s, studies were undertaken to determine whether removal of microaggregate debris from blood was clinically significant.<sup>107</sup> Several studies showed that microaggregate filtration of up to six units of blood during either hip or cardiac surgery provided no benefit. Collins *et al.*<sup>110</sup> concluded that the underlying clinical condition rather than the infusion of the microaggregate debris in blood led to the development of the respiratory distress syndrome reported earlier among patients undergoing massive transfusions. Microaggregate blood filters today are used mostly in conjunction with cardiopulmonary bypass pumps and reinfusion of shed autologous blood collected during or after surgery. With the widespread adoption of leukocyte reduction filtration, routine leukocyte-reduced red cell transfusions are no longer complicated by microaggregate debris because these filters can remove not only leukocytes but also the larger microaggregate particles. As an alternative to microaggregate filters, cell washing can be used to remove intraoperative microparticulates.<sup>111</sup>

### Circulatory overload

*Hypervolemia*, termed Transfusion Associated Circulatory Overload (TACO), is a possible consequence of transfusion in the care of patients with cardiac insufficiency, renal impairment, or already expanded blood volumes, such as patients with chronic anemia.

Moreover, patients with restricted blood volumes (e.g., infants and small children) are at risk of TACO if transfused blood is not reduced to an amount proportional to body mass and intravascular blood volume. The reported incidence of TACO varies widely depending on the method of data collection used. The risk of TACO increases with rapid infusion. The 2011 US Food and Drug Administration report on transfusion-related mortality indicated that TACO was the second most commonly reported cause of death next to TRALI, accounting for an average of 15% of reported deaths between 2007 and 2011.<sup>112</sup> According to the annual summary for fiscal year (FY) 2018 from the US Food and Drug Administration report, TACO is now the leading cause of reported transfusion-associated deaths for FY16 through FY18 and is currently the leading cause of transfusion-associated fatalities over the five-year reporting period (FY2014 - FY2018) accounting for 32% of all reportable deaths. Prior to FY2016, TRALI was the consistent leading cause of transfusion-associated fatalities.<sup>113</sup>

Circulatory overload increases central venous pressure, causes congestion of the pulmonary vasculature, and decreases lung compliance, manifesting as dyspnea, tachycardia, acute hypertension, and in the extreme, pulmonary edema and left- or right-sided heart failure. Other signs and symptoms of circulatory overload include tachypnea, dry cough, chest or throat tightness, jugular venous distention, and pulmonary rales.

Recent studies have shown that the proinflammatory cytokine IL-6 was increased in post-transfusion TACO patients, and fever was described to occur in one-third of the patients developing TACO.<sup>114-116</sup>

Laboratory measurements of circulatory overload include PaO<sub>2</sub>, atrial natriuretic peptide, and B-type natriuretic peptide (BNP).<sup>117-118</sup> B-type natriuretic peptide (BNP), and N-terminal pro-BNP (NT-pro-BNP) are cardiac neurohormones specifically secreted from the ventricles in response to volume expansion and pressure overload.<sup>119</sup> BNP has a reported 81% sensitivity for detecting circulatory overload in the appropriate clinical setting. However, BNP and NT-pro-BNP testing did not reliably distinguish TACO from TRALI and possible TRALI in a cohort of transfused critically ill patients.<sup>120</sup> Diagnosing TACO can be difficult and confounded by other concomitant pathology. There is no accepted clinical definition of TACO, and the symptoms of

TACO can overlap significantly with other transfusion reactions such as TRALI (Table 48.1).<sup>121-122</sup> Some rapid methods of differentiating the overlapping symptoms include blood pressure, response to diuretic therapy, white cell count, and heart auscultation for an S3 (third heart sound).

If symptoms of overload appear, the transfusion should be stopped, and intravascular volume reduction through diuresis should be instituted as needed (e.g., administration of 40 mg furosemide intravenously). The patient should be placed in an upright (reverse Trendelenburg) position, if possible, with supplemental oxygen as necessary.

Rapid transfusion of any blood component into a patient who is not actively hemorrhaging produces no benefit and can cause harm. As a general guide, infusion should be at a rate not to exceed 2–4 mL/kg/hour, and the rate should be lower (~1 mL/kg/hour) for patients at high risk of circulatory overload.<sup>123</sup> In neonates, a slower blood infusion rate increases the hematocrit and decreases cardiac demand without affecting pulmonary artery pressure. More rapid infusion rates are associated with decreased lung compliance and increased pulmonary airflow resistance.<sup>124-125</sup>

For patients with volume overload caused by medical reasons existing before transfusion, furosemide can be given prophylactically, and transfusion should proceed slowly. The rate of transfusion can be even further slowed, if necessary, by dividing a unit of RBCs or another component into smaller aliquots and transfusing a portion at a time over as much as four hours, the maximum allowable time a blood component should be kept outside blood-bank-monitored storage. For RBCs and thawed plasma, the unused portion can be stored in the blood bank at 1–6 °C for up to 24 hours while the initial aliquot is administered. Platelet aliquots can be sampled from a single apheresis platelet unit, and with this practice, donor exposures can be minimized. It is important that the transfusion of all or part of a blood component be completed within four hours and that any unused portion be stored under regulated blood bank conditions because of concerns about increased risk of bacterial contamination during improper storage. RBC units, apheresis platelets, and whole-blood-derived platelet pools can be further concentrated by means of centrifugation and plasma removal, if other measures to prevent volume overload are inadequate.

**Table 48.1** Features in TRALI and TACO

Feature	TRALI	TACO
Body temperature	Fever can be present	Fever may be present (one-third of patients) <sup>102</sup>
Blood pressure	Hypotension	Hypertension
Respiratory symptoms	Acute dyspnea	Acute dyspnea
Neck veins	Unchanged	Can be distended
Auscultation	Rales	Rales, S3 may be present
Chest radiograph	Diffuse, bilateral infiltrates	Diffuse, bilateral infiltrates
Ejection fraction	Normal, decreased	Decreased
PA occlusion pressure	18 mmHg or less	Greater than 18 mmHg
Pulmonary edema fluid	Exudate	Transudate
Fluid balance	Positive, even, negative	Positive
Response to diuretic	Minimal	Significant
White count	Transient leukopenia	Unchanged
BNP	<200 pg/mL	>1200 pg/mL
Leukocyte antibodies	Donor leukocyte antibodies present, and crossmatch incompatibility between donor and recipient	Donor leukocyte antibodies may or may not be present, and positive results can suggest TRALI even with true TACO cases

The typical patterns that would be expected for cases of transfusion-related acute lung injury (TRALI) or transfusion-associated circulatory overload (TACO) are represented. A given case of TRALI or TACO may lack some of the typical features. Also, a case of TRALI may have some features suggesting TACO or vice versa, and TRALI and TACO can be present together. The best strategy is to develop a full clinical profile of the case using the feature list above and determine which diagnosis is most supported. BNP: brain natriuretic peptide; PA: pulmonary artery.

## Toxic reactions resulting from blood manufacture or processing

### Hypotensive reactions

Hypotension may accompany various transfusion reactions including hemolytic and allergic reactions, septicemia, and TRALI, but isolated hypotension as a primary manifestation has not been considered a unique type of transfusion reaction. In the past several years, there have been several reports of transfusion reactions characterized primarily by hypotension, and it now appears that such reactions do require a separate category of transfusion reactions.<sup>126</sup> Hypotension has been reported among patients receiving bedside, leukocyte-reduced platelets who are also medicated with ACE inhibitors.<sup>127</sup> These reactions appear to be caused by generation of bradykinin in transfused blood just as it is being passed through negatively charged leukocyte reduction filters. The mechanism is believed to involve the formation of activated factor XIIa when factor XII, a contact factor, is exposed to the negatively charged filter surface. The filter surface can mimic exposed, negatively charged subendothelium, which is the natural activating stimulus for the contact factors of the intrinsic coagulation pathway after blood vessel damage *in vivo*. Factor XIIa converts prekallikrein to kallikrein, which cleaves high-molecular-weight kininogen to form bradykinin. The biologic activity of the infused bradykinin is prolonged in transfusion recipients who are also receiving ACE inhibitors (e.g., captopril and enalapril), which inhibit kininase II, the enzyme that breaks down bradykinin. The combination of bradykinin generation just as the blood is being infused with inhibition of the transfusion recipient's ability to break down bradykinin produces prolonged bradykinin activity conducive to hypotensive reactions.

These reactions are less likely with use of prestorage leukocyte-reduced blood components because the bradykinin is broken down rapidly in the component bag during storage before transfusion. Although hypotensive reactions have been reported more frequently with negatively charged bedside leukocyte reduction filters, they also have been rarely reported with positively charged filters. This can be explained in part by the possibility that patients taking ACE inhibitors may be more prone to hypotensive reactions in general because of their relative inability to rapidly break down bradykinin generated *in vivo* by any allergic mechanism. Hypotensive reactions to bedside leukocyte reduction among patients taking ACE inhibitors can be prevented by the use of prestorage leukocyte-reduced blood components or by means of temporary discontinuation of ACE inhibitor treatment.

Apheresis procedures are also associated with hypotensive reactions, and the literature has described hypotensive reactions in both adult and pediatric apheresis patients.<sup>128–129</sup> Apheresis may contribute to hypotensive reactions through several mechanisms including the potentiation of bradykinin-mediated effects by albumin and secondary to hypocalcemia.<sup>129</sup> Data suggest that calcium infusions can mitigate some atypical apheresis reactions, while withholding ACE inhibitor medications 24–48 hours before apheresis may also contribute to lessening reactions.

### Plasticizer toxicity

Plasticizers are chemicals used to make rigid polyvinyl chloride plastics more malleable. The traditional plasticizer for blood storage bags is di(2-ethylhexyl)phthalate (DEHP), which leaches over time from the plastic into the blood and blood components with increasing exposure. The DEHP metabolite, mono(2-ethylhexyl) phthalate (MEHP), also accumulates during storage.<sup>130</sup> Infusion of

blood that contains DEHP results in deposition of DEHP in various tissues; the greatest accumulation is in body fat. Results of some studies with animals have suggested that DEHP is toxic and may even be carcinogenic in large quantities.<sup>131</sup> Other studies with animals have shown that MEHP is associated with the formation of peroxisomes, indicating tissue alteration and toxicity. Although there have been no reports of transfusion-related plasticizer toxicity among humans, results of some *in vitro* experiments suggest that high concentrations of MEHP have a negative inotropic effect and can cause irregular contractions in isolated human myocardial cells. Some clinical data have described the production of antiplasticizer IgE in transfusion recipients and the incorporation of plasticizer into red cells during storage.<sup>132</sup> Despite the possible adverse effects of DEHP plasticizers, other data indicate that these substances stabilize red cell membranes and improve the morphologic features of platelets during storage.<sup>133–134</sup> No good evidence exists, however, of actual improvement in post-transfusion outcomes as the result of these effects.

Formulations for plastic blood bags were developed with plasticizers other than DEHP that have a decreased capacity to leach into plasma. For example, one polyvinyl chloride-based material is made with plasticizer butyryl tri-*n*-hexyl citrate (BTHC). Although BTHC also leaches into blood components, it does so at a significantly slower rate than does DEHP. It also provides an antihemolytic effect similar to that of DEHP.<sup>135</sup> Studies have shown this citrate-based plasticizer is suitable for the storage of both RBCs and platelets.<sup>136</sup>

### Dimethyl sulfoxide toxicity during infusion of cryopreserved progenitor cells

DMSO is a versatile solvent that has been used as the principal cryopreservative for mononuclear cells since the 1950s. It is widely used as a cryopreservative for marrow and PBSCs used in human hematopoietic progenitor cell transplantation. Despite this, DMSO is not approved by the FDA as a pharmacologic agent for intravenous administration, and guidelines for intravenous administration are obscure. Toxicologic studies, however, have established the general safety of intravenous DMSO infusion.<sup>137–138</sup> The metabolism of DMSO yields a characteristic harmless odor, described as a malodorous garlic or sulfur-like smell. Because of the exceptional solvent properties of the compound, DMSO is distributed throughout all tissues after administration. The two metabolites of DMSO are dimethyl sulf dioxide and dimethyl sulfide (DMS). Dimethyl sulf dioxide is an odorless compound excreted by the kidney, and DMS is excreted through the lungs and through other tissues and contributes to the characteristic odor.

The clinical toxicity of DMSO in marrow transplantation has been studied. Anaphylactoid symptoms attributable to the release of histamine and other mediators are common. Other toxic clinical signs and symptoms include hemolysis with hemoglobinuria, hyperosmolality, increased serum transaminase values, nausea, vomiting, abdominal cramping, fever, chills, tachypnea, cough, diarrhea, flushing, and headache.<sup>139–140</sup> Patients who have been conditioned with chemotherapy or who have smaller body mass (<70 kg), seem more likely to experience nausea and vomiting after infusion of DMSO-preserved cells. Cardiovascular toxicities include decreased heart rate and bradycardia, occasionally increased heart rate and tachycardia, ectopic heartbeat, heart block, hypertension, and other lesser blood pressure changes.<sup>139,141</sup> Some studies, however, raise the question whether there is any significant cardiovascular toxicity of DMSO.<sup>142</sup> It is possible that some

adverse effects attributed to DMSO may be caused by the cellular infusion itself.

The mechanism of the clinical toxicities associated with DMSO infusion has not been well established. Histamine receptor binding of DMSO, histamine release, direct vagal tonic effects, cold thermal vagal responses, and renal failure secondary to hemolysis explain many of the symptoms observed during cryopreserved cellular infusion.<sup>139,143</sup> Increases in thrombin-antithrombin complex,  $\beta$ -thromboglobulin, platelet factor 4, and von Willebrand factor caused by DMSO have been described.

Several measures can be taken to prevent or reduce DMSO toxicity. Antihistamine prophylaxis is recommended routinely before any administration of DMSO. Additional premedications to consider before infusion of hematopoietic stem cells cryopreserved with DMSO include a sedative such as lorazepam and/or hydrocortisone. Intravenous DMSO should be given as a 10–40% solution to avoid local irritation. The recommended maximum daily dose of DMSO is 1 g/kg/day. Slowing an infusion containing DMSO or increasing the time between infusions of multiple aliquots greatly diminishes DMSO-related toxicity, which appears to be a dose-dependent but short-lived response. However, because DMSO is toxic to thawed mononuclear cells, hematopoietic progenitor cells can tolerate exposure to 10% DMSO for only as long as one hour.<sup>144</sup> This limits how much the infusion rate can be slowed. Antiemetics and sedatives can help to ameliorate symptoms, and cellular products can be carefully washed before infusion to remove DMSO and other substances.

## Reactions in special transfusion settings

### Granulocyte transfusion reactions

Granulocyte transfusions remain in use as a treatment option for neutropenic patients because of improved granulocyte collection yields after donor treatment with steroids and granulocyte colony-stimulating factor (G-CSF). Febrile nonhemolytic transfusion reactions after granulocyte transfusion are common and to some degree expected. Severe reactions can be accompanied by pulmonary complications (e.g., TRALI, dyspnea, pulmonary infiltrates, and hypoxia), hypotension, and even cardiovascular collapse.<sup>145</sup> In a recent study of dexamethasone and G-CSF-stimulated granulocyte transfusions, 37% of patients (7% of transfusions) experienced chills, 32% of patients (7% of transfusions) experienced a fever, and 11% of patients (2% of transfusions) experienced hives or itching during a course of therapy.<sup>146</sup> Oxygen desaturation of greater than 3% occurred in 7% of transfusions, and severe desaturation of greater than 6% occurred in 3 of 11 patients experiencing oxygen desaturation. In addition, granulocyte transfusions carry further risk of leukocyte alloimmunization and cytomegalovirus infection.<sup>147</sup>

Concurrent administration of amphotericin B and granulocytes has been linked to severe pulmonary reactions, although the association has not been confirmed and remains in doubt. In a study that examines 195 granulocyte transfusions in 144 patients demonstrated no severe pulmonary toxicity from concomitant administration of granulocytes and amphotericin B.<sup>148</sup> Dyspnea as a side effect of granulocyte transfusion was equally common among patients receiving amphotericin B and those in a matched control group not receiving amphotericin B. It is safe to administer granulocyte transfusion and amphotericin B simultaneously without pulmonary toxicity.

Nevertheless, it is prudent to separate amphotericin B administration and granulocyte therapy by at least six hours to avoid confusion about the cause of a severe reaction, which can occur with either of these reaction-prone treatments. Because of the relatively high rate and severity of febrile, pulmonary, and allergic reactions, it is prudent to give premedication with acetaminophen and diphenhydramine to recipients of granulocyte transfusions. Hydrocortisone may be added as premedication in the treatment of patients with severe reactions who otherwise cannot tolerate granulocyte transfusion, although the immunosuppressive effects of this agent are unwelcome among patients who need granulocyte transfusions to fight serious and life-threatening infections. Granulocyte concentrates should be transfused slowly.

### Autologous transfusion reactions

A variety of reactions to autologous blood occur despite the complete compatibility. In a study involving 596 hospitals, the rate of reported FNHTRs to autologous blood was 0.12% and the rate of allergic reactions was 0.01% per transfused unit.<sup>149</sup> Such rates are approximately 5–10-fold lower than those reported for allogeneic units. The cause of autologous transfusion reactions has not been clearly established. Mechanisms in many cases presumably are the same as for allogeneic transfusions. For example, autologous units can be contaminated with bacteria as can allogeneic units, and contamination leads to febrile reactions or septic complications.<sup>150</sup> Because autologous donors are patients, not healthy volunteers, they may have various medical problems that put them at increased risk of bacteremia.

RBCs: red blood cells; TRALI: transfusion-related acute lung injury; TACO: transfusion-associated circulatory overload.

Accumulation of inflammatory mediators in blood component bags during storage, released from passenger leukocytes or platelets, may result in the infusion of pyrogenic substances. Autologous leukocytes, rather than allogeneic leukocytes, can also generate endogenous pyrogens. Allergic reactions may be provoked by histamine generation during storage of blood components or by chemicals leached from blood storage containers or filters. Moreover, autologous transfusions may contribute to volume overload and hypervolemic reactions through mechanisms identical to allogeneic transfusions.

### Summary

A variety of acute, nonhemolytic, and noninfectious reactions are reported after transfusion (Table 48.2). Many of these reactions have an immune basis and represent inflammatory or allergic responses to infused cells (e.g., many FNHTRs) or plasma (e.g., some FNHTRs, allergic, and anaphylactic reactions). Although urgent transfusion can be lifesaving, it is important to recognize that a large volume of blood components given too quickly can itself have adverse chemical or physical effects, such as hypothermia, hyperkalemia, hypokalemia, hypocalcemia, and circulatory overload. Some reactions are also caused by unintended consequences of blood storage conditions or processing, such as generation of bradykinin by the contact of blood with some filter surfaces, leaching of toxic chemicals from filters or containers, and use of the chemical DMSO during hematopoietic progenitor cell preservation. It is important that these reactions and toxicities be recognized rapidly by the patient care team and blood bank personnel so that appropriate treatment and preventive measures can be instituted quickly.

**Table 48.2** Transfusion Reaction Summary

Type	Cause	Signs and Symptoms	Treatment	Prevention
<b>Febrile, nonhemolytic frequency of FNHTRs</b> before leukocyte reduction 0.33–0.37% (red cells) and 0.45–2.18% (platelets), and after leukocyte reduction 0.15–0.19% (red cells) and 0.11–0.15% (platelets) <b>Allergic</b> overall frequency of 0.11%	Recipient antibodies against leukocytes or platelets antigens in donor blood components; cytokines in plasma or supernatant portion of stored components; undetected bacteria contamination of blood component Allergen is a soluble substance in donor plasma	Chills, fever (>1 °C increase in body temperature); rigors in severe reactions Localized or generalized urticaria, erythema and pruritus; if severe, may have laryngeal or facial angioedema, and hypotension Urticaria, flushing, angioedema, stridor, wheezing, tachycardia, hypotension, shock, abdominal pain, diarrhea, pelvic pain	Stop transfusion, notify physician and blood bank, maintain IV line, monitor vital signs; physician may order acetaminophen Hold transfusion, notify physician, monitor vital signs; physician may order antihistamines or restart of transfusion if mild urticaria clears and no other symptoms in 30 minutes Stop transfusion; maintain IV line; notify physician and blood bank, monitor vital signs; physician may order antihistamines, epinephrine, oxygen, IV crystalloid, or glucocorticoids	Premedicate with acetaminophen (or glucocorticoid for refractory cases); give leukocyte-reduced RBCs Premedicate with H <sub>1</sub> blocking antihistamine; add H <sub>2</sub> blocker or glucocorticoid for refractory cases; consider washed RBCs and platelets for repeated or severe reactions Premedicate with antihistamines and glucocorticoid; transfuse washed RBCs and platelets for recurrent reactions; use IgA deficient donors or washed RBCs and platelets for sensitized patients with IgA deficiency
<b>Anaphylactic or anaphylactoid</b>	Recipient antibodies to a soluble substance in donor plasma; infusion of plasma with IgA into IgA-deficient recipient with IgG anti-IgA antibodies	Dyspnea, orthopnea, systolic hypertension, headache, peripheral edema, coughing, cyanosis, fever may be present	Slow or stop transfusion; keep IV line open; notify physician; monitor vital signs and input and output; physician may order diuretics and oxygen	Transfuse slowly; use split units; consider premedication with diuretics; carefully monitor aged, debilitated, cardiac or pediatric patients
<b>Transfusion-associated circulatory overload (TACO)</b>	Blood volume too large or infusion too fast for compromised cardiovascular system	Decreased body temperature, chills, cardiac arrhythmia (ventricular fibrillation)	Slow or stop transfusion; use an approved blood warmer, blankets, and other patient warming techniques (warm lavages, lamps)	Transfuse slowly, use an approved blood warmer
<b>Hypothermia</b>	Core body temperature <35 °C caused by rapid infusion of cold blood products, such as RBCs, FFP, cryoprecipitate	Perioral or peripheral paresthesia, tingling, buzzing, teeth chattering, bed or chair moving, cramps, nausea, vomiting, arrhythmia, bradycardia, hypotension, prolongation of QT interval, tetany	Slow or stop transfusion; slow or stop apheresis procedure; give IV calcium chloride or gluconate (for PBSC apheresis: 0.5 mg Ca <sup>2+</sup> /1.0 mL ACD-A), or check ionized Ca <sup>2+</sup> and dose per results; monitor relief of symptoms; consider hypomagnesemia	More likely in pediatric and lightweight patients (<70 kg) and patients with liver dysfunction, renal failure, or less skeletal muscle; observe patients closely for any symptoms; give IV calcium (for PBSC apheresis: 0.5 mg Ca <sup>2+</sup> /1.0 mL ACD-A).
<b>Citrate toxicity</b>	Excessive infusion of citrate during apheresis procedure or massive or rapid transfusion; patients with liver failure are at increased risk	Hyperkalemia: cardiac arrhythmia, ECG changes—peaked T waves, prolongation of PR interval (if severe, flat or lost P wave), widened QRS, ventricular arrhythmia Hypokalemia: massive or rapid transfusion of citrate and metabolic alkalosis	Hyperkalemia: give calcium to protect against cardiac effect, alkalinize blood, D50 plus insulin, sodium polystyrene sulfonate; dialysis Hypokalemia: give potassium	Hyperkalemia: give fresh products (<7 days old), or washed products
<b>Electrolyte disorder</b>	Hyperkalemia: transfusion of older blood components or massive transfusion of RBCs	Hypokalemia: massive or rapid transfusion of citrate and metabolic alkalosis	Hypokalemia: give potassium	Hypokalemia: give Adsolpreserved RBCs (not Nutrice)
<b>Hypotensive</b>	Bradykinin generation with use of negatively charged bedside leukocyte reduction filters in patients taking angiotensin-converting enzyme (ACE) inhibitors; apheresis procedures using albumin, especially in patient's taking ACE inhibitors; see also citrate toxicity	Hypotension; sometimes also flushing, respiratory distress, nausea, abdominal pain, and loss of consciousness	Stop transfusion, notify physician and blood bank; support blood pressure	Avoid use of bedside leukocyte reduction filters in patients taking ACE inhibitors; use prestorage leukocyte-reduced components or discontinue ACE inhibitor before transfusion or apheresis procedure; if during apheresis, correct electrolyte disorder such as hypocalcemia
<b>DMSO toxicity</b>	Cryopreservative for bone marrow, PBSCs, Cord blood, donor lymphocyte infusions, or any frozen cellular component; toxicity with DMSO >1.0 g/kg/day	Flushing, nausea, vomiting, abdominal cramping, throat tightness and cough, hypotension, hypertension, arrhythmia, fever, chills, headache, hemoglobinuria, hyperosmolality, increased liver enzymes	Antihistamines; antiemetics; slow or stop the infusion; supportive care; wait between infusions for symptoms to clear	Antihistamines; sedative (lorazepam) and hydrocortisone, washed or plasma or volume depleted cellular infusions; antiemetics

IV: intravenous; RBCs: red blood cells; FFP: fresh-frozen plasma; PBSC: peripheral blood stem cell; ACD-A: acid-citrate-dextrose-adrenaline; D50: dextrose 50% in water; ECG: electrocardiogram; DMSO: dimethyl sulfoxide.

**Table 48.3** Overlapping Signs and Symptoms of Transfusion Reactions

Sign or Symptom	Possible Reaction	Most Likely > Less Likely Blood Component*
Fever, chills, and rigors	Febrile nonhemolytic	Platelets (especially septic)
	Septic	> RBCs > plasma
	Acute hemolytic	
Urticaria and pruritus	TRALI or TACO	
	Allergic	Plasma > platelets > RBCs
Dyspnea	Anaphylactic	
	TACO	Any
	TRALI	
Hypotension	Anaphylactic	
	Septic	Platelets > plasma > RBCs
	Hypotensive	
	Acute hemolytic	
	Anaphylactic	

\* Granulocytes would very likely cause febrile reactions and dyspnea. However, granulocytes are not listed in the table because they are infrequently transfused compared to other blood components.

Care providers who administer transfusions must recognize that some symptoms of transfusion reactions, such as fever, are non-specific and may be early manifestations of potentially life-threatening reactions, such as hemolysis or sepsis (Table 48.3). For that reason, the guiding rule regarding most transfusion reactions is to err on the side of conservatism and stop the transfusion immediately. Transfusion of a blood component that causes a reaction before complete infusion should not be restarted, with the possible exception of mild urticarial reactions. Several strategies are available to prevent repeated reactions among patients who are reaction-prone. These include

leukocyte reduction for the prevention of FNHTR, cell washing for the prevention of allergic and anaphylactic reactions and possibly some FNHTRs, and various premedication regimens.

## Disclaimer

The authors have disclosed no conflicts of interest.

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- A full reference list for this chapter is available at: <http://www.wiley.com/go/simon/Rossi6>
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## CHAPTER 49

# Transfusion-related acute lung injury and other respiratory-related transfusion reactions

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Respiratory compromise related to transfusion may be divided broadly into cardiogenic and noncardiogenic causes. Acute noncardiogenic pulmonary edema occurring immediately after transfusion of donor plasma known to contain leukoagglutinins was first described by Brittingham in 1957.<sup>1</sup> Scattered case reports of *allergic pulmonary edema* or *anaphylactic pulmonary edema* followed. In 1985, Popovsky and Moore published the first prospective study of this complication that they termed *transfusion-related acute lung injury* (TRALI).<sup>2</sup> They reported a much higher incidence of the complication than had previously been considered, and following their report, increasing numbers of individual cases and small case series were published. With the development of hemovigilance schemes in the late 1990s, it became apparent that TRALI was not only an important cause of transfusion-related morbidity but also, with the reduction in infectious complications of transfusion, a leading cause of transfusion-related mortality. Better understanding of the pathophysiology has led to more efficacious prevention strategies. The increased understanding and interest in TRALI also led, via active hemovigilance schemes, to improved recognition of cardiogenic dyspnea due to volume overload, now generally termed *transfusion-associated circulatory overload* (TACO).

### TRALI: clinical features

Clinical reports of TRALI describe sudden deterioration of lung function temporally related to blood transfusion. Acute lung injury with noncardiogenic pulmonary edema is common among critically ill patients and is generally considered to have a multifactorial pathogenesis. Many sick patients receive transfusion, especially after trauma. There is evidence that TRALI is a significant contributor to acute lung injury (ALI) and that plasma-rich components are particularly implicated. Many case reports describe severe clinical manifestations, including most of the early published reports. Thanks to a better understanding of the pathophysiology, consensus definitions, and hemovigilance schemes, milder cases are being recognized more often, including those with more subtle presentation.

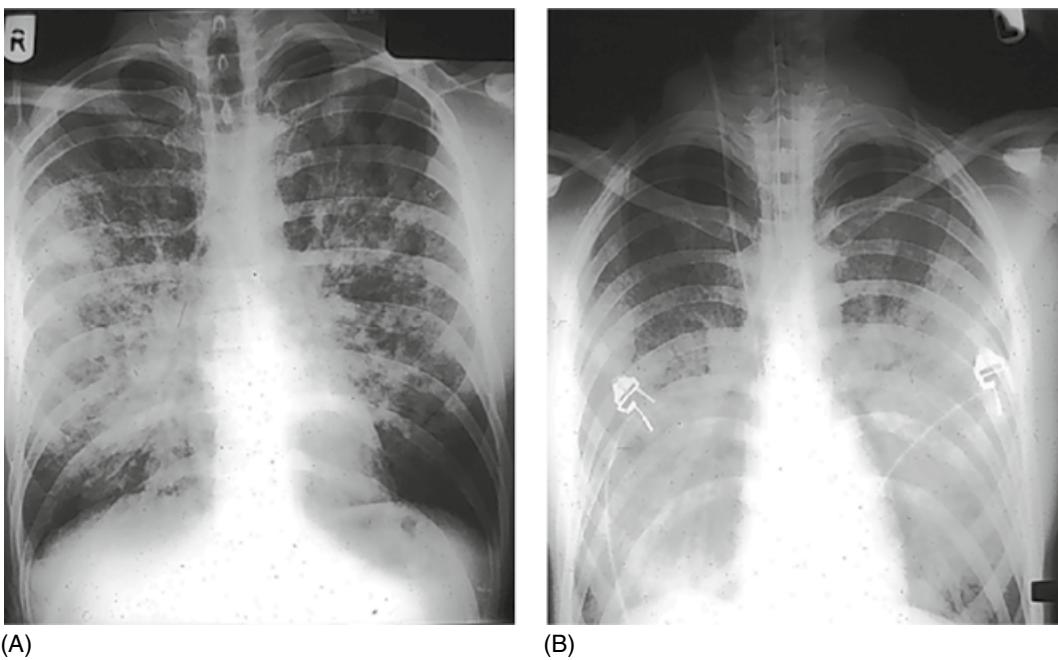
The 2019 consensus definition of TRALI eliminates “probable” and “possible” terminology in lieu of Type I and Type II.<sup>3</sup> In TRALI

type I, patients have no evidence of ALI prior to transfusion, while TRALI type II occurs in patients with preexisting, mild acute respiratory distress syndrome (ARDS) or risk factors for developing ARDS. TRALI symptoms quickly occur after cessation of transfusion: usually within two hours and almost always within six hours. A small number of reports suggest that the onset may rarely be delayed until 12 hours or more after transfusion.<sup>4</sup> Patients describe chest tightness, shortness of breath, and dry cough but may also experience nausea, dizziness, or rigors. On examination, the patient is hypoxic, often hypotensive, tachypneic, and tachycardic. On chest auscultation, widespread crepitations are heard. Rigors and fever are commonly reported but are not always present, and fever may develop hours later.

If the patient is mechanically ventilated, a typical finding is copious and frothy tracheal exudate, resembling whipped egg whites. Although nondiagnostic, anesthesiologists often remark on the quantity of this exudate, and it may be a hallmark of some severe cases of TRALI. Arterial blood gasses show hypoxia ( $\text{PaO}_2/\text{FiO}_2 \leq 300$  mmHg or oxygen saturation less than 90% on room air) and hypercapnia that are often severe.

Chest radiographs show bilateral infiltrates, typically in the “bat’s wing” pattern of acute respiratory distress syndrome (ARDS) (Figure 49.1). The edema from increased pulmonary capillary permeability is characterized as patchy or nodular shadowing mainly in the peripheral lung. The apices and costophrenic angles are spared and have the appearance of air bronchograms. In later stages or in severe TRALI, the chest radiograph may progress to a complete whiteout of the lung fields. Recovery of respiratory function starts as early as six hours after symptom onset in milder cases, but in some patients deterioration may continue for 24 hours or beyond.

Some authors have reported a slightly different clinical picture in which the chief signs are rigors and fever with transient respiratory dysfunction, hypertension rather than hypotension, and occurrence usually within 30 minutes of transfusion. Pulmonary edema is not always demonstrated radiologically, and recovery is within one or two hours. These reactions have typically been associated with cellular blood components. It has been suggested that these cases may represent a different, nonantibody-related etiology from the “classic” severe cases.<sup>5</sup> However, a similar spectrum of mild-to-moderate



**Figure 49.1** Chest X-rays from a 33-year-old man with severe TRALI, taken 2 hours (A) and 24 hours (B) following transfusion of FFP containing HLA class II antibodies. Note the "bat's wing" pattern of edema with sparing of the lung bases, the air bronchograms clearly visible on the first radiograph, and the more confluent airspace shadowing but continued basal sparing in the second X-ray.

reactions of this nature has also been documented with transfusion of plasma-containing antibodies to a neutrophil antigen, anti-HNA-2.<sup>6</sup>

### Pathophysiology

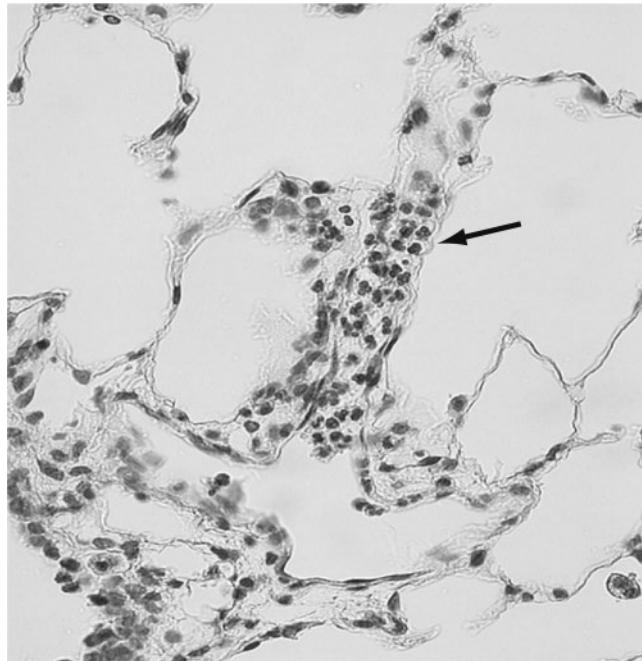
It is now understood that many cases of TRALI, including the most severe cases, are due to an antibody–antigen interaction between donor and recipient. Other mechanisms have been postulated and may be contributory to antibody-related cases or to other causes of lung damage. It is helpful to divide TRALI into antibody-related and nonantibody-related lung damage.

### Acute lung injury

ALI is the result of capillary endothelial leakage, allowing fluid to pass from the pulmonary vessels, through the interstitial space, and into the alveolar air space. Because this edema is distinct from hydrostatic edema caused by cardiac failure or volume overload, it is sometimes known as *nonhydrostatic edema*. Numerous stimuli have been suggested to contribute to the likelihood of developing nonhydrostatic pulmonary edema, including sepsis, trauma, aspiration of gastric contents, disseminated intravascular coagulation, and high tidal volume ventilation. In some cases of TRALI, the transfusion appears to be the only probable cause of the lung injury, whereas in other cases it may be only one of several possible factors present. Histopathology, clinical findings and experimental work have helped elucidate the stimulus present within the transfused blood and the mechanism of subsequent lung damage.

### Histopathology

Histopathologic findings from fatal TRALI cases are consistent with those for early ARDS: interstitial and intra-alveolar edema<sup>2,4,7–11</sup> with extravasation of neutrophils into the interstitial and air spaces (Figure 49.2).<sup>4,7,8,11</sup> Hyaline membrane formation and



**Figure 49.2** Sections of lung from a fatal case of TRALI. Note the presence of granulocytes in the capillaries (arrow indicates neutrophils).

destruction of the pulmonary architecture have also been reported.<sup>7,8</sup> A consistent finding in TRALI is the presence of increased numbers of neutrophils within the pulmonary capillary vasculature and other small pulmonary vessels.<sup>10,11</sup> On electron photomicrographs, neutrophils were degranulated and (focally) in direct contact with denuded stretches of the capillary wall. A positive correlation has been reported both between capillary

leukostasis and desquamated epithelial cells, as well as between the degree of capillary leukostasis and the amount of proteinaceous fluid within the alveolar air spaces.<sup>11</sup> From these observations, it appears that the neutrophil is a central culprit of lung damage. Following neutrophil sequestration in the early stages of TRALI, neutrophils and endothelial cells establish close contact within the pulmonary microvasculature. Activation of the neutrophils leads to endothelial damage and capillary leakage. The transit of proteinaceous fluid from the vessels into the air spaces results in acute pulmonary edema. In the later stage, especially of severe TRALI, neutrophils extravasate from the capillary into the alveoli where they induce further pulmonary injury.

### Evidence for an antibody-related etiology

The relationship between TRALI and leukocyte antibodies in donor plasma was first noted by Brittingham, who reported that leukoagglutinins present in the plasma of a multitransfused patient induced an acute pulmonary reaction when transfused to a volunteer.<sup>1</sup> Similarly, severe pulmonary edema occurred in a healthy volunteer who received an experimental gamma globulin concentrate, deliberately prepared from plasma that contained leukocyte- and monocyte-reactive antibodies.<sup>12</sup> It is likely that this preparation contained high levels of human leukocyte antigen (HLA) class II antibodies. In addition to these cases of TRALI resulting from experimental transfusion of plasma containing leukocyte antibodies, there are numerous case reports of TRALI in which a transfused unit has been found to contain antibodies reactive with recipient leukocytes. In two large series of TRALI, where pulmonary infiltrates were apparent in chest X-rays, leukocyte antibodies in the

donor of a transfused blood component were detected in 61–89% of cases.<sup>2,13</sup> Animal models have provided confirmation of this antibody-mediated mechanism of TRALI.<sup>14</sup>

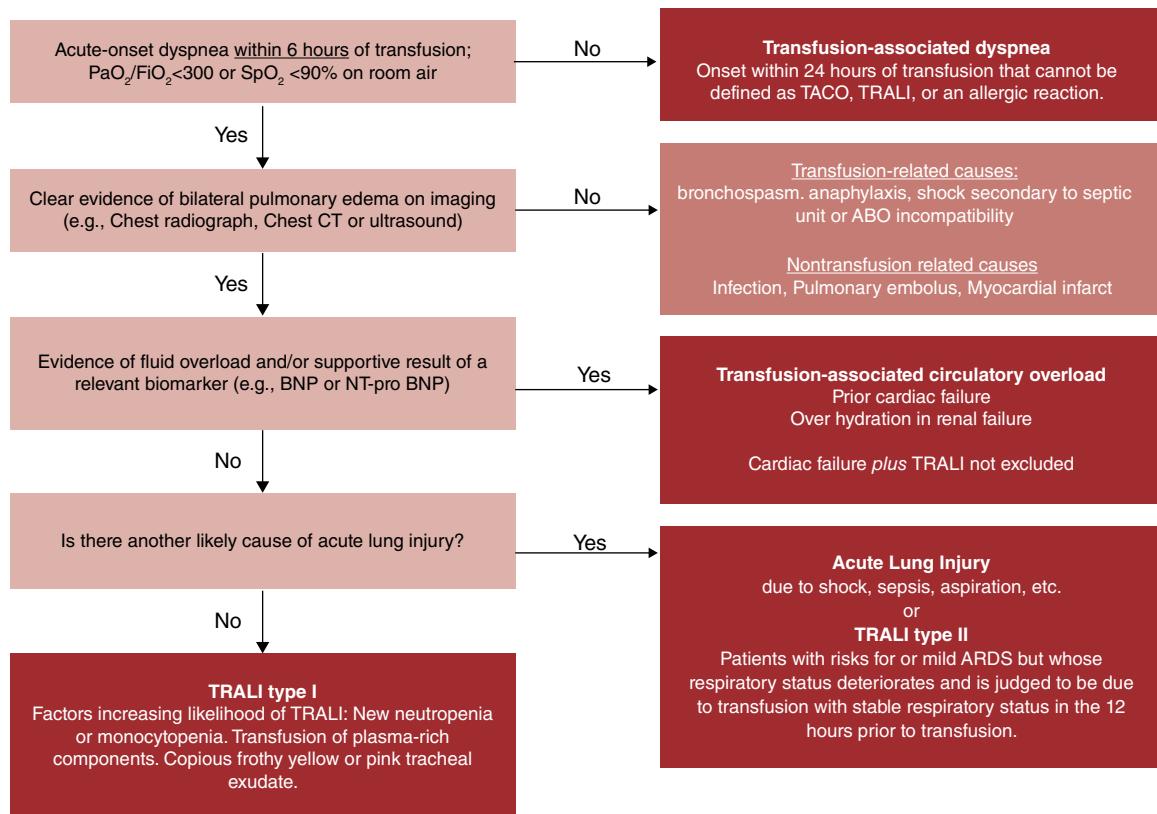
Alternative mechanisms leading to TRALI have been proposed and are discussed in this chapter, but the available evidence demonstrates that the predominant mechanism is donor leukocyte antibodies reacting with recipient antigens. Large clinical studies have corroborated these experimental findings, as have hemovigilance data obtained in countries where plasma from female donors (or female donors with a history of pregnancy) is no longer used for clinical transfusion.<sup>15–20</sup>

### Diagnosis

#### Respiratory dysfunction

New-onset respiratory dysfunction caused by pulmonary edema in a patient with recent transfusion should prompt consideration of TRALI. Significant respiratory dysfunction consistent with ALI can be defined as a decrease in transcutaneous oxygen saturation to less than 90% or an arterial  $\text{pO}_2$  of less than 60 mmHg while breathing room air or a  $\text{PaO}_2\text{--FiO}_2$  ratio of less than 300 mmHg.

Pulmonary edema can be demonstrated by clinical examination or chest X-ray. Alternative causes of sudden respiratory dysfunction, without edema, include transfusion-related problems (such as allergic reactions with bronchospasm, shock associated with a bacterially infected unit, or ABO incompatibility) and causes unrelated to the transfusion such as cardiac arrhythmias, infection, or pulmonary embolus (Figure 49.3).



**Figure 49.3** Flowchart for differential diagnosis of respiratory-related transfusion reactions.

## Physiologic measurements

Left atrial pressure or volume may be measured by pulmonary artery wedge pressure through a Swan–Ganz catheter, via esophageal Doppler ultrasound, or by transthoracic echocardiography. The goal of such measurements is to assess cardiac status in patients with suspected TRALI. High left atrial filling pressure or volume suggests cardiogenic pulmonary edema or fluid overload. Normal or low pressure or volume is consistent with noncardiogenic pulmonary edema. Low left atrial pressure indicates hypovolemia, a common finding in TRALI. Electrocardiography may also be helpful in detecting cardiac strain patterns or evidence of infarction.

Prior or new cardiac failure does not exclude the possibility of TRALI because both may be present in the same patient. However, cardiac failure does indicate that the use of diuretics, which are otherwise contraindicated in TRALI, may be beneficial.

## Consensus definition of TRALI

A consensus definition of TRALI was agreed upon in 2004 and revised in 2019.<sup>3,21</sup> These criteria do not include laboratory tests. A clinical diagnosis of TRALI is considered where a new episode of ALI has occurred within six hours of a blood component or derivative transfusion. Where no other cause of ARDS is found, the diagnosis of TRALI is designated Type I. Where another possible cause of ARDS is present, the diagnosis of TRALI is designated Type II (Table 49.1). Many cases of TRALI occur in patients with competing etiologies for ALI. Further recipient and donor investigation can help determine, often in retrospect, the probability of lung injury being entirely or partly due to transfusion.

## Laboratory tests

### Changes in circulating leukocytes

When leukopenia—in particular neutropenia and monocytopenia—occurs within the first hour after transfusion or is present before clinical signs of lung injury, transfusion is favored over other causes of ALI. The neutropenia is often followed by neutrophilia 5–6 hours later.<sup>1,22</sup> Monocytopenia is common, may be absolute, and may be more persistent than neutropenia.<sup>22,23</sup> Neither change is completely specific nor sensitive. The degree of neutropenia is likely to be greater with neutrophil-specific antibodies, such as anti-HNA-2, and with neutrophil-agglutinating antibodies, such as anti-HNA-3a and anti-HLA-A2, whereas monocytopenia alone is most likely to be seen with HLA class II antibodies.<sup>23</sup> Neutropenia is seen without evidence of lung damage with some neutrophil-specific antibodies.<sup>6,24</sup>

**Table 49.1** 2019 Consensus TRALI Definition

TRALI type I—patients who have no risk factors for ARDS and meet the following criteria:

- A   i   Acute onset
- ii   Hypoxemia:  $\text{PaO}_2/\text{FiO}_2 \leq 300$  or  $\text{SpO}_2 < 90\%$  on room air
- iii   Clear evidence of bilateral pulmonary edema on imaging (e.g., chest radiograph, chest CT, or ultrasound)
- iv   No evidence of left atrial hypertension or, if LAH is present, it is judged to not be the main contributor to the hypoxemia
- B   Onset during or within 6 hours of transfusion
- C   No temporal relationship to an alternative risk factor for ARDS

TRALI type II—patients who have risk factors for ARDS (but who have not been diagnosed with ARDS) or who have preexisting mild ARDS ( $\text{PaO}_2/\text{FiO}_2$  of 200–300), but whose respiratory status deteriorates and is judged to be due to transfusion based on:

- A   Findings as described in categories A and B of TRALI type I
- B   Stable respiratory status in the 12 hours prior to transfusion

## B-type natriuretic peptide and edema-to-serum protein concentration

A serum level of B-type natriuretic peptide (BNP) of  $<250$  pg/mL is consistent with ALI rather than cardiac failure, whereas a level of  $>250$  pg/mL or a twofold increase from a prior level is consistent with cardiac failure.<sup>25</sup> A low level is neither specific for ALI nor sensitive: many patients with ALI also have cardiac dysfunction. A high level may also be seen in renal failure.

A protein concentration in the pulmonary edema greater than 70% of the serum protein concentration is strong evidence in favor of a capillary-to-alveolus leak as seen in ALI from any cause.

## Incidence and epidemiology

### Variables affecting the incidence of TRALI

The frequency of reported TRALI depends upon variables affecting donors, recipients, and treating clinicians. Antibody-associated TRALI is the most common form and historically associated with blood from female donors with a history of pregnancy. Antibody-associated TRALI is both more common and more severe with transfusion of plasma-rich products, such as FFP or apheresis platelets. HLA antibodies have been found in approximately 15% of all female donors but are more common in those who have been pregnant more frequently or more recently,<sup>26,27</sup> and they are even more common with highly sensitive tests.<sup>28</sup> Human neutrophil antibodies are less common. Therefore, incidence of TRALI will depend, in part, on the donors recruited by the local blood center.

Lookback studies of regular blood donors whose antibody-containing plasma has been implicated in cases of TRALI suggest that many cases of TRALI are either overlooked or unreported. In addition, the infusion of an antibody to a recipient with a matching antigen does not necessarily result in TRALI.<sup>29,30</sup> Both inherited and acquired recipient variables as well as details of the transfusion will affect the likelihood of developing clinically apparent TRALI. Inherited variables have not been defined but will almost certainly include polymorphisms within immune response pathways. The presence of high levels of soluble HLA antigens in the recipient's plasma may be protective.<sup>31</sup> Acquired recipient variables include the presence of other lung insults and comorbidities. Transfusion variables include the titer and specificity of donor antibody, the volume of plasma infused, and the rate of infusion. Plasma-reduced RBCs transfused over 90 minutes rarely cause TRALI, whereas FFP given over 15 minutes—a more than 50-fold higher rate of plasma transfusion—is not uncommonly implicated.

Finally, the likelihood of diagnosing and reporting TRALI heavily depends on the treating physician's knowledge of the condition.

### Reported frequency of TRALI

One single-hospital prospective study estimated TRALI frequency for all blood components transfused without any donor selection as 1 in 5000 units.<sup>2</sup> A retrospective single-hospital study estimated the incidence as 1 in 7900 units of FFP only.<sup>22</sup> Hemovigilance data from voluntary reports in the United Kingdom found a reported incidence of approximately 1 in 30,000 units of FFP transfused.<sup>32</sup> Retrospective studies of ALI/TRALI in critically ill patients and a single prospective trial of TRALI after FFP infusion found that as many as 1 in 50–200 units of plasma from parous female donors could cause some respiratory dysfunction when compared to plasma from male donors.<sup>25,33</sup>

It is not possible to give a single figure for incidence, but the chance of female donor plasma containing an antibody matching a recipient antigen is greater than 1 in 50. Severe and clinically distinct TRALI probably occurs about 1 in 2500–4000 units of female donor plasma transfused. ALI in which transfusion is a contributory factor in a critically ill patient may be much more common.

## Management and outcome

### Management of the patient

The mainstay of treatment for TRALI is respiratory support using oxygen, alone, or in combination with either continuous positive airway pressure or mechanical ventilation. Fluid replacement is used in cases complicated by systemic hypovolemia. Severe cases often require central venous pressure or left atrial volume monitoring. Additional strategies include high-frequency ventilation and prone position ventilation. Diuretics are contraindicated because they worsen hypovolemia and hypotension.<sup>34</sup> Only in cases with concomitant fluid overload from cardiac failure (or other causes) are diuretics indicated.

There is limited evidence about the use of corticosteroids. When the diagnosis is made within six hours and the lung damage is severe, the use of high-dose steroids might *theoretically* limit further damage by inhibiting neutrophil activation. Prednisolone, methylprednisolone, and dexamethasone have all been used, but there is no evidence of efficacy. When the diagnosis is made later, high-dose steroids are unlikely to be helpful. In intractable cases, when maximal ventilation is insufficient, extracorporeal membrane oxygenation has been used to support the patient.<sup>35</sup> Plasmapheresis has also been utilized with the intent of removing the causative antibody.<sup>12</sup> Further transfusions should be given if clinically indicated, but plasma-rich products from female donors should be avoided.

### Clinical course and outcome

Most patients begin to improve within 24 hours of the initial symptoms. Milder cases improve as early as six hours. Chest X-rays usually show clearance of edema by 48–96 hours. The majority of patients recover without any long-term lung damage; however, damage in other organs, such as acute renal failure, is seen in more severe cases. It is unclear whether the organ injury seen in severe cases is due to hypoxia, hypovolemia, or damage to capillary beds in organs outside of the lungs.<sup>22</sup>

Mortality in different published series varies between 5% and 30%. This variation depends, in part, on how cases are ascertained. Where patients receiving transfusion were assessed prospectively for the complication, the incidence was higher, presumably because milder cases were more likely to be recognized, but the mortality was low (6%). Where reports depend on a physician recognizing and reporting the condition, the incidence is lower but mortality higher. As an example, the hemovigilance scheme in the United Kingdom reported seven deaths out of 28 possible, probable, or likely cases reported in 2002, a mortality of 25%.<sup>32</sup> Mortality is more likely in patients receiving larger volumes of single-donor plasma (including fresh-frozen plasma [FFP]) rather than RBCs and more likely in patients with more comorbidities. In 2020, only two cases of TRALI were reported to the UK SHOT system, with one fatality, giving the year a morality rate of 50%.<sup>18</sup>

### TRALI in neonates and children

TRALI has been reported in children with the same features as adult cases, including leukopenia, nonhydrostatic pulmonary edema, and hypovolemia. Some of the published reports attributed death of the child partly or wholly due to the transfusion reaction.<sup>36–38</sup> Age range of cases is from 0 months to 16 years, but few neonatal cases have been reported.<sup>38,39</sup> Use of blood components is more common in neonates than older children, and neonates also receive larger volumes of single-donor plasma relative to their weight. It is possible that neonates are less susceptible to TRALI.

A peculiar feature of pediatric TRALI is its occurrence after the use of directed donations from the mother. This practice has been documented to cause TRALI and can be expected to be high risk because of the high probability that the mother has leukocyte antibodies corresponding to her child's antigen specificity.<sup>40</sup> By the same rationale, directed donations from a wife to a husband, or of a leukocyte-containing component from a child to a mother or a husband to a wife, may be particularly likely to cause TRALI.

## Mechanisms of lung damage in TRALI

### Priming and activation of neutrophils

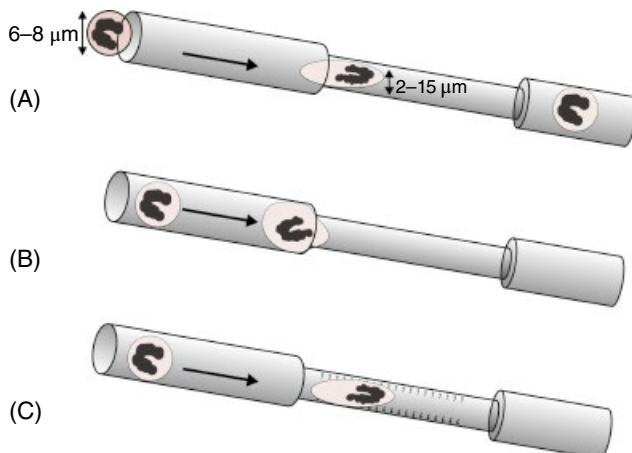
The dogma that TRALI is mediated by activated neutrophils has lost some of its cogency during recent years, mainly because TRALI still occurred in experimental animals depleted of neutrophils.<sup>41–43</sup> It appears reasonable to consider neutrophils as the major, but not the only, mediators in a process that also includes monocytes and the endothelium. Neutrophils activated by injurious agents respond by producing a range of highly toxic reactive oxygen species (ROS) and the release of preformed granular enzymes, proteins, and neutrophil extracellular traps (NETs).<sup>44,45</sup>

Neutrophils may be activated directly by one sufficiently strong stimulus, but activation more often requires two or more stimuli. The first “priming” stimulus potentiates the response to a second “activating” stimulus. A large percentage of patients who develop TRALI are sick, and there is *in vivo* evidence that surgical procedures and active infections induce neutrophil priming.<sup>46–48</sup> In response to priming agents, neutrophils become less pliable.<sup>49</sup> This “stiffening” augments mechanical retention of neutrophils within the pulmonary capillary bed, prolonging their passage through the lungs.<sup>50</sup> Prolonged, close contact between neutrophils and the endothelium provides a microenvironment in which transmembrane receptors and released mediators of each cell type can interact closely. Sequestered neutrophils, having been primed in the circulation, and endothelial cells can be activated by exogenous stimuli present in the blood bag. These transfused stimuli include antibodies, cytokines, and bioactive lipids.

Antibodies to neutrophil antigens involved in TRALI cases are able to prime and activate neutrophils in some cases without additional stimuli,<sup>14,51–53</sup> explaining why even completely healthy individuals can develop TRALI if the antibody stimulus is sufficiently strong.<sup>12</sup>

### Neutrophil passage through pulmonary microvasculature

The alveolar capillary bed is a complex, interconnected network of short capillary segments. The path of a neutrophil from arteriole to venule crosses eight or more alveolar walls and encounters more than 50 capillary segments. Approximately half of these pulmonary capillaries are narrower than the diameter of a spherical neutrophil (Figure 49.4). This forces neutrophils to slow and deform as they pass through narrow capillary segments. The



**Figure 49.4** The neutrophil's passage through the pulmonary microvasculature. Approximately 50% of all pulmonary capillaries surrounding the alveoli have a diameter smaller than the spherical neutrophil. (A) In order to pass through the capillary, neutrophils are forced to slow down and deform, assuming a "sausage shape" that allows transit through the capillary. (B) Neutrophil priming is associated with a decrease of deformability (called *stiffening*), which results in local trapping of the cell and a prolonged transit time. (C) Activation of the capillary endothelial cells results in the upregulation of surface ligands, which contributes to local trapping of the cell and a prolonged overall transit time.

transit time of neutrophils through the pulmonary microvasculature is mainly affected by the time it takes for neutrophils to deform, and slow transit accounts for significant accumulation of neutrophils in the lungs.<sup>54</sup> The pulmonary circulation normally contains about 30%, the "marginated pool," of total blood neutrophils. The stimulus-induced decrease in deformability appears to be more important than selectin-mediated rolling, a key mechanism of neutrophil adhesion in other capillary beds, but changes in surface receptors in primed neutrophils will also favor molecular adhesion to endothelial cells.<sup>54–56</sup> Under physiologic conditions, primed and locally trapped neutrophils migrate from the capillaries into the alveoli as part of a local inflammatory reaction. In TRALI, the primed and trapped neutrophil encounters a further activation signal in the form of transfused antibody or other transfused stimulus, and activates its microbicidal arsenal, inducing endothelial damage.

#### Activation of pulmonary endothelial cells

TRALI can also be triggered by activated pulmonary endothelium. In addition to constitutively expressed surface receptors, activated endothelial cells upregulate surface membrane receptors that facilitate neutrophil adhesion and priming. Primary activation of endothelial cells has been suggested as the mechanism responsible for TRALI after infusion of bioactive lipids.<sup>57,58</sup> More recently, antibodies recognizing proteins present on the endothelial surface (i.e., HNA-3a) were shown to directly interfere with endothelial function,<sup>42</sup> indicating that barrier breakdown leading to lung edema does not necessarily involve the activation of neutrophils. These experimental data are in line with a clinical report of an HLA-B44-negative patient transfused with blood containing anti-HLA-B44 antibodies who developed "half-sided" TRALI in his recently transplanted, HLA-B44-positive lung only.<sup>59</sup> These antibodies must have either reacted with transplant endothelium or, possibly, donor-type (alveolar) macrophages.

#### Activation of monocytes

In contrast to HNA and HLA class I, HLA class II antigens are usually not present on neutrophils (or endothelial cells), but are present on monocytes. Kopko *et al.* suggested a monocyte-dependent mechanism of TRALI, where HLA class II antibodies bind to monocytes, induce the release of neutrophil-activating mediators, and induce neutrophil activation.<sup>60</sup> Using human plasma with anti-HLA-DR7 and -DR52 specificity along with human neutrophils and monocytes in an ex vivo rat lung model, Sachs *et al.* showed that HLA class II antibodies can induce TRALI via such a multistep pathway, including the initial activation of monocytes and the release of interleukin-8 (IL8).<sup>61</sup> Subsequently, experiments in mice delineated that monocytes may also be involved in HLA class I-mediated TRALI because depletion of these cells suppressed HLA class I antibody-mediated TRALI, as did the blockage of MIP2 receptors, the murine analog of IL8.<sup>41,43</sup>

#### Platelets and TRALI

Studies to date have not clearly elucidated the role, if any, of platelets in the pathophysiology of TRALI;<sup>41,44,45,62–64</sup> however, some data support a pathogenic role of platelets in the development of TRALI.<sup>65</sup>

#### Neutrophil, monocyte, and endothelial cell interplay

The interplay between neutrophils, monocytes, endothelial cells, and, potentially, platelets contributes to lung damage. Neutrophils respond to monocyte and/or endothelial cell-derived mediators by activating and expressing integrins and by releasing proinflammatory mediators and granule contents. Released mediators activate endothelial cells, which, in turn, mobilize selectins, upregulate adhesion proteins, and produce inflammatory mediators; thereby, enhancing neutrophil and platelet adhesion and neutrophil, platelet, and monocyte activation. It is within this interplay that the lung barrier breaks down and allows transit of proteinaceous fluid and, later, of neutrophils into the alveolar space. Experimentally, other blood cells seem to attenuate this complex process.<sup>66,67</sup>

#### Mechanisms of lung injury by different mediators in transfused blood components

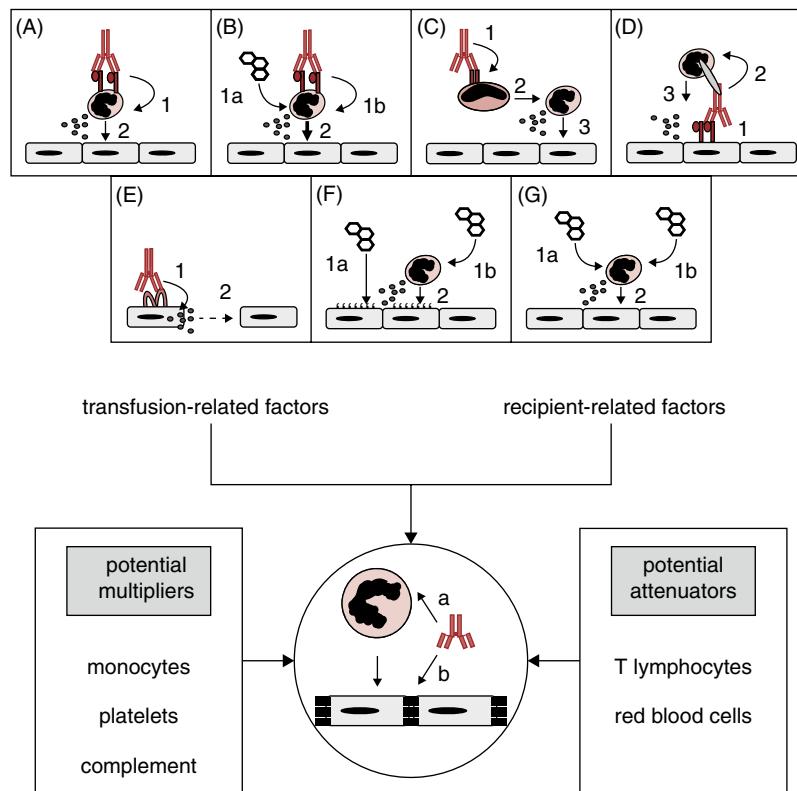
The exact pathway leading to lung damage associated with transfusion depends on the nature of the antibody or other stimulus and the interplay between it and the cellular components.

#### Antibody mediators of TRALI

Antibodies to neutrophil antigens, HLA class I, and HLA class II have all been clearly implicated in TRALI. Evidence from both hemovigilance schemes and laboratories specializing in TRALI investigation shows that the majority of cases (75–90%) are associated with HLA antibodies, and that about 50% of these antibodies are directed against HLA class II antigens.<sup>20</sup> Antibodies to neutrophil antigens are found less frequently, in about 10–25% of cases.

#### Antibodies to human neutrophil antigens (HNA)

The ability of HNA antibodies to induce TRALI has been shown in ex vivo models of lung injury.<sup>14,68</sup> In particular, antibodies with HNA-2 and HNA-4a specificity are capable of directly activating neutrophils, which appears to be the mechanism by which they induce TRALI (Figure 49.5A–B).<sup>14,51,69</sup> Of note, antibodies against HNA-3a can interact directly with endothelial cells because their cognate antigen CTL-2 is expressed not only on neutrophils but also on endothelial cells within lungs.<sup>42</sup> Binding of anti-HNA-3a to endothelial cells leads to the production of ROS within the



**Figure 49.5** Proposed mechanisms of TRALI. The activation of neutrophils and the subsequent release of toxic agents that harm the endothelium, predominantly ROS, are key mechanisms in TRALI. Epidemiological, clinical, and experimental data show that most TRALI reactions are induced by antibodies present in a transfused blood component. These antibodies recognize epitopes on the surface of (A, B) neutrophils, (C) monocytes, or (D, E) endothelial cells. Binding of HNA or HLA class I antibodies to neutrophils causes direct activation of the cell. (C) Binding of antibodies, especially of HLA class II antibodies, to monocytes has been proposed to induce the release of mediators that activate the neutrophil. (D) Experimental data demonstrate that neutrophils may also be activated indirectly when HLA class I antibodies are bound to the endothelium of the lung, where they can recruit neutrophils via their Fc receptors binding to the Fc parts of these antibodies. (E) Further evidence also indicates direct endothelial effects of antibodies, such as anti-HNA-3a. (F, G) Biologically active substances other than antibodies (e.g., bioactive lipids and CD40L derived from cellular blood components) are thought to cause some cases of TRALI. These substances are usually too weak to activate neutrophils directly, but may induce TRALI in concert with other factors that activate endothelial cells or neutrophils. See the text for details.

endothelium, causing the endothelial cells to lose cohesion, allowing fluid to shift to the alveolar space (Figure 49.5E). It is suggested that this direct mechanism of TRALI induction accounts for a higher rate of fatal cases of TRALI associated with HNA-3a antibodies.<sup>70</sup> The activation of endothelial cells by direct binding of HNA-3a antibodies also leads to cellular activation and neutrophil recruitment, indicated by the fact that these antibodies can induce (milder) TRALI in a murine model in the absence of neutrophils, but induce more severe TRALI when neutrophils are present,<sup>42</sup> corroborating the idea that once the process is started, multiple players become involved.

#### Antibodies to HLA class I antigens

HLA class I antibodies are frequent in the donor population. The presence of HLA class I antibodies in transfused plasma is a common cause of TRALI (Figure 49.5D). The cognate antigens recognized by these antibodies are expressed on all of the cell types that we understand to play a role in TRALI. HLA class I antibodies can directly bind to neutrophils and prime oxidases in both humans and rats.<sup>71,72</sup> Not surprisingly, there are anecdotal reports of TRALI associated with HLA class I antibodies, especially of anti-HLA-A2 specificity, which is directed against a commonly occurring HLA

antigen. However, HLA class I antibodies were not associated with increased risk of TRALI in several observational studies.<sup>15–17</sup>

Most TRALI animal experiments utilized anti-MHC (major histocompatibility complex) class I antibodies. Of note, only one of the tested monoclonal MHC class I antibodies induced TRALI in mice when infused (anti-H2K<sup>d</sup>, clone 34.1.2s), and results obtained with these experiments, albeit highly informative, should be interpreted cautiously, especially when it comes to their impact on transfusion medicine. The various experimental findings reported for HLA class I antibodies make it likely that numerous activation steps must occur before a reaction commences in a patient. This may involve antibody binding to neutrophils, endothelial cells, or monocytes. The fact that human antibodies—in contrast to the monoclonals used in animal studies—are polyclonal and differ in their binding characteristics and avidity may explain the lack of a clear association of HLA class I antibodies and TRALI in studies of donor risk factors.

#### Antibodies to HLA class II antigens

In contrast to HNA and HLA class I antigens, HLA class II antigens are usually not present on neutrophils (or endothelial cells) but are present on monocytes. Neutrophils and endothelial cells may express HLA class II following stimulation, but HLA class II

antigen expression was not found on vascular endothelium of pulmonary capillaries or intravascular neutrophils in a patient who experienced fatal TRALI caused by an HLA class II antibody.<sup>73</sup> Although a direct mechanism between HLA class II antibodies and neutrophils or pulmonary capillary endothelium cannot formally be excluded, monocytes appear currently as major target cells for these type of antibodies (Figure 49.5C).<sup>60,61,74</sup> Intact endothelium likely prevents transfused antibodies from accumulating in the alveolar space in sufficient concentration to induce release of cytokines or subsequent activation of neutrophils and/or endothelial cells, but where there is already some damage to the pulmonary endothelium, such a reaction may exacerbate ALI.

It remains possible that the detection of antibodies to HNA and HLA could be surrogates for antibodies to as-yet-unknown antigens on other cell types (e.g., on monocytes). Alloantibodies to these or other cells might explain some apparently antibody-negative cases.

### Inverse TRALI: transfusion of neutrophils

In most cases of TRALI, the causative agent for the pulmonary reaction are antibodies or neutrophil-priming agents present in the transfused component. However, TRALI has also been reported in alloimmunized patients receiving blood components that contain neutrophils. Viable neutrophils may still be present in blood components, and Popovsky and Moore estimated that 6% of observed TRALI cases were caused by antibodies present in the recipient.<sup>2</sup> Universal leukocyte reduction has likely reduced the incidence of such reactions from platelet concentrates (PCs) and red blood cells (RBCs). Inverse TRALI will remain of particular relevance to patients receiving granulocyte transfusions.<sup>39,75</sup>

### Nonantibody mediators of TRALI

#### *Biologically active lipids*

Blood components can accumulate intermediate metabolic products, including biologically active (bioactive) lipids, throughout their storage. These substances are breakdown products of membrane lipids, including lyso-phosphatidylcholines (lysoPCs), which prime respiratory burst reactions in neutrophils. The longer that RBCs are stored, the more proinflammatory mediators accumulate in the unit to be transfused. These neutrophil-priming agents do not develop in stored, acellular plasma because their generation depends on the presence of blood cells. The changes in stored RBCs may mediate antibody-negative cases of TRALI. Indeed, the administration of supernatant from stored (but not fresh) human RBCs caused TRALI in ex vivo and in vivo animal models of TRALI (Figure 49.5F and 49.5G).<sup>57,71</sup> The metabolic products that accumulate are able to activate endothelial cells in the pulmonary microvasculature through upregulated ICAM-1 expression causing increased PMN adherence and subsequent BLT2 receptor-mediated activation of protein kinase C.<sup>76</sup>

Biologically active breakdown products were also investigated in the setting of platelet transfusions. In a cross-species study, supernatant of aged human platelets, but not of fresh platelets, caused ALI in an ex vivo rat lung model.<sup>58</sup> In a syngeneic rat model, whole aged rat platelet suspensions, but not fresh ones, led to neutrophil adherence and pulmonary edema. However, when LPS was used to mimic patient-related risk factors, stored platelets no longer induced TRALI despite an increased lysoPC content.<sup>77</sup>

In summary, there is currently no clear evidence on whether lysoPCs (or other bioactive lipids)<sup>78</sup> are involved in TRALI or not. It

should be noted that cumulative data from three large clinical trials are not supportive for a role of RBC storage time and/or lysoPC content as relevant risk factors for TRALI.<sup>15–17</sup>

#### *CD40 ligand (CD40L)*

The contribution of CD40L to TRALI is incompletely defined. Like bioactive lipids, CD40L is another breakdown product that can prime neutrophils. CD40L is platelet-derived, proinflammatory mediator found in both cell-associated and soluble (sCD40L) forms. It is found in PCs and accumulates throughout product storage.<sup>79</sup> sCD40L binds to CD40 present on the surface of monocytes, macrophages, and neutrophils.<sup>80</sup> CD40L–CD40 interaction causes neutrophil priming and may be associated with TRALI following platelet transfusions because platelet units involved in TRALI cases were found to have higher concentrations of sCD40L. In vitro, human microvascular endothelial cells preincubated with LPS experienced severe damage when sCD40-primed neutrophils were added, whereas unprimed neutrophils did not induce such damage.<sup>80</sup> Others have questioned the role of CD40L in TRALI.<sup>81</sup> For instance, patients with TRALI after cardiac surgery did not differ in their sCD40L levels from controls.<sup>82</sup>

#### *Immunoglobulins*

IgG has been postulated to activate neutrophils in a patient with osteopetrosis being treated with gamma interferon, granulocyte stimulating factors, and monocyte colony-stimulating factors.<sup>36</sup> The patient had very low levels of endogenous IgG1 and IgG2 and developed severe lung injury shortly after transfusion of platelets from an untransfused male donor. No leukocyte antibodies could be found in either donor or recipient. It is suggested that transfused IgG binding to the neutrophils, which were already primed by interferon and colony-stimulating factor, was sufficient to cause neutrophil activation and lung injury. This case may be considered a good example of “multiple hit” TRALI.

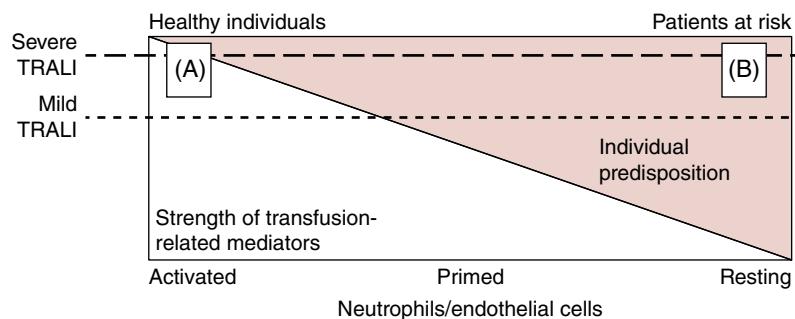
Reports of lung injury following intravenous IgG infusion are rare and seem to be associated with high doses or concentrates prepared intentionally with a high level of leukocyte antibodies.<sup>12,83</sup> It is possible that antibodies are both diluted and neutralized during the preparation of the pooled product, as suggested for pooled pathogen-inactivated plasma.<sup>84,85</sup>

#### **Multiple-hit/threshold theory of TRALI causation**

The two-hit theory of TRALI suggests that the first hit is the patient’s underlying clinical condition and that the second hit is delivered by the transfused blood product.<sup>65</sup>

Most patients receiving transfusion and especially transfusion of plasma have significant comorbidities, which may result in priming or activation of neutrophils or cause damage to pulmonary endothelium. It has been suggested that TRALI will be more common in patients with certain chronic conditions such as alcohol abuse, tobacco use, and systemic inflammatory disorders, as well as patients with acute insults such as shock and liver surgery.<sup>65</sup> Early reports noted that most patients with TRALI had recently undergone surgery and suggested that this in itself was a sufficient stimulus in many cases.

The second hit is delivered by the transfused product. In 80% of the cases, the second hit is an antibody against the patient’s leukocytes: either anti-HLA class I, anti-HLA class II, or anti-HNA. Some experimental evidence for the “two-hit” or “multiple-hit” theory has been provided by studies on bioactive lipids described earlier.<sup>58</sup> The multiple-hit theory has been further developed by Bux



**Figure 49.6** The Bux–Sachs TRALI threshold model. The TRALI threshold model proposes that a certain threshold must be overcome to cause a TRALI reaction. The threshold of mild TRALI, in which oxygen supply is sufficient, is lower than that of severe TRALI, in which patients require mechanical ventilation (horizontal lines). In order to overcome this threshold, numerous factors must act in concert. These factors can be summarized as strength of exogenous transfusion-related mediators (white wedge) and individual predisposition of the patient (lavender wedge). The individual predisposition covers both constitutive (genetic) factors and dynamic or acute influences (i.e., acute infection or trauma). A strong exogenous transfusion-related mediator, such as a strong neutrophil-specific antibody, will precipitate a TRALI reaction even if the influence of the individual predisposition is low (example (A), in an otherwise healthy recipient). In contrast (example (B), an individual “at risk,” e.g., a septic patient with an activated pulmonary endothelium), a relatively mild exogenous transfusion-related mediator with low neutrophil-priming activity will be sufficient to overcome the threshold. Source: Bux and Sachs (2007).<sup>86</sup>

and Sachs,<sup>86</sup> who suggest that neutrophil and endothelial cells are central to the pathogenesis of TRALI and that activation of these cells requires sufficient stimuli from one or more sources to reach a certain threshold, at which point full activation and lung damage will ensue (Figure 49.6). Depending on the degree of neutrophil or endothelial cell response, the lung damage can be mild or severe with corresponding clinical effects.

This theory of TRALI holds that given sufficiently strong stimulus to endothelial and neutrophil activation, lung damage can occur in an otherwise healthy individual. Evidence for this comes from reports of TRALI in transfused volunteers and plasma transfusions used for clinical reasons in otherwise healthy individuals. These cases are uncommon.

### TRALI and transfusion in critically ill patients

Clinical evidence for the “multiple hits” theory of TRALI has been provided by studies of critically ill patients who are known to be susceptible to ALI. Retrospective studies have suggested a relationship between the amount of blood transfused and morbidity and mortality. All these studies are confounded by the factor of how much blood the patient required also being a marker for severity of illness. The Transfusion Requirements in Critical Care (TRICC) trial was both prospective and randomized; it found a higher incidence of pulmonary edema and of ALI in patients receiving more transfusion with relatively plasma-rich components.<sup>87</sup> Gajic *et al.*<sup>88</sup> studied ALI developing in critically ill patients during mechanical ventilation. They found a strong association with transfusion of plasma, but not with red cell transfusion, the age of transfused red cells, the leukocyte content of transfused red cells, or platelet transfusion. Further studies identified the transfusion of female plasma as being particularly associated with the development of ALI in keeping with an antibody-mediated mechanism.<sup>89</sup> A prospective study in intensive care patients comparing male donor and parous female plasma found similar results.<sup>33</sup> The incidence of ALI associated with transfusion in these studies was of the order of 1 in 50–200 units of female plasma transfused, a far higher incidence than the reported incidence of TRALI in other circumstances. These findings reaffirm the Bux–Sachs threshold model of TRALI in which

highly susceptible patients subject to multiple insults to neutrophils and endothelial cells develop lung damage after a relatively mild additional stimulus from transfusion.

### Donor investigation

Strategies of donor investigation vary among blood centers. The aim of investigation is twofold: to help establish the diagnosis and to allow appropriate management of implicated donors. A typical scheme adapted from Su and Kamel<sup>90</sup> is shown in Figure 49.7. Investigation of donors is undertaken only when a diagnosis of TRALI is considered probable or possible. Donations transfused within the six hours before the development of lung injury are considered to be under suspicion. Where the number of possibly implicated donors is small (four or fewer), all donors should be investigated. Where the number of possibly implicated donors is greater, the high-risk donors with a history of pregnancy or transfusion are investigated initially. If a donor or donors are found with an antibody that matches a cognate antigen, investigation of the lower risk donors is not undertaken. If no donor is implicated in the initial investigation, lower risk donors can be further tested in those cases that are considered to be probable TRALI. A further selection policy is to initially investigate only those donors of plasma-rich components. Investigations are undertaken at a qualified laboratory with adequate sensitivity and specificity for HLA class I and II antibodies, and HNA antibodies. Recipient HLA and HNA typing may be performed prospectively or only when a donor antibody is found. Tests for recipient antibodies may be undertaken when the lung injury followed transfusion of granulocyte concentrate or following nonleukocyte-reduced component transfusion in which no match can be identified between donor antibodies and recipient antigens. When a nonantibody mechanism is suspected, or if an antibody-related etiology has been largely excluded, tests on the residual donation for abnormal lipids or other bioactive substances may be carried out at a research or reference center.

### Management of implicated donors

Management of implicated donors varies. It is possible to permanently defer all donors who are implicated in a case of TRALI by virtue of having leukocyte antibodies of any specificity. This would

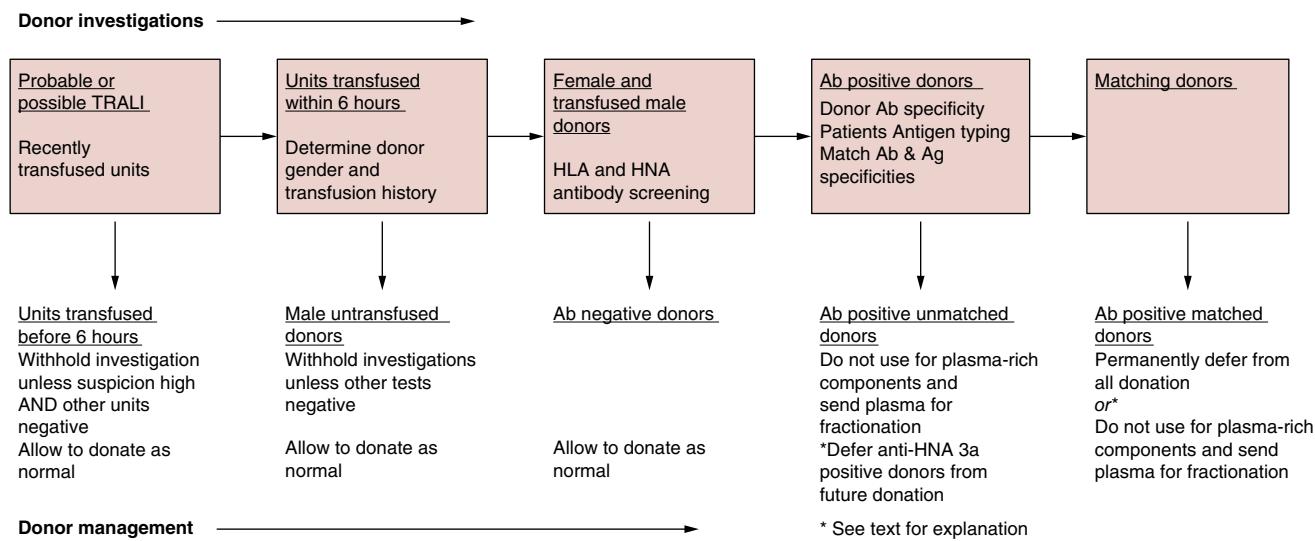


Figure 49.7 Flowchart for investigation and management of donors.

undoubtedly exclude many donors unnecessarily, and the logical extension of such a policy is that all donors with leukocyte antibodies should be deferred whether or not they have been possibly implicated in a case of TRALI. Alternatively, only those donors with antibody matching a recipient antigen are excluded from future donation. This policy assumes that some feature of the antibody in that donor makes them more likely to have caused TRALI in the index case, and thus to cause TRALI with further donations. Finally, it is possible to defer donors who are either possibly or definitely implicated only from donation of plasma-rich components. In addition, some centers automatically permanently defer any donor found to have anti-HNA-3a because of the high frequency of the antigen in the recipient pool (95%) and the common association with severe TRALI.

## Prevention

The majority of severe TRALI cases are related to antibodies. Therefore, prevention is largely aimed at reducing the likelihood of causative antibodies being transfused. There are several strategies in practice.

### Avoidance of unnecessary transfusion of plasma

This includes unjustified transfusion of plasma components such as FFP and unnecessary transfusion of plasma associated with RBCs. Use of FFP considerably varies among countries, and it has been demonstrated that use to correct minor coagulopathies is ineffective and unnecessary.<sup>91,92</sup> Avoidance of unnecessary FFP transfusion will reduce the potential for transfusion of units containing antibodies with the potential to cause TRALI. RBCs in additive solution containing less than 20 mL residual plasma are uncommon causes of TRALI, and in those recorded cases there have usually been multiple antibodies in the donor plasma matching cognate antigens in the recipient. Whole blood donations, particularly from high-risk donors (parous female or previously transfused donors), can be processed into RBCs in additive solution with minimal remaining plasma. Similarly, the use of platelet additive solution (PAS) and pathogen inactivation reduces the

volume of plasma within platelets units. While evidence is still emerging about the incidence of TRALI in pathogen-reduced platelet products, in one study of nearly two thousand units of pathogen-reduced platelet components, there were zero instances of TRALI.<sup>93</sup>

### High-risk donor exclusion

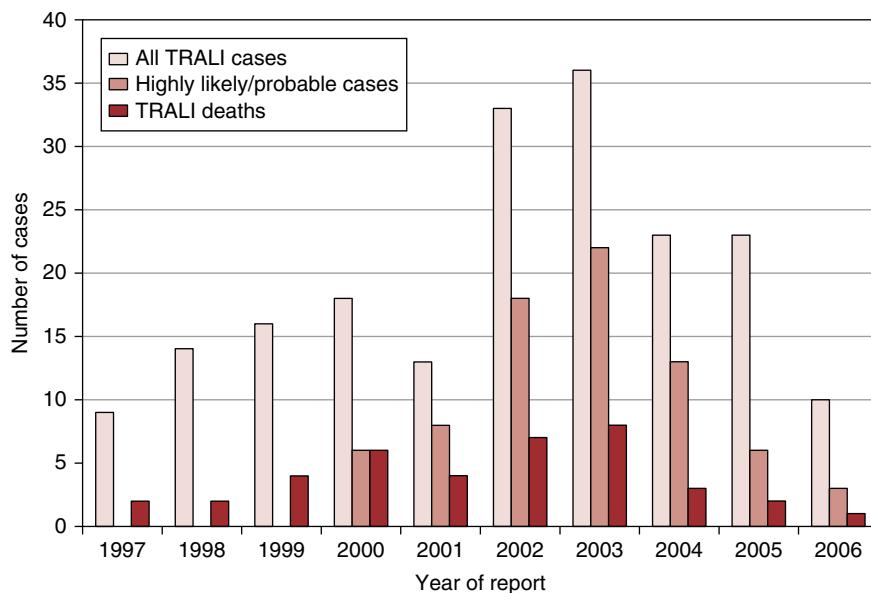
The most straightforward form of this policy is to exclude female donations of plasma-rich components. Some individual blood centers have followed such policies at the loco-regional level for years.<sup>94</sup> The effectiveness of this intervention was demonstrated in the United Kingdom, where such a policy was instituted nationally in 2003. An active hemovigilance scheme clearly showed a decrease both in the incidence of probable TRALI and in associated deaths over the next three years (Figure 49.8).<sup>95</sup> Further developments of this policy either exclude only those females with a history of pregnancy or exclude any donor with a history of blood transfusion or organ transplantation. Evidence of the efficacy of excluding female donor plasma comes from many countries, including those in North America and in the Netherlands, Germany, and Australia.

### Antibody testing

By testing donors for HLA and HNA antibodies, it is possible to exclude all donors with detectable antibodies, or those with antibodies of certain specificities or titer, either from all blood donation or just from donation of apheresis-collected, plasma-rich components. This strategy is attractive in that unnecessary exclusion of valuable donors is avoided but it also depends on the sensitivity and specificity of the antibody tests. It has been used with apparent success in some centers.<sup>96</sup> However, the cost of screening all patients will be prohibitive for some blood collection centers.

### Use of pooled plasma

Pooled solvent/detergent-treated plasma does not appear to cause TRALI. It is hypothesized that this is because of dilution of antibodies, neutralization of HLA antibodies by soluble HLA



**Figure 49.8** Cases of TRALI reported to the UK hemovigilance scheme from 1996 to 2006. After 1998, all cases were considered by an expert panel and classified as highly likely, probable, possible, or unlikely. From 1996 to 2003, case ascertainment rose steadily with increasing knowledge of the condition among physicians. From October 2003, a policy to procure FFP from only male donors was introduced. Following the change, there was a decrease in reports, and a marked decline in highly likely or probable TRALI and in deaths from TRALI (note that cases were recorded by the year that the report was made and that many cases documented for 2004 occurred in 2003). Source: Data from Dr. C Chapman on behalf of SHOT UK (<http://www.shotuk.org>).

antigens in the plasma of other donors, and subsequent removal of the immune complexes in processing.<sup>84,85</sup> Extensive use of solvent/detergent-treated plasma in Europe has not been associated with TRALI.<sup>97,98</sup>

#### Nonantibody TRALI and inverse TRALI

Leukocyte reduction decreases the production of cellular activation or breakdown products that have been implicated in TRALI. It also will prevent the rare but distinct cases of inverse TRALI related to bystander granulocyte transfusion.

### Other respiratory-related transfusion reactions

#### Transfusion-associated circulatory overload

TACO has become increasingly recognized through hemovigilance schemes. Like TRALI, there is a temporal connection to transfusion; however, in contrast to TRALI, the pathogenesis is essentially increased pulmonary capillary pressure leading to hydrostatic pulmonary edema. Patients particularly at risk are the small and elderly, those with preexisting cardiac disease and those with relative fluid overload such as some patients with renal failure. Obstetric cases are also reported and may be related in part to the increased blood volume during pregnancy or the cardiac effects seen in preeclampsia. Transfusion of any fluid can lead to cardiac overload. Where blood transfusion is involved, all components have been implicated, and unlike TRALI, high-volume plasma component transfusion is not over-represented among cases. There is no clear evidence that red cells themselves are implicated other than their volume being more restricted to the intravascular compartment. It remains possible that stored red cells with relatively poor oxygen delivery characteristics may contribute to cardiac ischemia and thus cardiogenic edema, but there is no clear evidence in favor of this hypothesis.

#### TACO: definition

The hemovigilance working party of the International Society of Blood Transfusion revised their definition of TACO in 2018.<sup>99</sup> The chief priority of the revision was to adopt a standard definition to improve recognition, reporting, and research with the ultimate goal of reducing adverse events associated with transfusion (Table 49.2).<sup>99</sup>

**Table 49.2** International Society of Blood Transfusion Definition of TACO (2018)<sup>99</sup>

- Cases of TACO are characterized by acute or worsening respiratory compromise and/or evidence of pulmonary edema during or up to 12 hours after transfusion and presence of a total of three or more of the criteria below
- Acute or worsening respiratory compromise
  - Evidence of acute or worsening edema based on either clinical examination and/or radiographic chest imaging and/or another noninvasive assessments of cardiac function
  - Evidence for cardiovascular system changes not explained by the patients underlying medical condition, including development of tachycardia, hypertension, widened pulse pressure, jugular venous distension, enlarged cardiac silhouette and/or peripheral edema.
  - Evidence of fluid overload including any of the following: a positive fluid balance; response to diuretic therapy, e.g., from diuretic therapy or dialysis combined with clinical improvement; and change in the patient's weight in the peri-transfusion period
  - Supportive result of a relevant biomarker, e.g., an increase of B type natriuretic peptide level (e.g., BNP or NT-pro BNP) above the age-group-specific reference range and greater than 1.5 times the pretransfusion value. A normal post-transfusion NP level is not consistent with a diagnosis of TACO; serial testing of NP levels in the peri-transfusion period may be helpful in identifying TACO.

Source: Adapted from Wiersum-Osselton et al. (2019).<sup>99</sup>

### TACO: epidemiology

Heart failure and pulmonary edema worsened or caused by transfusion have been recognized for many years. Patients with severe anemia due to vitamin B<sub>12</sub> deficiency were particularly known to be susceptible to sudden cardiac decompensation after transfusion. The rising incidence of cardiac overload due to transfusion was first highlighted by hemovigilance data from Quebec in 2010.<sup>100</sup> Subsequently, with increased recognition and reporting, it has emerged as a major cause of transfusion-related morbidity and mortality in many countries, including Ireland and the United Kingdom. Reports from the UK hemovigilance system (SHOT UK) show an increase in reports from 18 in 2008 to 139 in 2019.<sup>18</sup> And although more cases are being recognized, prospective surveys suggest a higher rate still, perhaps as high as 1%.<sup>25,101</sup> The incidence-per-unit is higher after red cell transfusion than with plasma or platelets alone, although this may be due to the patient's underlying illness and indication for transfusion rather than a specific effect of component type. Morbidity and mortality may be difficult to ascribe only to blood transfusion in a vulnerable patient who may also receive other fluids, but there is no doubt that transfusion appears to be a factor in many cases. The majority of patients developing TACO are elderly with a history of cardiac disease, respiratory disease, renal failure, or a combination of these conditions. A small number have no known cardiorespiratory comorbidities. Though patients are typically elderly with known cardiac failure, younger previously fit patients may also be affected.<sup>102</sup> Others have identified an unexpectedly high incidence in obstetric patients, perhaps related to the expanded blood volume seen in pregnancy.<sup>103</sup>

The diagnostic criteria outlined by the International Society of Blood Transfusion's working group on hemovigilance have not yet been adapted to evaluate TACO in pediatric patient populations. The reported incidence of TACO in pediatric patients receiving blood has varied from 1.5% to 4% in the literature. Part of this variation is a result of different study designs, but some of the studies used alternative definitions of TACO, highlighting the need of consistent definitions and diagnostic criteria.<sup>104</sup>

### TACO: diagnosis

Diagnosis is by identification of hydrostatic pulmonary edema as in Figure 49.3 and accompanying text on the differential diagnosis of TRALI/ALI and TACO/cardiac failure. Hypertension is one more characteristic of TACO, compared to the hypotension typifying TRALI. It is not impossible for a patient to have both volume overload due to poor fluid management and a capillary leak whether due to TRALI or to ALI from other causes. Radiographically, cardiogenic edema typically shows upper lobe venous distension and edema in the perihilar and basal areas. Edema in overhydration or renal failure is typically perihilar and shows no air bronchograms as seen in TRALI.

### TACO: treatment

Treatment is that of cardiac failure or volume overload, putting the patient in an upright position, and giving oxygen and diuretics, fluid restriction, and even phlebotomy. Continuous positive airway pressure or mechanical ventilation may be necessary, and in patients with marked renal failure, dialysis with removal of fluid may be used.

### TACO: prevention

The best preventive measure for TACO is avoiding unnecessary transfusion. Transfusion is rarely necessary in the absence of active bleeding and should be avoided in patients with otherwise correctable chronic anemia. Pretransfusion assessment for risk factors for TACO, such as low body weight, fluid overload, or poorly controlled heart failure, may allow a careful transfusion protocol with diuretic coverage and close observation.

Hypertension, tachypnea, tachycardia, and reduced oxygen saturation are all signs of cardiac overload. Early recognition may allow reduction in transfusion rate, discontinuation of transfusion, or use of oxygen and diuretics to halt or reverse the developing volume overload.

### Respiratory consequences of allergic transfusion reactions

Allergic transfusion reactions (ATRs) are some of the most common transfusion reactions and are usually mild; however, some ATRs are severe and can present with respiratory compromise and/or anaphylaxis. Similar to TRALI, plasma-containing products are the most often implicated. Reactions are usually within minutes of starting the transfusion and almost always occur within four hours. ATRs can be caused by preformed IgE antibodies in the recipient against donor plasma antigens, but most cases are idiopathic. Localized angioedema in the airway can cause bronchospasm and stridor.<sup>106,107</sup>

### Transfusion-associated dyspnea (TAD)

TAD is defined by increased breathlessness not meeting the criteria for TRALI or TACO or an allergic reaction and occurring within 24 hours of transfusion. Respiratory distress should be the most prominent clinical feature and should not be explained by the patient's underlying condition or any other known cause.<sup>105</sup> It is important to record and classify such reactions, but given the uncertainty as to whether there is a specific pathophysiology, their clinical usefulness in managing transfusion is uncertain.<sup>106</sup>

### Conclusion

It has taken over 50 years for the full extent of TRALI to be appreciated as a major but preventable adverse outcome of transfusion. Female donor exclusion has proven a highly effective preventive strategy. Loss of donor plasma for transfusion has not been a major problem partly because more rational and evidence-based use of plasma has reduced demand. TACO typically occurs in patients with cardiac failure, expanded blood volume due to pregnancy, renal failure, or other causes. With the reduction in TRALI-related deaths and introduction of hemovigilance schemes, TACO has emerged as an important cause of transfusion-related morbidity and mortality.

### Disclaimer

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## CHAPTER 50

# Transfusion-associated graft-versus-host disease

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Transfusion-associated graft-versus-host disease (TA-GVHD) is a rare but almost uniformly lethal complication of blood transfusion. Although the graft-versus-host disease (GVHD) that occurs after allogeneic bone marrow transplantation (BMT) and TA-GVHD shares some clinical similarities, GVHD after BMT is not uncommon and often responds positively to immunosuppression.<sup>1</sup> The much rarer TA-GVHD, however, is associated with destruction of the recipient's bone marrow, does not usually respond to immunosuppressive therapy,<sup>1</sup> and is generally fatal.<sup>2</sup> Because there are no effective treatments for TA-GVHD, the management of this complication focuses almost entirely on prevention by irradiation or other means of inactivation of residual T cells found in cellular blood components (whole blood, red blood cells [RBCs], granulocytes, and platelets) intended for transfusion to susceptible recipients. Over the past 20 years, the use of irradiated products for high-risk patient populations has reduced the incidence of TA-GVHD in the Western world and Japan to almost undetectable levels. In this chapter, we review the pathophysiology and incidence of TA-GVHD, characteristics of blood transfusion recipients that make them susceptible to the development of TA-GVHD, and strategies to prevent, diagnose, and treat TA-GVHD.

### Pathophysiology of TA-GVHD

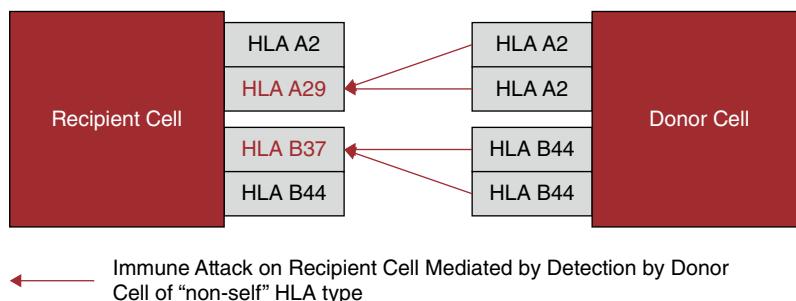
The development of TA-GVHD requires infusion of viable allogeneic donor ("non-self") lymphocytes, which in rare circumstances can proliferate and attack recipient ("self") tissues. Unlike BMT-associated GVHD, in which the transplanted donor hematopoietic cells in the engrafted marrow are the cells that mediate the attack on host (recipient) tissues, TA-GVHD results in the destruction of both the host tissues and host bone marrow by donor T lymphocytes that proliferate in the recipient following transfusion. It is the host bone marrow aplasia that occurs only in TA-GVHD that primarily drives its lethality.

The biologic basis for the differentiation of "self" and "non-self" involves immune recognition of specific cell surface proteins, most importantly human leukocyte antigens (HLA) expressed on the surface of both immune and nonimmune cell types. In most

situations, transfused donor lymphocytes contained within cellular blood components are identified and eliminated by the host immune system due to their expression of donor "non-self" HLA proteins. In individuals with intact immune systems, donor lymphocyte clearance appears to occur within a week;<sup>3</sup> this clearance likely occurs more quickly in donor lymphocytes from irradiated blood products that lack ability to proliferate. However, in rare situations, transfused donor lymphocytes are not recognized as "non-self" by the recipient immune system. If the transfused donor lymphocytes are sufficiently viable to proliferate within the recipient, TA-GVHD can result.

Given its rarity, the precise circumstances needed to induce TA-GVHD are not known. Transfusion-associated microchimerism (TA-MC) is a condition that occurs after transfusion of cellular blood components, in which a small number of donor allogeneic lymphocytes proliferate within a host and remain detectable for years. TA-MC is associated with perhaps 10% of patients transfused after sustaining traumatic injury, but is not known to have any clinical sequelae.<sup>4</sup> Distinguishing the factors that influence why T-lymphocyte engraftment sometimes results in severe disease (TA-GVHD), and sometimes results in no disease manifestations (TA-MC), has been the subject of ongoing investigation, but remains unknown. However, understanding immunomodulatory circumstances that allow TA-MC may also be revealing for the potential setup for TA-GVHD, given that there may be overlapping factors influencing prolonged donor lymphocyte survival and proliferation.

In many cases, the presence of severe acquired or congenital immune deficiency in the transfusion recipient serves as a prerequisite to the development of TA-GVHD. TA-GVHD, however, has also been reported to occur in the absence of a recognized immune deficiency, particularly in situations where the blood donor is homozygous for HLA antigens for which the recipient is heterozygous.<sup>2</sup> In this scenario, all HLA antigens expressed by the donor are also expressed by the host, but the host expresses HLA antigens that are not expressed by the donor. This can result in *unidirectional tolerance*, where donor lymphocytes are spared from attack by the host immune system, whereas host tissues are left vulnerable to attack by donor lymphocytes and their progeny (Figure 50.1).



**Figure 50.1** Unidirectional tolerance occurs when donor lymphocytes mediate an attack on host tissues while simultaneously being viewed as “self” by the host immune system. In this example, recipient cells expressing HLA A29 and HLA B37 antigens are attacked by donor lymphocytes that do not express these antigens; however, donor-derived HLA A2 and HLA B44 expressing cells are not targeted by the recipient immune system because these antigens are also expressed by recipient cells. The scenario depicted is similar to a case report by Benson *et al.*<sup>7</sup>

### Incidence of TA-GVHD

As mentioned previously, TA-GVHD has become an extremely rare phenomenon over the past 20 years. In the United Kingdom, more than 55 million cellular blood components have been transfused since 1999 (Table 50.1). During that interval, there have been 14 cases of reported TA-GVHD with only one case reported since 2002. Similar data compiled by the US Food and Drug Administration (FDA) identified only three cases of fatal TA-GVHD in the United States from 2005 to 2018, during which time more than 210 million cellular blood components were transfused (Table 50.2). In both the United Kingdom and the United States, widespread use of blood component irradiation in at-risk populations has contributed to the low incidence of TA-GVHD.

In Japan, there is substantially less HLA diversity than in the United States or the United Kingdom. In addition, Japan historically has a high rate of directed-donor blood collections where relatives donate blood for other family members.<sup>5–6</sup> As a result, Japan has had a higher reported rate of TA-GVHD with a large proportion of TA-GVHD case reports published by Japanese authors.<sup>5,6</sup> Indeed, in Japan prior to the routine irradiation of blood components, TA-GVHD had a calculated expected incidence of 1 in 874 transfusions when donor and recipient were unrelated, and 1 in 102 when donor and recipient were a mother-child pair.<sup>5</sup> Routine irradiation of

cellular blood components was introduced in Japan in 1998, and there were no cases of TA-GVHD reported to the country’s hemovigilance network between 2000 and 2013.<sup>2</sup> Similar scenarios are found in other countries where there is substantially less HLA diversity among the donor-recipient population.

### Clinical scenarios associated with TA-GVHD

#### Patients without recognized immunodeficiency syndromes

Prior to the widespread irradiation of cellular blood components in Japan, the reported rate of TA-GVHD in Japan was higher than in Western Europe or the United States. The Japanese medical literature describes numerous cases of TA-GVHD in patients without a recognized immunodeficiency.<sup>44</sup> Uchida *et al.* reported that, among 66 confirmed cases of TA-GVHD in Japan from 1992 to 1999, 65 of the patients were not considered by the study authors to be immunosuppressed.<sup>2</sup> The majority of these patients received transfusions either during the treatment of solid tumors or for surgical or traumatic bleeding episodes.<sup>2</sup> Similarly, Juji *et al.* reported 96 cases of TA-GVHD among immunocompetent patients who underwent cardiac surgery in Japan between 1981 and 1986.<sup>45</sup>

**Table 50.1** UK Reports of Cellular Blood Component Infusions and TA-GVHD to the Serious Hazards of Transfusion Hemovigilance Network, 1996–2019

Year	Number of RBCs Transfused (UK)	Number of Platelets Transfused (UK)	Reports of TA-GVHD (UK)	References
2010–2019	17,565,378	2,701,141	1	21–30
2000–2009**	22,035,873	2,316,377	1	12–20
1996–2000*	10,304,047	1,090,647	12	8–11
Total	49,905,298**	6,108,165**	14	

\* Universal leukodepletion introduced in 1999.

\*\* Excluding 2017, when individual transfusion data were not reported by SHOTUK.

**Table 50.2** Reports of TA-GVHD to US FDA Compared to Annual Estimates of Transfusion of Cellular Blood Components, 2005–2018

Year	Annual Average of RBCs Transfused (USA)	Annual Average of PLTs Transfused (USA)*	Cumulative Reports of TA-GVHD (USA)	References
2011–2018*	12,220,250	2,067,000	1	35–43
2005–2010*	14,832,000	1,876,000	2	31–34

\* Apheresis equivalent units.

There are several factors that appear to play a role in the pathogenesis of TA-GVHD among patients without recognized immunodeficiency syndromes. One contributing factor is likely the relative HLA homogeneity in Japan, where many of the cases were reported. A study of 655 Japanese found the frequency of the most common HLA haplotype to be 7.5%, and the second most common HLA haplotype had a frequency of 5%. In contrast, the same study found the frequency of the most frequent HLA haplotype among US Caucasians to be only 4%, and the most common HLA haplotype among Italians was identified in only 2.2% of the population.<sup>5</sup> It is likely that the lack of HLA diversity among the Japanese population resulted in unidirectional tolerance more frequently in Japan than in other parts of the world, helping to explain the historically high levels of TA-GVHD in Japan prior to widespread irradiation of cellular blood components. However, the anticipated incidence of TA-GVHD based on rates of HLA similarity is much lower than the actual reported incidence of TA-GVHD,<sup>1,46</sup> indicating that a lack of HLA diversity is only one of many factors that influence the pathogenesis of TA-GVHD.

Another factor that may contribute to TA-GVHD in some patients is trauma or major surgery, particularly cardiac surgery.<sup>47</sup> Although the mechanism of immune suppression, if any, contributing to TA-GVHD in these patient populations is not known, it has been reported that hospitalized patients, compared to healthy volunteers, are less likely to generate antibodies after exposure to allogeneic, minor blood group antigens via transfusion.<sup>48</sup> Consequently, it is possible that although hospitalization and surgery help to prevent alloimmunization to minor red blood cell antigens, they simultaneously increase the risk of developing TA-GVHD due to incompletely understood changes to the immune system that result from trauma or cardiac surgery. This hypothesis may also help explain the reported relatively high (10%) incidence of TA-MC seen after transfusion following a traumatic injury.<sup>4</sup>

Besides cardiac surgery and HLA similarity between donor and recipient, other factors that have been associated with TA-GVHD in immunocompetent individuals have also been described, including transfusion of recently collected (<72 hours old) whole blood or cellular components<sup>44,49</sup> and use of blood collected from first- or second-degree relatives.<sup>44</sup> These factors likely enhance the probability of developing TA-GVHD by either enhancing the viability or dose of transfused lymphocytes (e.g., using fresh or nonleukoreduced or nonirradiated blood components) or increasing the likelihood of HLA similarity between donor and recipient (e.g., using blood from close family relations).

### **TA-GVHD in immunosuppressed patients**

Although cases of TA-GVHD are reported among patients without recognized immunodeficiency, many more reported cases involve patients who are profoundly immunosuppressed.<sup>46</sup> Among immunosuppressed patients, those considered at highest risk for the development of TA-GVHD include patients with severe T-cell immunodeficiency syndromes (e.g., severe combined immunodeficiency or DiGeorge syndrome), patients undergoing allogeneic or autologous bone marrow transplant, patients diagnosed with Hodgkin's lymphoma or aplastic anemia on immunosuppressive therapy, neonates, a fetus receiving intrauterine transfusions, and patients being treated with purine analogs (fludarabine, cladribine, or deoxycoformycin) or alemtuzumab (anti-CD52).<sup>46,50,51</sup> Although rare cases of TA-GVHD have been reported in patients after organ transplantation, patients undergoing treatment for solid tumors, or for patients with either non-Hodgkin's lymphoma

or acute leukemia (without stem cell transplantation), the risk is generally considered to be lower compared to the first groups listed above.<sup>50,51</sup> No cases of TA-GVHD attributed only to immunodeficiency caused by HIV/AIDS (in the absence of other conditions listed above) have been reported in the medical literature to date.<sup>50,51</sup> A described murine model of TA-GVHD suggests that functional recipient CD8 T-lymphocytes and natural killer cells have a particularly important role in the suppression of TA-GVHD development.<sup>1</sup> Recipient mouse CD4 T-lymphocyte depletion increased the threshold for viable donor lymphocytes needed to induce TA-GVHD, which may in part explain the apparent lack of susceptibility of HIV/AIDS patients to this complication.<sup>52</sup> AABB guidelines, as well as national guidelines from the United Kingdom and Australia, address the importance of ensuring that all cellular blood products used in high-risk settings are appropriately irradiated.<sup>50,51,53</sup>

The last known TA-GVHD case in the United Kingdom occurred in 2012. In that case, two intrauterine transfusions were administered to a fetus (hemoglobin 4.4 g/dL) at 21 weeks gestation who was anemic due to a maternal parvovirus infection. The transfusions were performed using a total of 33 mL of nonleukoreduced, nonirradiated blood that had been collected from the mother.<sup>23,54</sup> Although cellular blood components (including whole blood) intended for intrauterine transfusions are generally leukoreduced and irradiated in the United Kingdom, these product modifications were not made in this case due to the emergent need for the transfusions. At 32 weeks gestation, the fetus was delivered hydropic and pancytopenic. Two months after birth, a bone marrow biopsy revealed an aplastic marrow, and maternal engraftment was detected by chimerism studies. HLA typing performed on the mother found her to be HLA homozygous and showed unidirectional tolerance for her child. The neonate was diagnosed with TA-GVHD and died despite attempted allogeneic stem cell transplantation using stem cells collected from the mother.

### **Diagnosis of TA-GVHD**

It is important to emphasize that the incidence of TA-GVHD is extremely low and thus cases will only be detected if there is clinical awareness that the diagnosis is a possibility. Historically, the most common reported signs and symptoms of TA-GVHD were fever, maculopapular rash progressing to generalized erythema, pancytopenia, bone marrow aplasia, diarrhea, and hepatitis; these generally occurred within 1–2 weeks of transfusion but can be seen up to 30 days after transfusion.<sup>2,6</sup> The case fatality rate for TA-GVHD approaches 100%.<sup>2,8–10,12,23,31,34</sup> The relatively long latency period between transfusion and the development of symptoms (up to 30 days) is another reason why cases may not be recognized clinically. In addition, if patients are critically ill at the time of their transfusion, it may be difficult to differentiate the clinical signs of TA-GVHD from their underlying illness. For these reasons, TA-GVHD may be underdiagnosed.<sup>55</sup> Nonetheless, a diagnosis of TA-GVHD should be considered in any patient with fever, erythema, cytopenias, gastrointestinal symptoms, and hepatitis within 30 days of a transfusion with nonirradiated cellular blood products (whole blood, RBCs, granulocytes, or platelets). It is notable that reports of TA-GVHD have never been attributed to transfusions with plasma, cryoprecipitate, factor concentrates, albumin, intravenous immunoglobulin, or previously frozen, deglycerolized RBCs. This is presumably due to the absence of sufficient viable donor lymphocytes in these products.<sup>50</sup>

If TA-GVHD is suspected clinically, confirmation should be obtained by testing for donor chimerism in the patient. Donor lymphocytes can be differentiated from host lymphocytes by measuring differences in restriction fragment length polymorphisms or numbers of short tandem repeats between the donor and the host, although modern molecular pathology laboratories may choose a more modern approach to identity testing. Consultation with a molecular pathology specialist is recommended to choose the optimal testing approach at a given hospital. In most situations, molecular pathologists will essentially recommend the same molecular assays that they routinely employ to detect donor-recipient chimerism in patients who have undergone allogeneic stem cell transplantation.<sup>55</sup> One strategy is to biopsy both affected and unaffected patient tissues and to compare the results to samples obtained from the blood donor (if available).<sup>50</sup> In older reports, detection of the Y chromosome was used to diagnose TA-GVHD resulting from a blood transfusion from a male donor to a female recipient.<sup>56</sup> Today, this approach is considered to be relatively insensitive in most circumstances, and would be of no use in cases of TA-GVHD suspected in male recipients, in patients who have undergone a sex-mismatched stem cell transplant or in cases involving the transfusion of a blood component collected from a female donor.

### **Prevention of TA-GVHD**

Although the introduction of universal leukoreduction is associated temporally with a reduction in the reported incidence of TA-GVHD, cases of TA-GVHD have been reported in patients receiving leukoreduced (but nonirradiated) cellular blood transfusions, and neither AABB standards nor other national standards accept leukoreduction alone as an adequate preventive measure of TA-GVHD in vulnerable populations.<sup>57</sup> In addition, the minimum dose of lymphocytes required to cause TA-GVHD in humans is not precisely known, and it may be influenced by factors that are not likely to be fully known prior to transfusion. These factors include the degree of HLA match between donor and recipient, the viability of the remaining transfused lymphocytes, and the degree of immunosuppression of the recipient. Consequently, leukoreduction alone is not considered to be sufficient prophylaxis against TA-GVHD; irradiation of cellular blood components is the only widely recognized method to prevent TA-GVHD in all cellular blood components.<sup>50,51,53,58</sup>

In the United States, in order to render lymphocytes contained in RBCs, platelets, granulocytes, or whole blood incapable of engraftment, 25 Gy must be directed at the center of the blood component being irradiated, with a minimum of 15 Gy at any part of the bag, if the intended recipient of the product is at high risk for TA-GVHD.<sup>53</sup> Of note, standards in the United Kingdom require a minimum dose of 25 Gy, with no more than 50 Gy delivered to any portion of the bag.<sup>50</sup> Special, radiation-sensitive labels are used to verify and permanently document that a blood product was irradiated. The expiration date of irradiated RBCs is shortened to 28 days from the date of irradiation, or the product's original expiration, whichever comes first.<sup>53</sup> In contrast, platelet component expiration is not affected by irradiation due to the short shelf life of platelets. Blood products that are inadvertently irradiated more than once or at a dose not in accordance with

standards should generally be discarded. It must be noted that bone marrow, peripheral blood stem cells, or donor lymphocytes infused as part of a hematopoietic stem cell transplant program must *never* be irradiated.

Of note, platelets that are treated with ultraviolet light-based pathogen reduction technology generally do not need to be irradiated because the pathogen inactivation process induces strand breakage of lymphocytic DNA, rendering any residual lymphocytes incapable of engraftment or proliferation.<sup>59-63</sup> This process has the additional benefit over standard irradiation in that it also effectively eliminates the ability of most infectious agents to replicate, reducing the risk of transfusion-transmitted infections. Blood banks using pathogen-reduced platelets should follow the guidance of their national regulatory authority regarding the need for irradiation. In the United States, the package insert for the psoralen/UVA-light-based pathogen inactivation system for platelets states that the pathogen inactivation process is "an alternative to gamma irradiation for prevention of TA-GVHD".<sup>64</sup> At present, pathogen inactivation systems are not available for clinical use with RBCs, whole blood, or granulocytes. It is anticipated that, in the coming years, pathogen reduction approaches to RBCs, and whole blood that obviate the need for routine irradiation will become widely available.

Most blood banks or blood centers use <sup>137</sup>Cs,<sup>60</sup> Co, or X-rays as a source of ionizing radiation.<sup>58</sup> In order to remove sources of ionizing radiation from hospital blood banks or blood centers that could be used for domestic terrorism, the US government is actively facilitating the elimination of irradiators that use <sup>137</sup>Cs or <sup>60</sup>Co and replacing them with X-ray-based irradiators.

Due to the rarity of cases of TA-GVHD, and the limited availability of irradiation in hospitals in some rural areas of the United States, there is not universal consensus on the specific medical conditions that require the use of irradiated cellular blood products, beyond directed donations, intrauterine transfusions, and neonatal transfusions. Some of the most common indications for irradiation are summarized in Table 50.3. As universal irradiation of all cellular blood products is not practiced in most countries, patients with TA-GVHD risk factors are occasionally transfused with nonirradiated blood.<sup>65</sup> Fortunately, the incidence of TA-GVHD is still very low among patients with risk factors who should have received irradiated blood. For example, in the United Kingdom between 2006 and 2010, there were 389 instances where patients inadvertently received nonirradiated cellular blood components when irradiation was indicated. This cohort included 178 patients undergoing purine analog therapy, 68 patients with lymphoma (including Hodgkin's lymphoma), and 44 patients undergoing stem cell transplant. None of these patients developed TA-GVHD as a consequence of their transfusion.<sup>65</sup> It was recently reported that from 1999 to 2018 in the UK, there were 1478 instances where leukoreduced, but nonirradiated cellular blood products were administered when irradiated components were indicated. None of these patients developed TA-GVHD.<sup>28</sup> Possible explanations include lack of HLA similarity between donors and recipients or decreased lymphocyte viability due to the storage time of the blood component before transfusion. In situations where irradiated red cell products would be indicated but are not available, some guidelines recommend preferential use of older (>14-day-old) products.<sup>66</sup> Regardless, if a

**Table 50.3** Stratification of Risk for Development of TA-GVHD Based on Medical Condition, Component Infused, and Medication Exposures

Highest Risk Association	Lower-Risk Association	No Known Risk
<p><i>Medical Conditions</i></p> <ul style="list-style-type: none"> <li>-Stem cell transplant</li> <li>-Aplastic anemia</li> <li>-Neonatal status</li> <li>-Intrauterine status</li> <li>-Hodgkin's lymphoma</li> <li>-Severe cellular immunodeficiency syndromes</li> </ul> <p><i>Blood Components</i></p> <ul style="list-style-type: none"> <li>-Nonirradiated whole blood</li> <li>-Nonirradiated RBCs</li> <li>-Nonirradiated platelets</li> <li>-Nonirradiated granulocytes</li> <li>-Nonirradiated cellular components used for intrauterine transfusions</li> <li>-Nonirradiated "fresh" (&lt;72 hours from collection) cellular blood components</li> <li>-Nonirradiated cellular components collected from first- or second-degree relatives, or in transfusion within population with limited HLA diversity (e.g., Japan)</li> </ul> <p><i>Medications</i></p> <ul style="list-style-type: none"> <li>-Purine analogs (e.g., fludarabine, cladribine, and deoxycoformycin)</li> <li>-Purine antagonists (e.g., clofarabine)</li> <li>-Alemtuzumab (anti-CD52)</li> </ul>	<p><i>Medical Conditions</i></p> <ul style="list-style-type: none"> <li>-Acute leukemia</li> <li>-Solid organ transplantation</li> <li>-Solid tumors</li> <li>-Non-Hodgkin's lymphoma</li> <li>-T cell malignancies</li> <li>-Patients requiring trauma resuscitations or cardiac surgery</li> </ul> <p><i>Blood Components</i></p> <ul style="list-style-type: none"> <li>-Plasma</li> <li>-Clotting factor concentrates</li> <li>-Cryoprecipitate</li> <li>-Albumin</li> <li>-Intravenous immunoglobulin</li> <li>-Irradiated whole blood</li> </ul> <p><i>Medications</i></p> <ul style="list-style-type: none"> <li>Other cytotoxic or immunomodulatory agents (e.g., ATG or rituximab)</li> </ul>	<p><i>Medical Conditions</i></p> <ul style="list-style-type: none"> <li>-HIV/AIDS</li> <li>-Congenital humoral immunodeficiencies</li> </ul> <p><i>Blood Components</i></p> <ul style="list-style-type: none"> <li>-Irradiated RBCs</li> <li>-Previously frozen, deglycerolized RBCs</li> <li>-Irradiated platelets</li> <li>-Irradiated granulocytes</li> <li>-Freeze-dried plasma</li> </ul> <p><i>Medications</i></p> <ul style="list-style-type: none"> <li>Noncytotoxic, nonimmunomodulatory agents (e.g., antibiotics)</li> </ul>

patient at risk for TA-GVHD is transfused with a nonirradiated cellular blood component, they should be monitored for signs of TA-GVHD for 30 days.

### Treatment of TA-GVHD

There are only rare reports of patients surviving TA-GVHD. In one case, recovery was attributed to treatment with anti-CD3 monoclonal antibody and cyclosporine.<sup>67</sup> Another case of TA-GVHD resolved spontaneously, although the patient's skin biopsy did not show several classic histologic features of TA-GVHD despite the presence of Y-chromosome DNA in the sample of this female patient.<sup>68</sup> However, overall, one of the distinguishing characteristics of TA-GVHD, compared to post-bone-marrow transplant GVHD, is that TA-GVHD does not usually respond to immunosuppressive therapy.<sup>1</sup> This is highlighted in one systematic review that showed similar rates of immunosuppressive therapy used in both survivors and nonsurvivors of TA-GVHD.<sup>69</sup> Bone marrow transplant has been performed in rare cases, several of which resulted in patient survival.<sup>69</sup> However, the quick time course from diagnosis of TA-GVHD to death significantly hinders the ability to find an appropriate BMT donor. Unfortunately, treatment is typically limited to supportive care and the case fatality rate approaches 100%.

### Conclusion

TA-GVHD is a rare but almost uniformly fatal consequence of transfusion with cellular blood components. Although the incidence of TA-GVHD in the United States, the United Kingdom, and Japan has become very low in the past 10–15 years, there are still sporadic case reports. Therefore, vigilance—in terms of both avoidance of unnecessary transfusions and irradiation of blood components given to high-risk patients who are susceptible to TA-GVHD—must continually be borne in mind.

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## CHAPTER 51

# Transfusional iron overload

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## Introduction

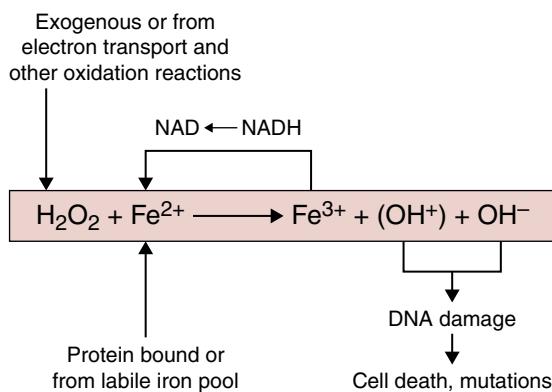
Tissue iron overload inevitably results in patients who receive regular red blood cell (RBC) transfusions for congenital or acquired anemias. Iron is initially sequestered in normal storage sites, namely the liver and monocyte-macrophage (reticuloendothelial) system, but as these tissues become increasingly saturated, iron begins to deposit in other organs such as the endocrine glands and the heart. The body lacks an effective means of eliminating this excess iron, and without therapy, cirrhosis, heart disease, diabetes, and other disorders develop; death is usually the result of cardiac failure. Transfusional iron overload, with its resulting morbidity and mortality, is an important health issue in patients of all ages. Children with thalassemia major (TM), or other inherited disorders such as Diamond-Blackfan anemia (DBA), congenital dyserythropoietic anemia (CDA), and congenital aplastic anemia, are dependent on regular red cell transfusions to maintain their well-being. Children with sickle cell disease (SCD) who are found to be at increased risk of stroke based on transcranial Doppler (TCD) evaluation, or who have developed silent infarcts in the brain, may be placed on a regular transfusion program to reduce the risk of progressive neurologic disease.<sup>1,2</sup> Adults with severe aplastic anemia (SAA) or myelodysplasia syndrome (MDS) may also become dependent on regular red cell transfusions. In addition, with chemotherapeutic regimens becoming more intense and myelosuppressive, patients undergoing treatment for various forms of cancer, particularly leukemias and lymphomas, may also receive a large number of red cell transfusions. Transfusions around stem cell transplantation may lead to iron overload when the indication is non-hematologic or worsen premorbid transfusional hemochromatosis. With advances in the medical management of many of the conditions enumerated above, including improved chelation regimens, patients also have a better survival probability, thus increasing the number of individuals in whom transfusional iron overload requires close medical attention. Most long-term experience in the management of transfusional iron overload comes from studies in patients with TM. We now understand that the pathophysiology of iron loading is different based on the underlying condition, and consequently the management must be adapted to minimize complications of iron toxicity. There has been much debate on the relevance of transfusional iron overload in MDSs yet without consensus on who requires treatment

or on the benefits of such therapy.<sup>3</sup> There are no uniform guidelines for assessment and management, and individual tailoring of therapy is recommended.

## Pathophysiology

Iron is an essential nutrient required by every cell. It is present in the human body in hemoglobin, myoglobin, and several mitochondrial respiratory enzymes. It serves as a carrier for oxygen and electrons and acts as a catalyst for a variety of oxygenation reactions. It is able to perform these functions in part because of its ability to reversibly and readily cycle between its ferrous and ferric forms. This very property also makes it potentially toxic, capable of producing free radicals, which can cause cellular damage (Figure 51.1).

The normal body iron concentration is approximately 40–50 mg Fe/kg body weight (~4–5 g in total); women have lower amounts, and men somewhat higher.<sup>4</sup> Three-quarters of this iron, about 30 mg Fe/kg, is contained in the circulating red cell compartment as hemoglobin, and approximately 5–6 mg Fe/kg is present in functional form in a variety of heme compounds (myoglobin and cytochromes) and iron-dependent enzymes. The remainder (5 mg Fe/kg in women and 10–12 mg Fe/kg in men) is held in reserve in the two primary storage forms, ferritin and hemosiderin, in the liver, bone marrow, spleen, and muscle, readily available when required for erythropoiesis. Normally, iron balance is maintained by controlling iron absorption: iron stores and iron absorption are reciprocally related so that as stores increase, absorption declines, and these processes are closely managed by the iron regulatory "hormone" hepcidin,<sup>5</sup> which plays a critical role in iron metabolism. It regulates the absorption of iron from the gastrointestinal tract through its effect on levels of ferroportin, the protein present on the basolateral membrane which is the primary iron exporter from cells. Red cells are normally broken down by cells of the monocyte-macrophage system, and the iron contained in hemoglobin is usually returned via transferrin to the erythroid precursors in the bone marrow, for recycling into new red cells. Hepcidin controls the release of iron from macrophages, an effect also mediated by ferroportin. In the normal host, when the body has adequate or increased amounts of iron, hepcidin is upregulated, iron absorption from the intestine is inhibited, and iron is



**Figure 51.1** Iron-induced oxidative tissue damage caused by free radicals generated through the Fenton reaction.<sup>92</sup> These reactive oxygen species (ROS) affect all tissues, but the heart and endocrine organs may be particularly susceptible.

sequestered in its storage sites, the macrophages and hepatocytes. The body lacks any effective mechanism for the excretion of excess iron. Iron exchange is limited so that the adult male absorbs and loses only about 0.01 mg Fe/kg/day.

Iron overload may result from repeated blood transfusion, excessive iron absorption from the gastrointestinal tract, or a combination of these processes.<sup>4</sup> In patients with severe congenital anemias, such as the thalassemias, DBA, or CDA, death from severe anemia in infancy is averted by a regular transfusion program that, if adequate, allows for normal growth and development during the first decade of life. Individuals with MDS or SAA usually begin transfusions later in life. As in normal individual, macrophages process senescent red cells (transfused in these patients) and extract iron from heme. With no effective means for the excretion of this iron, there is gradual tissue accumulation of this iron, which eventually exceeds the body's capacity for safe storage. As the amount of iron increases, there is virtually no change in the amount that is contained within the functional or transport (transferrin-bound) pools, and almost all of the excess iron is stored. Initially, the iron is sequestered in the cells of the monocyte-macrophage system and the liver (hepatocytes and Kuppfer cells), both normal storage tissues, but with continued accumulation, iron spills over and is deposited in other tissues, including the heart, pancreas, and other endocrine organs. Transfusion-dependent individuals may be broadly categorized as those having ineffective erythropoiesis, such as individuals with thalassemia, some MDS, or some forms of sideroblastic anemia; those with effective erythropoiesis, such as individuals with sickle cell anemia or hereditary spherocytosis; and those with decreased or absent erythropoiesis, such as patients with other myelodysplastic syndromes, SAA, or DBA. The "effectiveness" of erythropoiesis, with the ability to incorporate iron into hemoglobin in mature red blood cells, is a critical determinant of the pathophysiology of iron loading in transfusion-dependent patients. Once transfused red cells are phagocytosed by macrophages, hepcidin levels determine the fate of iron released from heme. If hepcidin levels are high, more iron remains sequestered in these storage cells, and less is released for potential deposit in other tissues. Iron stored in the hepatocyte is similarly retained. In contrast, if hepcidin levels are low, more iron is released from these cells and may be available for deposition in other parenchymal tissues. Thus, hepcidin helps determine the partition and internal redistribution of the excess

iron between the monocyte-macrophage and parenchymal sites,<sup>5</sup> another crucial factor in tissue toxicity.

Ineffective erythropoiesis (IE) and, to a lesser degree, anemia result in a downregulation of hepcidin expression and result in enhanced absorption of iron from the intestine.<sup>6</sup> Patients with TM or thalassemia intermedia (TI), paradoxically, have relatively low levels of hepcidin even in the presence of iron overload, as a result of their marked ineffective erythropoiesis. In patients with SCD, who have intact erythropoiesis, hepcidin expression varies inversely with erythropoietic drive,<sup>7</sup> with untransfused patients excreting less hepcidin in the urine. Similarly, patients with DBA or SAA, who have almost no erythropoiesis, likely have appropriately elevated hepcidin levels when iron loading occurs. Thus, patients with TM or TI not only have higher iron burdens because of increased intestinal absorption, but also have a greater risk of parenchymal tissue deposition than the latter groups. In fact, patients with TI who have received very few or no red cell transfusions may have profound iron overload as a result of marked hepcidin inhibition<sup>8</sup> as a result of their ineffective erythropoiesis. In patients with TM or TI, regular transfusion suppresses ineffective erythropoiesis to some extent, and hepcidin levels may rise somewhat, but they remain inappropriately low in relation to the higher body iron burden, resulting in iron loading through gastrointestinal absorption. A recently described hormone, erythroferrone (ERFE), has been shown in mice to suppress hepcidin expression.<sup>9</sup> When erythropoiesis is active, even if ineffective, maturing erythroid precursors may produce ERFE, which could suppress hepcidin leading to iron mobilization from stores and deposition in tissues. In thalassemic mice, another growth differentiation factor (GDF15) has been identified, which is secreted during erythroblast maturation and suppresses hepcidin production.<sup>10</sup> Its role in human disease is not completely clear. Therefore, variability in the hepcidin response to the underlying erythropoietic status produces differences in the partition and distribution of the excess iron, even when the volume of red cells transfused is similar. This likely explains the variability of iron deposition in different transfusion-dependent conditions.

Although hepcidin levels are critical in modifying the pathophysiology of iron loading in transfusion-dependent patients, several other variables are important in determining the morbidity related to the increased body iron burden. These include (1) the age at which transfusion therapy began, (2) the duration of transfusion therapy, and (3) the initiation and maintenance of effective chelation. Additional modifying factors include other genetic determinants, alcohol use, coexisting viral hepatitis, and other drugs and medications that the patient may be taking. The complex interplay between the underlying disorder and these factors in each individual plays a key role in the pathophysiology of iron toxicity.

Even though the body has the ability to safely store increased amounts of iron, deposition in nonstorage tissues eventually occurs as the body iron burden rises. Tissue toxicity depends on three factors: (i) the inherent sensitivity of the tissue to iron loading, (ii) the amount of iron in that tissue, and (iii) the duration of time for which that tissue remains exposed to abnormally high levels of iron. Without treatment, patients with thalassemia, who begin transfusions in infancy, develop growth retardation, diabetes, and other endocrine disturbances in the first decade of life, and death owing to iron loading of the myocardium occurs during the second decade. Patients with SCD, who receive regular transfusions to prevent the occurrence of strokes, may begin these transfusions later in childhood, and may already achieve normal growth and sexual maturation before the threshold for tissue damage is reached.

A comparative study of regularly transfused patients with TM and SCD with similar transfusional iron burdens showed that the latter group had fewer cardiac and endocrine complications.<sup>11,12</sup> The SCD group began regular transfusions at a later age, and had been transfused for approximately half as long as the thalassemia patients, possibly contributing to the observed differences in clinical manifestations. It has been reported that patients with DBA who begin transfusions in early infancy may have higher cardiac iron burdens and may be at higher risk for cardiac mortality.<sup>13</sup> Patients with MDS who begin transfusions in adulthood may not have any endocrine abnormalities, although the iron may contribute to some extent to the diabetes or sexual dysfunction that these older individuals may have. These observations emphasize the relevance of the age at which transfusions are begun, particularly with regard to growth, development, and sexual maturation. As the duration of transfusion increases, severe iron loading may occur in all transfusion-dependent patients who are not effectively chelated, and diabetes and cardiac disease may develop, the latter remaining the major cause for morbidity in all of these patients.

In recent years, there has been much interest and some controversy surrounding iron overload in patients with MDS, specifically the role in pathophysiology and the need for treatment. The major cause of siderosis in these individuals is mostly red cell transfusions, but ineffective erythropoiesis may play some role. Iron deposition in the different organs is not as consistent as it may be in TM, and the role of iron in organ dysfunction is also not clear. Excess iron has been demonstrated in the liver after just 20 units of packed RBCs, and in the heart after 75 units.<sup>14</sup> Iron has been demonstrated in the pituitary and pancreas of these individuals as well,<sup>15</sup> and although hepcidin levels are variable, they are usually lower in low-risk MDS patients.<sup>16</sup> However, the removal of iron may improve trilineage erythropoiesis and reduce transfusion requirements,<sup>17,18</sup> with postulated mechanisms including reduction in oxidative stress, increase in erythropoietin, and inhibition of nuclear factor κB (NF-κB).<sup>19</sup>

Iron overload has also been described in the setting of stem cell transplantation, with several studies showing that the incidence of iron overload in these patients is generally high,<sup>20</sup> with higher burdens conferring a poorer prognosis on patients with MDS or acute myeloid leukemia undergoing transplantation,<sup>21</sup> possibly related in part to an increased risk of invasive fungal infections.<sup>22</sup> More prospective studies related to iron in this setting are underway.

As the body accumulates iron from transfused red cells, chelation therapy is usually begun to prevent deposition and damage to parenchymal tissues. Just as the rate of uptake of iron by these tissues is influenced by transfusion parameters and hepcidin levels, the variability of the chelation regimen also plays an important role. The timing of initiation, choice of chelator and dosing, and compliance with the regimen are some of these variables. When transfusion and chelation are occurring simultaneously, there is likely a complex and dynamic balance between transfusional iron loading and chelator-induced iron purging.<sup>23</sup> Different organs may take up iron at different rates and likely have different thresholds for when tissue damage occurs. It has been demonstrated that in transfused and chelated patients with thalassemia and SCD, the relationship between the liver iron concentration and myocardial iron levels is nonlinear and variable.<sup>24</sup>

In summary, the pathophysiology of iron metabolism in the condition underlying the transfusion-dependent anemia, as well as the factors directly related to the initiation, intensity, duration, and effectiveness of both the transfusion regimen and the chelation

regimen, contributes to the pathology of transfusional iron overload.

Storage iron is present in the body predominantly in two high-molecular-weight forms, ferritin and hemosiderin. Both forms are stained on pathological specimens by Prussian blue, via the Pearl's reaction. Hemosiderin is insoluble and deposited in aggregates of varying sizes, whereas ferritin is soluble and more homogeneously distributed within cells. Under physiologic conditions, iron is distributed approximately equally between these storage forms, but as the body's iron load increases, the proportion stored as hemosiderin increases dramatically. A small amount of iron is present in a low-molecular-weight intracellular pool, likely the most toxic form of iron, because of its propensity for producing free radicals, which cause oxidative damage. It is likely that the ferritin fraction is in closer equilibrium with the low-molecular-weight iron pool.<sup>25</sup> As the magnitude of iron excess grows, some stored iron may be mobilized into the circulation from macrophages or hepatocytes. As transferrin is increasingly saturated, some plasma nontransferrin-bound iron (NTBI) may appear. This iron may be more easily taken up by parenchymal tissues that do not normally store iron, leading to toxicity. Thus, it is critical to ensure that effective chelation therapy is ongoing to bind this iron and prevent its entry into cells, thereby avoiding organ damage.

Histopathological examination of the liver in mild transfusional iron overload reveals predominant Kupffer cell iron deposition, with preserved hepatocyte architecture and no fibrosis. As iron loading continues, the hepatocytes also demonstrate increased iron uptake, and there is some disruption of the architecture and some scarring and fibrosis. Until this point, these changes are reversible with appropriate chelation. With massive overload, there is bridging fibrosis and progression to cirrhosis, which may or may not be reversible.<sup>26</sup> Myocardial iron distribution is heterogeneous, with more in the left heart than the right, the ventricles than the atria, and the epicardium than the endocardium.<sup>27</sup> It has been suggested, based on studies in the iron-loaded gerbil model, that iron in myocardial fibers may cause impaired repolarization of the myocytes,<sup>28</sup> which interferes with generation and orderly propagation of the electrical impulse and may lead to a variety of arrhythmias. With progressive loading, fibers may not contract normally, and a restrictive cardiomyopathy with impaired filling and diastolic dysfunction may result, eventually leading to congestive heart failure. Heart failure is reversible in part, and with improved chelation, cardiac function may be recovered substantially.<sup>29,30</sup> Therefore, long-term monitoring of transfused and chelated patients should include serial measurement of myocardial iron deposition as well as the liver iron concentration.

### Transfusional iron burden

The total amount of iron that enters the body via red cell transfusion may be estimated. As the magnitude of the iron burden is critical in the development of complications, it is of great importance to maintain accurate records, in each patient, of the amount of red cells transfused. The total amount of transfused red cells is calculated as the total amount of blood in milliliters multiplied by the hematocrit of each unit (as a percentage) divided by 100. Each milliliter of red cells contains 1.08 mg of iron, and the total amount of iron introduced ( $K_{in}$ ) is calculated as

$$K_{in} = (\text{total amount of red cells transfused [in mL]}) \times 1.08$$

Alternatively, if the exact volume is not available, the iron burden may be estimated from the number of units transfused. Each unit of transfused red cells contains 200–250 mg of iron. Using one of these methods, the total iron burden may be calculated. Transfusion-dependent patients usually require 200–300 mL/kg/year of blood, an amount equivalent to 0.25–0.40 mg Fe/kg/day.

With no physiologic means of excreting this excess iron, and the inapplicability of phlebotomy in patients who are regularly transfused, these individuals must receive iron chelation therapy in order to facilitate excretion. A minimum chelator-induced excretion of 0.25–0.40 mg Fe/kg/day would maintain iron balance in such individuals. Higher levels of excretion would be necessary to produce a negative iron balance.

Iron balance may be monitored during chelation therapy to assess the efficacy of such treatment. The total body iron ( $U_s$ ) at any point in time  $t$  may be extrapolated from the liver iron concentration (in mg Fe/g dry weight [dw]) using the formula published by Angelucci *et al.*:<sup>31</sup>

$$U_s(t) = 10.6 \times \text{liver iron concentration} \times \text{body weight (in kg)}$$

For patients who are being chelated as well, there is an ongoing change in the body iron balance as the iron entering the body through transfused red cells is removed by the chelator. In these situations, it is important to monitor the total body iron excretion between measurements of liver iron concentration. This may be calculated based on the amount of red cells transfused ( $K_{in}$  = iron introduced in mg) and on the changes in total body iron between measurements of liver iron concentrations at baseline ( $t_0$ ) and a later time ( $t$ ), expressed as milligrams of iron excreted per day:<sup>32</sup>

$$\text{total body iron excretion} = \left( K_{in} + [U_s(t_0) - U_s(t)] \right) / (t - t_0)$$

### Clinical features

Although iron deposition in tissues begins soon after the initiation of regular transfusions, signs and symptoms of iron toxicity usually occur after a few years of iron loading, when the usual storage sites for iron have been almost saturated, and there is deposition in other tissues that do not normally store iron. Organs suffer damage, often irreversible, resulting in significant morbidity and sometimes an early death, most commonly as a result of cardiac failure.<sup>33,34</sup> A cross-sectional study of patients with thalassemia born after 1970 in Italy found that 7% had heart failure, 6% had diabetes, 11% had hypothyroidism, and 55% had hypogonadism.<sup>35</sup> A third of these patients had died, the cause of death in 68% being heart failure. Such data are not available for other transfusion-dependent anemias or MDS.

Liver disease of transfusional iron overload may be manifested as hepatomegaly; abnormalities in function; fibrosis, which may lead to scarring, bridging fibrosis and micronodular regeneration, and cirrhosis; and hepatocellular carcinoma.<sup>36,37</sup> Patients may be asymptomatic, or have mild-to-moderate icterus with progression, symptoms, and signs of cirrhosis and hepatic failure.

Heart failure remains the leading cause of mortality in transfusional iron overload. Unfortunately, symptoms do not appear until the myocardium has large amounts of deposited iron, and routine testing by conventional echocardiography and Holter monitoring early in the course almost always yields normal results. With

progressive deposition, patients manifest symptoms. Early signs may include a variety of arrhythmias, including supraventricular or ventricular tachycardias, premature of extra systoles, heart block, and atrial fibrillation. Contractile dysfunction affects diastole early,<sup>38</sup> likely the reason that conventional echocardiography, which mainly assesses systolic function, indicates “normal” ejection fractions. The onset of cardiac failure is late, and often sudden, and it is an ominous sign. Both complications are often refractory to treatment. Therefore, the prevention of myocardial iron loading should be one of the main thrusts of therapy.

In patients who begin receiving transfusions in early childhood, the developing endocrine organs are particularly susceptible to iron deposition if effective chelation is not initiated at the appropriate time. The anterior pituitary is most often involved, resulting in slow growth, delayed sexual maturation, and often infertility, the latter being the result of direct iron deposition in the gonads as well. Supplemental growth hormone or sex hormone therapy is often required to treat these complications. The pancreatic islet cells are also susceptible to the toxic effects of iron. Impaired insulin secretion and abnormal glucose tolerance usually precede the development of insulin-regulated diabetes and may occur in highly loaded individuals at any age.<sup>39,40</sup> Older males may experience dysfunction in sexual performance, and females may develop secondary amenorrhea related to gonadal iron deposition and disturbances in sex hormone secretion. Thyroid dysfunction, although uncommon, may also manifest at any age.<sup>36</sup>

Other manifestations include arthropathy and skin pigmentation, which are directly related to iron deposition in these tissues. Patients with marked hemosiderosis appear bronzed initially, and with progression may appear darker, or grayish. Complicating the manifestations of iron toxicity are the clinical signs and symptoms of chelator side effects, which are described further in this chapter.

### Measurement of iron burden

The goal of therapy for individuals with transfusional iron overload is to maintain iron balance at low levels of tissue iron, thereby preventing the development of overload and complications. When there is little or no ineffective erythropoiesis, simply keeping track of the volume of packed red cells transfused would provide a measure of the body iron burden in transfused patients who are not being chelated. However, different degrees of IE and the concomitant use of chelation change the balance of iron coming in and out of the body, and thus a regular monitoring of iron levels is required to assess the efficacy of the chelation regimen. In addition, different tissues load iron at different rates, and the balance between loading via transfusion and removal by chelation is also a determinant in the differential deposition of iron. Thus, monitoring iron deposition in any one organ is not representative of the others. The ideal means of monitoring iron overload would be to periodically assess tissue iron levels in each of the different organs affected using a technique that is safe, accurate, noninvasive, and valid over a wide range of concentrations. Magnetic resonance (MR)-based methods meet almost all of these criteria, and have become accepted as the standard for these tissue iron measurements, although access to centers offering them is still limited.

The reference method for assessing the liver iron concentration (LIC) (normal <1.6 mg/g dry weight) has been the quantitative chemical estimation of nonheme iron in liver tissue obtained by biopsy. To obtain accurate results by this method, several requirements must be met: the patient should not have cirrhosis or focal

lesions, the biopsy must weigh at least 1 mg dw (or 4 mg wet weight), and it should be processed using strictly iron-free methods. There may also be sampling variability since the iron deposition may be heterogeneous in some cases. Serial biopsies would ideally allow monitoring of not only the rate of iron loading and the efficacy of chelation therapy in the liver, but also the detection of the development of, and the rate of progression of, fibrosis or scarring. Since liver biopsy is an invasive procedure with considerable risks, biopsy is only recommended now for the monitoring of progression of fibrosis or hepatitis.

Instead, magnetic resonance imaging (MRI) techniques have been developed to noninvasively measure the liver iron concentration. Hemosiderin and ferritin iron affect the relaxation of hydrogen atoms present in the nuclei of tissue water molecules. Initial limitations in the technology have been overcome, and more sensitive instruments capable of measuring more rapid signal decay (very short relaxation times) are now used to quantify hepatic and cardiac iron concentration. Two widely used MRI techniques are (1) a gradient echo T2-based sequence, which measures the signal intensity ratio of liver to skeletal muscle,<sup>41</sup> and (2) a stimulated spin-echo R2-based sequence scan ([www.resonancehealth.com/rh\\_ferriscan](http://www.resonancehealth.com/rh_ferriscan); Ferriscan).<sup>42</sup> Correlation between these magnetic measurements and tissue iron content as assessed by biopsy has improved over time, and this method now provides a safe, accurate, and noninvasive means of assessing LIC.<sup>41–43</sup> The relationship between estimates of liver iron concentration by MR methods and the serum ferritin has been variable.

The liver iron concentration (LIC) has been used as an indicator of the body's total iron burden. In patients with TM, who had been cured by stem cell transplantation, phlebotomy was used as a means of removing excess iron that had accumulated from years of red cell transfusions. A serial decline in the liver iron concentration correlated well ( $R = 0.98$ ;  $P < 0.001$ ) with the amount of iron removed by phlebotomy, supporting the idea that the liver iron concentration is a reflection of the total body iron load.<sup>25</sup> It has also been demonstrated that whereas patients with thalassemia who had liver iron concentrations of 6–13 mg Fe/g dw had an almost twofold risk of progression of fibrosis; in those with liver iron concentrations of 13–41 mg Fe/g dw, the hazard rate was almost 9.<sup>44</sup> Furthermore, coexisting infection with the hepatitis C virus (HCV) independently increased the risk for progression of fibrosis threefold. These observations confirmed the results of previous studies that had reported that LIC  $>7$  mg Fe/g dw is associated with an increased risk of hepatic fibrosis, diabetes, and other complications of iron overload. Based on these data, it has been suggested that maintaining LIC in the “ideal range” of 3–7 mg Fe/g dw in regularly transfused and chelated patients should minimize iron deposition in nonstorage parenchymal sites and prevent significant toxicity.<sup>45</sup> In addition, individuals who are heterozygous for hereditary hemochromatosis may be asymptomatic with LIC in that range. However, more recently it has been suggested that maintaining LIC closer to the upper limit of normal is more ideal in TM patients, but there are only limited data to support this.<sup>46</sup> Since the correlation between hepatic and cardiac iron concentrations is not linear,<sup>24</sup> independent assessment of myocardial iron should be performed.

Although the liver iron concentration can be measured directly by biopsy or noninvasive means, direct tissue quantification of myocardial iron is not feasible. A surrogate for cardiac iron burden, myocardial T2' (decay of transverse magnetization as a result of spin–spin relaxation and magnetic field inhomogeneity) (normal  $> 20$  milliseconds), also measured by MRI, has been shown to

correlate with function<sup>47–50</sup> and is currently used in several centers. Iron concentrations in the myocardium are much lower than those in the liver, and the iron is distributed heterogeneously in different areas of the heart. A multiecho spin-echo T2' technique is the most widely used method of assessing myocardial iron. All individuals with clinical cardiac disease had T2' measures of  $<20$  milliseconds, and all subjects with T2' values above 20 milliseconds had normal cardiac function.<sup>47</sup> An algorithm has been developed to assess risk for iron-induced cardiac disease, and abnormalities in both contractile and electrical function, based on T2' values.<sup>50</sup>

More recently, methods for assessing iron deposition in other tissues such as the pancreas and anterior pituitary have been described with some correlation with endocrine function in these two organs.<sup>51,52</sup> However, these techniques are not universally available, and current ideal monitoring includes periodic assessment of hepatic and myocardial iron deposition only. Such serial measurements would be an accurate and reliable means of monitoring the progression of iron loading and the efficacy of chelation therapy.

Magnetic biosusceptometry has been validated to be a safe, accurate, and noninvasive method of quantitatively measuring the liver iron concentration over a wide range of body iron burdens. The principle is that storage iron (both hemosiderin and ferritin) is paramagnetic: That is, when a steady magnetic field is applied, these molecules will induce a field change that is proportional to the number of iron atoms present. An extremely sensitive sensor, or SQUID (superconducting quantum interference device), measures this response. A strong linear correlation ( $R = 0.99$ ) between liver iron concentration determined by biopsy, and by SQUID susceptometry, has validated this technique,<sup>53</sup> but the use of this method has been limited because of the complexity and high cost of building, installing, and maintaining the instrument. Currently, only four centers around the world are using this technique. Other limitations include use in individuals who are obese, are very small, or have metal implants or devices that may not be removed prior to the study. In addition, susceptometric methods cannot be used to quantify iron in other organs that may be loaded with iron, such as the heart, pancreas, and other endocrine organs. A new high transition temperature device, cooled using liquid nitrogen and with better resolution of the signal from surrounding tissues, is under development,<sup>54</sup> but the complexity of the device and its maintenance have led to increased reliability on magnetic resonance methods.

Traditionally, the serum ferritin level has been used as an indirect estimate of the body iron burden. Although several studies have shown a significant correlation between the serum ferritin level and the liver iron concentration estimated by biopsy in patients with thalassemia,<sup>55,56</sup> the coefficient of correlation in most studies was poor, with marked scatter around the correlation line. A recent study in patients with SCD also showed poor correlation between serum ferritin levels and the liver iron concentration measured in biopsy tissue in patients with SCD.<sup>57</sup> There are several reasons for this variability in the serum ferritin level, which do not necessarily reflect a change in the body iron burden. Ferritin is well known to be an acute phase reactant, and serum levels may be increased by infection, inflammation, hepatic dysfunction, tissue injury, or hemolysis, and decreased when there is chronic hypoxia or a deficiency of ascorbic acid. Thus, it is at best an indirect marker of iron overload; and, although it is practical and easy to measure, it is not reliable as an indicator of the total body iron burden or as a parameter for the monitoring of chelator efficacy, and does not in any way inform of the differential iron loading that may exist between the liver and the heart.

Without the wide availability of noninvasive measures of hepatic and cardiac iron, there is still reliance on the serum ferritin level as a means of monitoring the iron burden and the efficacy of chelation. However, it may actually be misleading in individual patients, remaining stable in the face of rising body iron burden and ongoing deposition in the heart and endocrine organs. Similarly, measurements of serum iron, transferrin or transferrin saturation, and urinary iron excretion do not provide a reliable indication of the level of body iron stores. Plasma nontransferrin-bound iron (NTBI) has been suggested to be a marker of the “toxic iron pool,” but its measurement is very complex and is currently still used largely for research purposes only.<sup>58,59</sup> These limitations in serum measurements of iron-related moieties underscore the need for assessment of iron in specific tissues. While not optimal, it may be used to follow trends, and when there is a consistent trend, an MRI could be performed to assist with decisions related to the transfusion regimen.

As discussed in this chapter, there is variability in iron deposition between the heart and the liver, as a result of the changing balance between transfusional iron loading and chelation.<sup>23,24</sup> Thus, optimal monitoring of iron burden should include assessment of both the hepatic iron concentration by biopsy, magnetic resonance, or susceptometry as well as myocardial iron using the surrogate T2\* estimate. These noninvasive monitoring methods are not yet widely available, but special effort should be devoted to securing such testing for patients in order to chelate them effectively and prevent the development of complications.

## Management

The primary goal of chelation therapy is to prevent tissue deposition of excess iron, thereby preventing organ damage and resulting morbidity and mortality. Maintaining safe levels of tissue iron requires achieving a balance between the amount of iron entering the body and that being removed by chelation. Unlike in patients with hereditary hemochromatosis, phlebotomy to remove excess iron is not an option for those with transfusion dependence, although it may become an option after the patient has successfully undergone stem cell transplantation or once therapy has been completed in case of patients treated with intensive chemotherapy regimens. If transfusion therapy is ongoing, iron-overloaded patients must be treated with a chelating agent capable of sequestering iron and permitting its excretion from the body. As discussed previously, a minimum chelator-induced excretion of 0.25–0.40 mg Fe/kg/day

is necessary to maintain iron balance in regularly transfused individuals. In less regularly transfused individuals, such as those on intensive chemotherapy regimens or those with thalassemia intermedia or sickle cell syndromes, a lower daily iron excretion rate may be tolerated. Monitoring the rate of iron loading by keeping a record of units transfused, as well as periodic assessments of tissue iron levels, is extremely helpful in tailoring chelation therapy. The ideal chelator should form a high-affinity 1:1 uncharged complex with iron; be able to chelate intracellular iron; be orally effective, with a long half-life, high chelator efficiency, and low toxicity profile; and be effective in removing iron from, and preventing deposition in, all organs that may be affected by long-term usage. Such a chelator is not yet available. Chelators in current use are described in brief in the “Chelation therapy” section; more detailed reviews of chelation are available elsewhere.<sup>60</sup>

## Chelation therapy

### Chelating agents

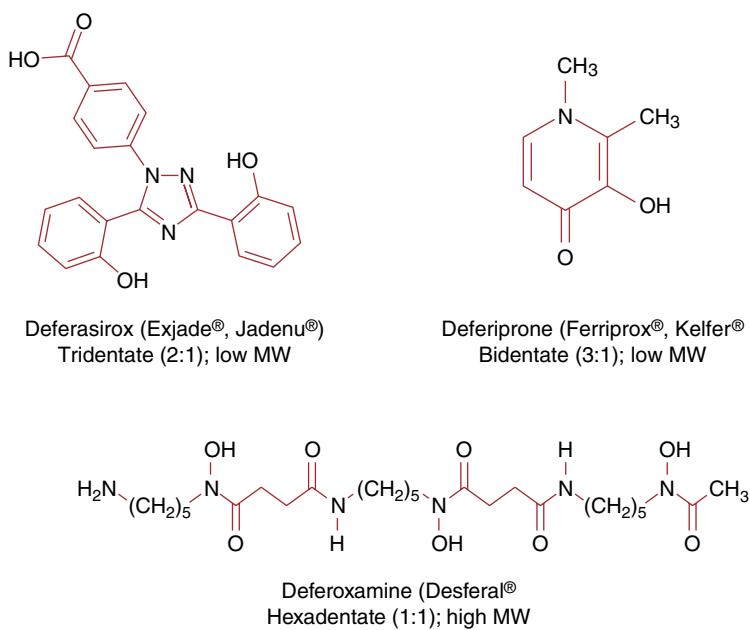
Three iron chelators are in clinical use currently. One of these, deferoxamine, has been the mainstay for approximately four decades; and the other two, deferiprone and deferasirox, have been approved for clinical use in the past 10 years. Table 51.1 shows a comparative evaluation of these agents.

### Deferoxamine B mesylate (DFO, Desferal<sup>®</sup>)

Deferoxamine B mesylate (DFO, Desferal; Figure 51.2), a naturally occurring trihydroxamic acid produced by *Streptomyces pilosus*, was first introduced almost 40 years ago and has been found to be a generally safe and effective means of managing iron overload, ameliorating or preventing iron-induced organ damage, and reducing morbidity and mortality. In fact, patients with TM who were effectively chelated survived significantly longer than those whose chelation was ineffective.<sup>61</sup> Deferoxamine is a hexadentate chelator that forms a charged 1:1 complex with iron that does not readily enter or leave cells and is excreted almost equally by the liver in bile and by the kidneys. Unfortunately, deferoxamine is not absorbed intact when taken orally and has a very short half-life (approximately 15 minutes); it must be administered by subcutaneous infusion using a portable syringe pump over 8–12 hours daily for maximal chelation efficiency.<sup>62</sup> It has been shown that levels of NTBI (the form of iron that more easily enters parenchymal cells) rise in the plasma within 16 hours of the end of the infusion,<sup>62</sup> and maintaining chelator levels in the body is critical to prevent toxic effects of this iron. Patients who receive the drug in this

**Table 51.1** Chelators in Current Clinical Use

	Desferrioxamine	Deferasirox	Deferiprone
Structure, iron binding (chelator:iron)	Hexadentate (1:1)	Tridentate (2:1)	Bidentate (3:1)
Half-life			
Route of administration, frequency	20–30 minutes Parenteral IV or SQ, usually SQ over 8–12 hours, 5–7 nights per week	10–16 hours oral, once daily	1.5–2.5 hours oral, in three divided doses
Iron excretion	Urinary and fecal	Mostly fecal	Mostly urinary
Dose range	30–60 mg/kg	10–40 mg/kg	75–100 mg/kg
Usual chelation application	First or second choice as single agent, or in combination	Usual first choice now, and in combination	Usually second choice, and in combination
Reported efficacy	Efficient hepatic and cardiac iron removal	More efficient clearance of hepatic iron, also effective in cardiac iron removal	More efficient in removing cardiac iron, less so for hepatic iron clearance
Adverse events	Local and allergic reactions, optic neuritis, sensorineural hearing loss	Gastrointestinal upset, rash, hepatic and renal toxicity, proteinuria	Neutropenia/agranulocytosis, gastrointestinal upset, arthralgia/arthropathy, hepatotoxicity
Dose adjustment required if	Impaired renal function	Hepatic or renal toxicity	Hepatotoxicity or neutropenia



**Figure 51.2** Chemical structure of different chelator molecules.

manner often have pain, swelling, and redness at the site of infusion, and may develop injection abscesses. Rare toxicities occur when deferoxamine is present in excess of chelatable iron, including effects on hearing (usually high-frequency hearing loss) and vision (leading to blindness in severe instances).<sup>62,63</sup> For all of these reasons, compliance with this regimen is not very good, and morbidity from organ deposition and dysfunction is often the result. The dosage range for deferoxamine is between 30 and 50 mg/kg administered over 8–12 hours by subcutaneous infusion 5–7 times a week.<sup>63</sup> In severely loaded individuals, such as those with iron-induced cardiac disease,<sup>29</sup> it may be administered by continuous intravenous infusion, although dosing above 50 mg/kg/day does not increase iron excretion further<sup>62</sup> and may result in toxicity. Although cardiac function may improve relatively quickly (2–3 months), such continuous therapy may have to be continued for many months or even years before there is a substantial decline in the liver iron concentration or improvement in the myocardial T2\*.<sup>30</sup> Though a review by the Cochrane Collaboration concluded that deferoxamine is still the recommended first-line agent for chelation in patients with thalassemia,<sup>64</sup> given the cumbersome nature of its administration, this agent is now mostly used in combination with one of the oral agents.

#### **Deferiprone (1,2-dimethyl-3-hydroxypyridin-4-one, DFP, L1)**

Deferiprone (Figure 51.2) is a bidentate chelator that forms an uncharged 3:1 complex with iron, enabling it to easily cross membranes and remove toxic iron from cells. It is effective orally, but has a short half-life (<2 hours) and must be taken 3–4 times a day. Most of the chelated iron is excreted in the urine. It is less effective than deferoxamine in reducing the liver iron concentration, but it has been suggested to have greater effectiveness in removing myocardial iron.<sup>65</sup> A retrospective study comparing TM patients who had been treated with deferiprone long term showed that there were significantly fewer cardiac events as compared with patients who had been treated with deferoxamine alone.<sup>34</sup> Serious idiosyncratic complications have been reported with its use, including neutropenia (up to 5%) and agranulocytosis (up to 0.5%), the development

of an erosive arthropathy (from 5 to >20%), and the development of a neurologic syndrome of cerebellar and psychomotor retardation.<sup>66</sup> In spite of adherence to the suggested weekly monitoring of blood counts, one death from agranulocytosis has been reported.<sup>67</sup> Although a Canadian study described progression of hepatic fibrosis in several patients who were on this drug long term,<sup>68</sup> a subsequent study did not confirm this finding.<sup>69</sup> Deferiprone is prescribed at 75–100 mg/kg/day divided into three doses, with careful monitoring of blood counts every week. The ability of this drug to move across the cell membrane led to the idea of using it in combination with deferoxamine, with deferiprone acting as a siphon to bind iron and transport it extracellularly. Although one study showed that patients treated with combination deferoxamine and deferiprone showed significantly greater improvements in T2\* than those on deferoxamine alone,<sup>70</sup> the significance of this finding is limited by the small number of subjects and the short duration of evaluation. Although approved for use as an iron chelator, a recent independent review by the Cochrane Collaboration<sup>71</sup> concluded that deferiprone is a second-line agent for iron chelation in thalassemia. The disadvantage of this agent in that it had to be given three times a day resulting in some reduced compliance has now been ameliorated to some extent, with a twice daily preparation now being available.

#### **Deferasirox (ICL 670Q, Exjade®, DFX)**

Deferasirox (Figure 51.2) is a tridentate N-substituted bis-hydroxyphenyltriazole chelator that forms a 2:1 complex with iron that is primarily excreted in bile. With a plasma half-life of 12–16 hours, once-daily administration means the drug is present in the circulation throughout the day, enabling constant effective scavenging of iron. There are three preparations available, a dispersible tablet (Exjade®), a film coated tablet (Jadenu®), and sprinkles for children (also Jadenu®). At the suggested starting dose of 20 mg/kg (Exjade®) or 14 mg/kg (Jadenu®), most patients are not able to achieve negative iron balance, although this dose appears adequate to maintain iron balance in well-chelated individuals.<sup>72</sup> At higher doses, it is able to cause negative iron balance (reduction in liver iron concentration) but may result in a higher incidence of hepatic

and/or renal toxicity. Other side effects include gastrointestinal upset, rash, and, rarely, neutropenia.<sup>72</sup> Patients who are on this agent should have careful monitoring of hepatic and renal function, and appropriate dose adjustments should be made. Studies have shown that deferasirox is able to consistently remove myocardial iron as well as deferoxamine, and higher doses of 30–40 mg/kg/day (Exjade<sup>®</sup>) or 21–28 mg/kg/day (Jadenu<sup>®</sup>) may be an effective way of reducing the cardiac iron burden and improving function.<sup>73</sup> Because it is the most conveniently administered iron chelator, with once-daily dosing, it has become the first choice of many hematologists for initiating chelation. However, a recent Cochrane Collaboration review<sup>74</sup> concluded that there was not enough evidence to support this in thalassemia, and stated “If a strong preference for deferasirox is expressed, it could be offered as first-line option to individual patients after a detailed discussion of the potential benefits and risks.” Deferasirox has been used with good efficacy in patients with SCD,<sup>75</sup> but there are as yet no long-term studies of this agent in patients with MDS and transfusional iron overload. In clinical practice, given the once a day administration, wide therapeutic index, and good safety profile, this has become the first-line therapy for most patients with transfusional iron overload.

Newer agents in the desferrithiocin family<sup>76,77</sup> have shown promise in the animal model. These are tridentate chelators that initially showed significant nephrotoxicity in the rodent and primate models, but, with structural modifications, these have not been an issue. Phase I/II human trials have been initiated, with little human data available to date.

### Initiation of chelation

Without data to support a lower range, the optimal range recommended for maintaining the liver iron concentration is 3–7 mg Fe/g dw,<sup>78</sup> as discussed previously. When this range is reached after regular transfusion, chelation therapy should be initiated. There are no immediate clinically apparent consequences of not instituting chelation when the patient reaches this range, or even higher levels of iron overload, because such complications usually result at higher levels and after more prolonged loading. The goal of therapy is to prevent these complications, and chelation should be instituted well before they occur. Based on calculations in the “Transfusional iron burden” section, the range for initiation of chelation is usually reached after approximately 15 transfusions (or a total of ~180 mL/kg of packed RBCs). If records of transfused volumes are not available, the liver iron concentration, as measured by biopsy or noninvasive methods such as MRI or SQUID, should be used in making the decision. Usually in children in whom iron is required for growth, aggressive chelation could potentially reduce the amount of available iron, and a conservative liver iron concentration of 4–5 mg/g dw is usually used as the threshold for initiating chelation (Table 51.2). In the past, a serum ferritin level of 1000 ng/mL was used as the threshold for initiation of chelation, and some models have been proposed more recently<sup>79</sup> to suggest that 800 ng/mL should be the threshold in nontransfusion-dependent thalassemia patients. However, the correlation between the liver iron concentration and serum ferritin was not strong. An MRI assessment of LIC is strongly recommended if possible so as to provide a baseline to which subsequent measures could be compared to evaluate efficacy of the chelation regimen. Cardiac and endocrine organ iron deposition occurs late, and initial measurements are not indicated. Although an oral chelating agent would be much preferred by patients compared to a parenterally administered one, as discussed previously, there is still not enough experience or data with regard

**Table 51.2** Guidelines for Chelation Therapy

Current Guidelines For Chelation	
Initiation of chelation	LIC 3–7 mg Fe/g dry weight, or total transfusion volume ~180 mL/kg packed red cells, or ~15 transfusions
When to begin	Deferoxamine (Desferal <sup>®</sup> ) 35–40 mg/kg by subcutaneous infusion over 8–12 hours 5–7 times/week Deferasirox (Exjade <sup>®</sup> ) 20–30 mg/kg/day on empty stomach, daily
Choice of chelator	<b>Intensification (in conjunction with expert consultation and advice)</b>
Indication	1. LIC >15 mg Fe/g dry weight, with T2* >20 milliseconds, no clinical cardiac disease 2. LIC 3–15 mg Fe/g dry weight, with T2* 10–20 milliseconds, no clinical cardiac disease 3. LIC >15 mg Fe/g dry weight, with T2* 10–20 milliseconds 4. LIC 3–15 mg Fe/g dry weight, with T2* <10 milliseconds Deferoxamine 50 mg/kg/day For 1 and 2: by subcutaneous infusion over 8–12 hours daily For 3 and 4: by continuous infusion via central venous catheter Deferasirox For 1 and 2: 30–40 mg/kg/day For 3 and 4: 40 mg/kg/day Combination therapy (see Table 51.3) Multiple combinations are in use; usually deferasirox or deferiprone added to a lower dose/less frequent base deferoxamine regimen
Regimen	

LIC: liver iron concentration.

to the long-term use of deferasirox or deferiprone as a first-line agent, and these agents are only recommended if deferoxamine is contraindicated. However, given the complexity of parenteral administration of deferoxamine and the ease of administration and proven efficacy of deferasirox, an increasing number of hematologists are initiating chelation with the latter. Deferiprone remains the choice for patients who have tried one of the others and have had to discontinue because of toxicity.

There is much controversy as to when patients with MDS should begin chelation, or if they even need chelation.<sup>3,80,81</sup> Several centers have reported their experience,<sup>82–84</sup> but good data to inform the development of a guideline are lacking. Some studies are underway to gather such information, and there are likely to be some clearer directions in the future. With preexisting bone marrow disease, deferoxamine or deferasirox may be the safer options, but some centers have used deferiprone<sup>85</sup> with success.

### Compliance

Compliance with chelation therapy is the primary determinant of morbidity and survival in such patients, and the responsibility of the medical team caring for such patients in reinforcing this cannot be emphasized enough. If compliance with chelation is poor, iron loading exceeds removal and iron accumulates first in the liver and then in the other parenchymal tissues, including the heart. A meta-analysis of 18 studies of patients with thalassemia confirmed that noncompliance results in significant cardiac and endocrine morbidity, and increased risk of death from heart disease.<sup>86</sup> When transfusion and chelation are begun in early childhood, it is usually the responsibility of the parent to ensure that the chelation regimen is followed. In adolescence and young adulthood, when this responsibility shifts to the patient, various distractions and life-changing

**Table 51.3** Combination Chelation Therapy

Combination	Deferasirox (DFX) and add desferrioxamine (DFO)	Desferrioxamine (DFO) and add deferiprone (DFP) OR deferiprone and add desferrioxamine	Deferasirox (DFX) and add deferiprone (DFP)
Documented efficacy	Improvement in both LIC and cardiac T2*	Improvement in cardiac T2*, cardiac function (LVEF), and decrease in LIC	Case series with improvement in both LIC and cardiac T2*
Synergy Indication	Unknown Rising LIC despite DFX at max dose, or DFX dose limited by toxicity	Yes Worsening cardiac T2* despite DFO/DFP at maximum dose or to reduce DFO infusions per week	Unknown For worsening cardiac T2* despite DFX at max dose or DFX dose limited by toxicity
Regimens described	DFX 20–30 mg/kg with DFO 30–40 mg/kg, simultaneously or sequentially	DFO 30–40 mg/kg with DFP 75–100 mg/kg, in a variety of combinations	DFX 20–40 mg/kg with DFP 75–100 mg/kg, concomitantly or alternating

events may result in poor compliance. Often, a lack of physical symptoms in spite of significant iron overload lulls such individuals into a false sense of wellness and invulnerability. This is a particularly critical time for all caregivers to monitor these patients very carefully. Similarly, older individuals who have begun regular transfusion later in life may feel that it is unnecessary to chelate because the onset of symptoms may be several years later. Education and constant reinforcement are necessary to ensure compliance and reduce morbidity and mortality. Providing patients with accurate estimates of their tissue iron levels may be a powerful motivator, especially when achievable targets for LIC and cardiac T2\* are set.

Patients on chelation should be vigilant for symptoms and signs of infection, particularly if they have undergone splenectomy. Because almost all microorganisms require iron to thrive, the mobilization of iron sequestered in stores could allow such microbes to run rampant. Therefore, chelation should be temporarily suspended if there is any suspicion of a bacterial or fungal illness, until this is appropriately managed.

### Intensification of chelation

Noncompliance resulting in a positive iron balance and a rise in the liver iron concentration, or shortening of the T2\*, would necessitate changes in the chelation regimen to prevent progression of organ damage. The compliance record should be closely examined, and consultation with an expert in the management of iron overload should be obtained, before intensification of chelation is undertaken. Dosage of deferoxamine may be increased to a maximum of 50 mg/kg/day, beyond which there seems to be no additional increment in urinary iron excretion.<sup>62</sup> Higher doses have also been associated with the development of a pulmonary syndrome.<sup>87</sup> Increasing the frequency of subcutaneous administration to seven days per week is a more effective strategy (Table 51.2). For patients with symptomatic heart disease, continuous intravenous chelation with deferoxamine at 50 mg/kg/day has been shown to be effective in reversing heart failure, improving cardiac function, and reducing the liver iron concentration more effectively<sup>18</sup> (Table 51.2). Generally, an indwelling catheter is required because deferoxamine may cause sclerosis of peripheral veins. Close monitoring for toxicity and efficacy is necessary. Because noncompliance is the primary cause of severe loading, this regimen may not be acceptable to the patient. Studies using 40 mg/kg of deferasirox have shown some efficacy. Various combinations of chelators have been used with reported efficacy in the thalassemias,<sup>29,49,70,88,89</sup> but there are little data in other conditions. However, these reports have not consistently shown efficacy or benefit of a single combination. Some recommendations are made in Tables 51.2 and 51.3. Longer prospective studies with larger numbers of patients are required before a spe-

cific combination therapy can be recommended. Intravenous deferoxamine as described above should be considered the base of the regimen for any patient with a severe iron burden, corresponding to a liver iron concentration above 15 mg Fe/g dw even in the absence of clinical cardiac deposition, and for those with myocardial T2\* values below 10 milliseconds.

### Monitoring while on chelation

Once chelation is begun, regular noninvasive assessment of iron deposition in the heart (MRI T2\*) and liver (MRI R2 or SQUID) every 12–18 months is recommended (Table 51.4) to ensure the efficacy of chelation and give warning of rising levels, which may be associated with significant iron toxicity. When chelation is being initiated, an MR-determined LIC is helpful to establish a baseline, which would help evaluate the success of ongoing chelation. A cardiac T2\* is usually not necessary for a few years after chelation has been initiated if compliance is good and the LIC remains in the desired range. If, however, the LIC rises due to ineffective chelation and intensification of the regimen is being considered, a cardiac T2\* should be obtained. Given the discordance between LIC and cardiac iron loading while on transfusion and chelation,<sup>24</sup> cardiac T2\* should be obtained regularly for monitoring. As previously discussed, higher LIC values predispose to progression of fibrosis,<sup>44</sup> and keeping the LIC in the ideal range of 3–7 mg Fe/g dw is important to prevent this from occurring. A liver biopsy may be indicated if there is coexisting hepatitis, or liver dysfunction suggestive of fibrosis or progression to cirrhosis. Adjustments in the chelation regimen should preferably be based on changes in the LIC or the cardiac T2\*. More frequent measurements may be indicated after such changes have been made to ensure optimal efficacy of the regimen.

In addition to monitoring iron burden and adherence to chelation therapy, organ function must also be monitored for the development of abnormalities related to the transfusion and chelation regimen (Table 51.4). The ideal method for assessing cardiac function is magnetic resonance assessment of chamber dimensions, ventricular filling, and ejection fractions. This would provide indicators of systolic as well as diastolic function. Conventional echocardiography does not provide adequate assessment of diastolic function, and it is suboptimal because cardiac dysfunction in iron-induced cardiac disease is mainly diastolic and systolic function is preserved until late in the course of the iron-induced restrictive cardiomyopathy. Normal results often lead to a false sense of well-being and noncompliance with chelation. Serial measurements may provide useful information on trends and may signal the need for closer evaluation by MRI.<sup>90</sup> Despite preserved global function, tissue Doppler echocardiography-detected regional wall motion

**Table 51.4** Recommended Comprehensive Evaluation of Regularly Transfused and Chelated Individuals

System	Test	Frequency
Monitoring of iron load	LIC by biopsy, MRI, or SQUID Cardiac T2*	Every 12–18 months; more frequently if high iron burden and on intensive chelation; cardiac T2* may be less frequent if stable values and no upward trend in LIC
Cardiovascular	MRI for assessment of function (echocardiography with tissue Doppler if MRI not possible)	MRI as above, echocardiography annually when MRI is not done, especially if some residual cardiac dysfunction
Hepatobiliary	Holter monitoring	Annually
	Bilirubin levels and AST/ALT monitoring HBV, HCV serology	Every transfusion visit if on deferasirox or if active HBV or HCV infection; less frequent if on deferoxamine Annually
Hematologic	CBC with differential	Weekly if on deferiprone
Renal	BUN and serum creatinine	Every transfusion visit if on deferoxamine or deferasirox
Endocrine	Thyroid and parathyroid function	Annually
	Bone Density	Annually
	Glucose tolerance	Annually
	hGH, testicular and ovarian function	Based on age and clinical indications
Other	HIV serology	Annually
	Vision and hearing	Annually if on deferoxamine
	Pulmonary function	Every two years

LIC: liver iron concentration; SQUID: magnetic susceptometry.

abnormalities may represent an early sign of cardiac disease.<sup>91</sup> These special echocardiographic techniques may be of limited use, but annual magnetic-resonance-based cardiac function assessment, performed at the same time as T2\* measurement, is the ideal method of monitoring. Holter monitoring for the development of arrhythmias is also recommended annually. Regular assessment of hepatic and renal function should also be part of comprehensive care. In addition to screening for transfusion-associated viral infections, frequent assessment is also indicated to monitor for chelator toxicity. Comprehensive annual endocrine evaluations, including thyroid and parathyroid function, bone density assessment, glucose tolerance, and gonadal function testing, are recommended, especially for growing children and adolescents. Vision and hearing should be evaluated annually in patients on deferoxamine. Testing of pulmonary function is also recommended, although not as frequently. Monitoring for chelator toxicity should also be followed, as shown in Table 51.4. Guidelines for monitoring of iron overload and its management in patients with thalassemia have been developed and may be referenced at <https://www.thalassemia.org/thalassemia-management-checklists-now-available-download>.

### Novel Approaches to reduce transfusion burden in transfusion-dependent individuals

Novel agents targeting various pathways in erythropoiesis and iron regulation have been developed in recent years, with the goal of ameliorating IE and reducing the toxicity of iron in transfusion-dependent patients. Several approaches have been used:

#### 1 Erythroid maturation agents

Luspatercept (ACE-536) is a recombinant fusion protein comprising a modified extracellular domain of the human activin receptor type IIB fused to the Fc domain of human IgG1. The molecule acts as a trap binding select transforming growth factor (TGF) β superfamily ligands, resulting in a block of SMAD2/3 signaling, thus enhancing erythroid maturation. Randomized double blinded placebo controlled phase 3 clinical trials were conducted in transfusion-dependent thalassemia patients (BELIEVE trial),<sup>93</sup> and transfusion-dependent patients with very low, low, or intermediate risk MDS (MEDALIST trial).<sup>94</sup> Both trials had positive outcomes with a statistically significant reduction in transfusion requirement. In a proportion of TDT patients,

there was a reduction of 33% in transfusion requirement and a proportion of MDS patients were able to remain transfusion free for periods of eight weeks or longer. Based on these data, the drug has been approved for use in these specific populations.

#### 2 Pyruvate kinase (PK) activators

Inducers of PK may increase red cell ATP levels and increase survival. In addition, increased PK in the red cell precursors in the bone marrow may enable them to manage the oxidative stress better, improving the IE that is seen in thalassemia and PK deficiency. Phase 2 studies in PK deficiency have shown increased hemoglobin levels in nontransfusion-dependent patients<sup>95</sup> and a reduction in transfusion requirements (presented in abstract form only) in regularly transfused patients. Clinical trials are planned in TDT and NTDT patients as well with the goal of reducing transfusion needs in TDT patients and increasing hemoglobin levels in the latter group.

#### 3 Inducers of fetal hemoglobin (HbF)

IMR-687, a potent, specific, and highly selective small molecule, inhibits phosphodiesterase (PDE) 9, thereby increasing cyclic guanosine monophosphate (cGMP), leading to increased fetal hemoglobin production in preclinical models of sickle cell disease.<sup>96</sup> Clinical trials are underway in TDT patients with the goal of reducing transfusion requirements. Benserazide is a peripheral decarboxylase inhibitor shown to increase HbF levels in animal models.<sup>97</sup> An early phase trial is investigating safety and efficacy in NTDT patients.

#### 4 Agents affecting the hepcidin pathway

In animal models, hepcidin mimetics have been shown to improve the efficacy of erythropoiesis and increase levels of hemoglobin. Clinical trials of two such agents were undertaken.<sup>98</sup> The trial of LPJ401 in TDT (NCT03381833) assessed the efficacy of the agent in reducing cardiac iron and was terminated early for futility. A trial of PTG-300 (NCT03802201) to reduce transfusion burden in TDT is ongoing, but little data have been shared publicly. Antisense oligonucleotides (ASO) and small interfering RNA (siRNA) targeting TMPRSS6 have been shown to increase hepcidin, reduce iron, and thus improve ineffective erythropoiesis and RBC survival in mouse models of β-thalassemia.<sup>99,100</sup> Both approaches are now progressing to clinical trials in thalassemia.

## Summary

Iron overload is a significant cause of transfusion-related morbidity and mortality. With improved survival of patients with inherited or childhood transfusion-dependent anemias, older individuals with marrow failure, and cancer patients who have undergone intensive chemotherapeutic treatments or stem cell transplantation, the prevalence of this entity is likely to continue to grow. There is evidence to suggest that the reduction of iron burden in individuals with MDS and patients undergoing stem cell transplantation may be beneficial and result in better outcomes. More research is underway in those fields. Newer technologies are now available for the noninvasive assessment of the body iron burden, making it easier to quantify the amount of excess storage iron in different tissues and to monitor the efficacy of chelation regimens. New oral drugs have made it easier for patients to be compliant with the chelation therapy that must be instituted in order to prevent tissue iron deposition and ameliorate the toxicity that would result from such deposition. As more information becomes available on the long-term use of deferasirox and deferiprone, combination therapy with deferoxamine may change the way chelation is prescribed. Research continues on the development of

newer oral chelators. In addition, various strategies to reduce transfusion burden have been employed, some with success already, and others in clinical trials. These developments hold promise for a new era of effective diagnosis, monitoring, and treatment of transfusional iron overload.

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## **SECTION VIII**

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# **Cellular and tissue transplant technologies**

## CHAPTER 52

# Hematopoietic growth factors

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## Introduction

Over the past several decades, the major hematopoietic growth factors have been identified, purified, and therapeutic products made. These hematopoietic growth factors affect the growth and differentiation of stem cells and later progenitor cells of all lineages. Several of these molecules play important roles in patient care and are widely used. After a brief review of general principles of hematopoietic growth factor function, this chapter focuses on the clinically relevant hematopoietic growth factors that affect erythroid, myeloid, and megakaryocyte differentiation, specifically erythropoietin (epoetin alfa, epoetin beta, darbepoetin alfa, and methoxy polyethylene glycol-epoetin beta), G-CSF (filgrastim, tbo-filgrastim, pegfilgrastim, and their biosimilars), GM-CSF (sargramostim), and thrombopoietin (romiplostim, eltrombopag, avatrombopag, lusutrombopag, and hetrombopag). The general biology and clinical use of each in adults are described as well as their role in transfusion medicine. With the large proliferation of biosimilar versions of initial products, focus is devoted to the studies with the initial molecules with the reader being asked to recall that the regulatory approval for each biosimilar brings with it the indications of the initial product. It is beyond the scope of this review to address each individual study for each molecule currently approved. Furthermore, discussion is limited to products available in North America.

## General principles of hematopoietic growth factors

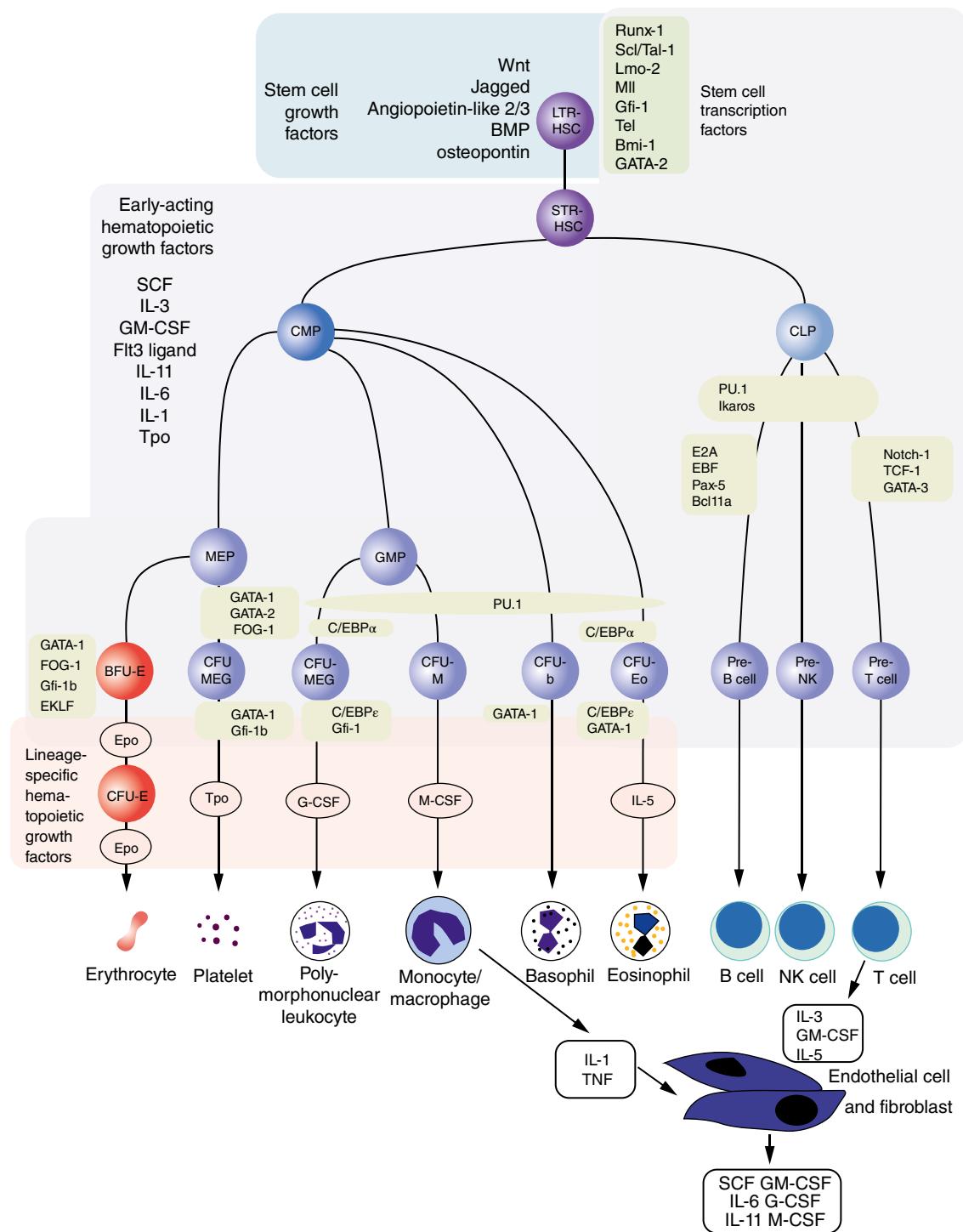
Pluripotential stem cells give rise to all of the final differentiated blood cells (Figure 52.1). The molecular differentiation steps for some of these precursor cells have been described and in general involve a random (“stochastic”) process in which lineage-specific differentiation occurs at multiple stages. For example, a micro-RNA (miR-150) binds to and downregulates c-Myb which causes the common erythroid–megakaryocyte precursor to undergo megakaryocytic, not erythroid, differentiation.<sup>1</sup> But the subsequent survival of cells at each differentiation stage is determined by the presence of specific hematopoietic growth factors; if the stage- or lineage-specific hematopoietic growth factor is absent, the cells undergo programmed cell death. For example, erythroid burst-forming cells

(BFU-E) will continue their divisions as long as erythropoietin is present; if absent, BFU-E cells undergo programmed cell death.

In this complicated hierarchy of cell division, differentiation, and apoptosis, the hematopoietic growth factors can be generally grouped into those that are early acting, whose circulating levels are not altered and affect multiple lineages (interleukin [IL] 3, IL-6, IL-11, GM-CSF, and stem cell factor), versus those that are late acting, whose levels are modulated and affect single lineages (erythropoietin, G-CSF, and M-CSF). Thrombopoietin (TPO) is somewhat of an exception in that it is necessary for the viability of multiple lineages but specifically amplifies megakaryocyte progenitors. All of these hematopoietic growth factors are active at very low concentrations and work via specific cell surface receptors thereby promoting the viability of the target cells.

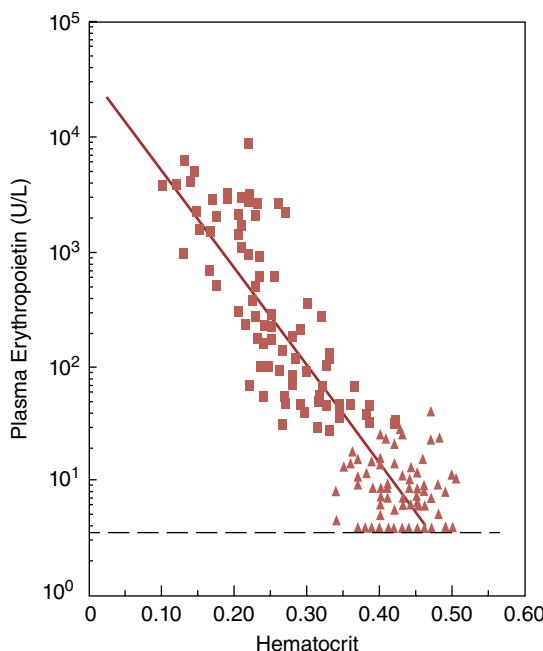
For the clinically relevant hematopoietic growth factors, G-CSF, erythropoietin, M-CSF, and thrombopoietin, a number of general physiological principles should be noted:

- The circulating level of each factor in normal physiology is inversely proportional to the mass of the differentiated cell it regulates, e.g., as the hemoglobin, absolute neutrophil count (ANC), monocyte count, and platelet count fall, the respective levels of erythropoietin, G-CSF, M-CSF, and TPO rise.<sup>2–4</sup>
- The normal physiologic response is usually “log-linear,” i.e., erythropoietin levels increase exponentially as the hemoglobin declines on a linear scale (Figure 52.2).<sup>2,5</sup>
- Circulating levels of each factor are determined by the relative rates of its production and metabolism. For erythropoietin, there is a precise mechanism that regulates its production with a rather fixed rate of renal clearance. For thrombopoietin and M-CSF, levels are primarily regulated through clearance by the platelet<sup>6</sup> or macrophage,<sup>7</sup> respectively. These cells have very high affinity receptors for their regulatory factor, and bind, internalize and degrade it.<sup>8</sup> There is little effect on the rate of production. Finally, G-CSF is regulated by both production and clearance. Mature neutrophils bind and clear G-CSF,<sup>9</sup> but many cells can increase G-CSF production when stimulated.<sup>10</sup> In normal basal physiology, G-CSF production is relatively constant.
- This normal physiologic response may be altered in pathologic states.



**Figure 52.1** Scheme of normal hematopoiesis. The many different stages of differentiation of the myeloid, erythroid, and megakaryocyte lines are illustrated along with their relevant early and late acting hematopoietic growth factors. BFU: blast-forming unit; CFU: colony-forming unit; CLP: common lymphoid progenitor; CMP: common myeloid progenitor; EPO: erythropoietin; G-CSF: granulocyte colony-stimulating factor; GM-CSF: granulocyte-macrophage colony-stimulating factor; GMP: granulocyte-myeloid progenitor; IL: interleukin; LTR-HSC: long-term repopulating hematopoietic stem cell; M-CSF: monocyte/macrophage colony-stimulating factor; MEP: megakaryocyte-erythroid progenitor; SCF: stem cell factor; STR-HSC: short-term repopulating hematopoietic stem cell; TNF: tumor necrosis factor; TPO: thrombopoietin. From Sieff and Zon.<sup>183</sup> Reproduced with permission of Elsevier Inc.

- In anemia of chronic disease, renal erythropoietin production is decreased.<sup>2</sup>
- In acute infection, there is a marked increase in G-CSF production.
- In liver failure, thrombopoietin production is markedly decreased.<sup>11</sup>
- The clinical effect of pharmacologic administration of a hematopoietic growth factor also operates on an exponential dose-response curve. In a healthy subject, linear increases in any blood cell require an exponentially greater dose of the specific hematopoietic growth factor.<sup>12,13</sup>



**Figure 52.2** Log-linear relation between erythropoietin (EPO) level and hematocrit. EPO levels were determined for normal blood donors (triangles) and patients with anemia (squares; those with renal disease, rheumatoid arthritis, or solid tumors were excluded). Dashed line denotes limits of detection for this assay. Note that EPO does not begin to rise until hematocrit falls below ~36, suggesting that other factors (i.e., androgens) account for the higher values. Reprinted from Erslev<sup>2</sup> with permission of Elsevier Inc.

### Erythroid growth factors

Ever since the initial studies by Paul Carnot in 1906, it has been known that blood contained a “hémopoïétine” that had the ability to stimulate red cell production.<sup>14,15</sup> Decades after identifying a factor in the urine that stimulated red cell production,<sup>16</sup> erythropoietin (EPO) was finally purified and a recombinant form FDA approved in 1989.<sup>17,18</sup> EPO was the first hematopoietic growth factor available for clinical use and paved the way for the development of many other hematopoietic growth factors.

### Structure, function, and physiology

Erythropoietin is made as a 193 amino acid precursor, of which 27 amino acids are cleaved off to produce a 166 amino acid form that loses a terminal arg166 to give the final 165 amino acid (MW = 30,400 Da) heavily glycosylated (30% carbohydrate) protein. It is made by a single gene (7q22.1) and produced by interstitial peritubular fibroblasts in the kidney with maybe a small amount being made by the liver. Using an efficient renal sensor of hypoxia (hypoxia inducible factor [HIF]), circulating EPO levels are primarily determined by its rate of transcription. HIF is a dimeric protein made of  $\alpha$  and  $\beta$  subunits that binds to a promoter region of the EPO gene and increases its rate of transcription. However, the HIF $\alpha$  subunit undergoes proline hydroxylation in the presence of oxygen and is degraded by the proteasome. So, with normoxia, HIF $\alpha$  is rapidly degraded and transcription of the EPO gene reduced; with hypoxia, HIF $\alpha$  survives and increases transcription. EPO has no storage form and once made is immediately secreted into the circulation and cleared by the kidney.

EPO binds to and activates preformed, inactive, dimeric EPO receptors (Figure 52.3) that are present on pronormoblasts,

basophilic normoblasts, polychromatophilic, and orthochromatophilic normoblasts, but not on reticulocytes or mature red cells. When EPO is present, these precursor cells do not undergo apoptosis but survive and produce mature red cells.

The usual log-linear relationship between the EPO level and the hematocrit (Figure 52.2) seen in disorders such as iron deficiency is blunted in conditions historically referred to as “anemia of chronic disease” (e.g., diabetes, cancer, renal failure, and inflammation).

### Clinically available erythroid growth factors

#### Epoetin alfa (Epogen, Procrit); biosimilar epoetin alfa-epbx (Retacrit)

This 165 amino acid (MW = 30,400 Da) glycosylated protein (Figure 52.3) is made in CHO cells and is available in vials and pre-filled syringes in a wide variety of doses. Epoetin alfa is FDA approved for:

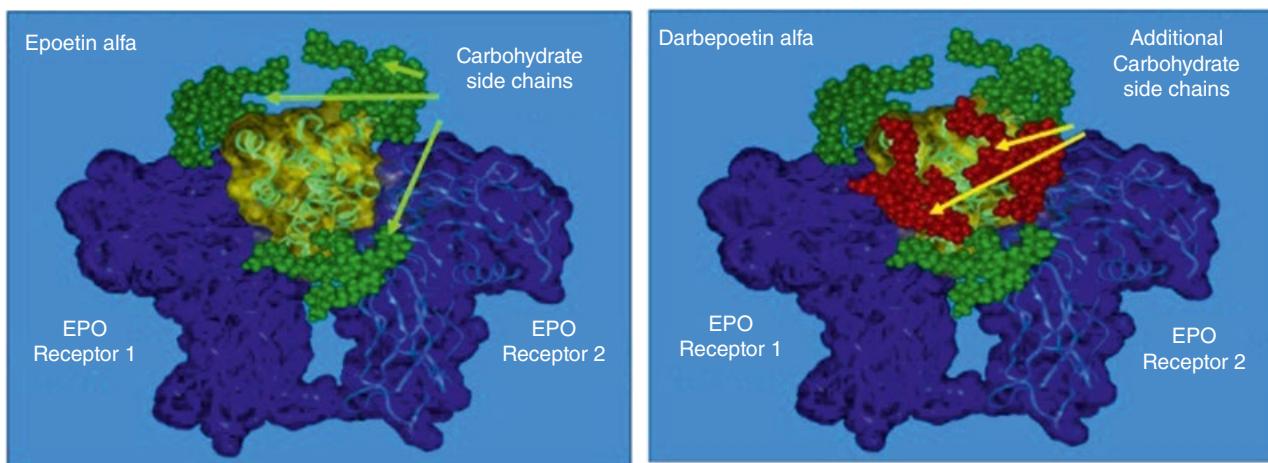
- The treatment of anemia due to chronic kidney disease (CKD) in patients on dialysis and not on dialysis.
- The treatment of anemia due to the effects of concomitant myelosuppressive chemotherapy, and upon initiation, there is a minimum of two additional months of planned chemotherapy.
- The treatment of anemia due to zidovudine in patients with HIV infection.
- Reduction of allogeneic RBC transfusions in patients undergoing elective, noncardiac, and nonvascular surgery.

The starting dose of epoetin alfa is usually 50–100 U/kg tiw for renal failure patients, 100 U/kg tiw for HIV patients, 40,000 units weekly or 150 U/kg tiw for chemotherapy patients (40,000 units per week has become a standard for oncology patients),<sup>19</sup> and either 300 U/kg daily for 15 days or 600 U/kg weekly for preoperative surgery patients with anemia. The intravenous route is recommended for patients on hemodialysis. Subsequent doses are adjusted every three to four weeks based upon response. Despite the recommendation above for patients on hemodialysis, this drug is more effective when given subcutaneously than IV.<sup>20</sup> Drug levels after subcutaneous administration rise at 2 hours, peak at 18 hours, with a T1/2 of 8 hours. All patients should be evaluated for iron status before and during treatment to maintain adequate iron repletion.

#### Darbepoetin alfa (Aranesp)

Darbepoetin alfa was designed from understanding how epoetin alfa is cleared from the circulation. Epoetin alfa has many isoforms due to variations in the number of sialic acids present (Figure 52.3).<sup>21,22</sup> Isoforms with the maximum 14 sialic acids have a prolonged half-life whereas those with 8 sialic acids have almost no biologic effect. This led to the hypothesis that if the number of sialic acids on epoetin alfa was increased, its T1/2 would be even longer. By creating two additional N-linked glycosylation sites on epoetin alfa, darbepoetin alfa was created and contains a maximum of 22 sialic acids versus the 14 sialic acids on epoetin alfa (Figure 52.3). Adding these sialic acids minimally reduced binding to the EPO receptor, but markedly increased the T1/2 from 8.5 to 25.3 hours, thereby increasing the biologic effect. Darbepoetin alfa (MW = 39,000 Da) is made in CHO cells and is 51% carbohydrate. It comes in vials and pre-filled syringes with various doses. It is FDA approved for:

- The treatment of anemia due to chronic kidney disease (CKD) in patients on dialysis and patients not on dialysis.
- The treatment of anemia due to the effects of concomitant myelosuppressive chemotherapy, and upon initiation there is a minimum of two additional months of planned chemotherapy.



**Figure 52.3** Binding of epoetin alfa and darbepoetin alfa to the erythropoietin (EPO) receptor. Epoetin alfa has three N-linked carbohydrate chains with a maximum of 14 sialic acids. It has a MW of 30,400 Da and is 40% carbohydrate. Darbepoetin alfa has five N-linked carbohydrate chains with a maximum of 22 sialic acids. It has a MW of 39,000 Da and is 51% carbohydrate. Both bind to and activate the preformed dimeric EPO receptor. Figure courtesy of Prof. David Kuter.

The starting dose for patients with CKD on dialysis is 0.45 mcg/kg intravenously or subcutaneously weekly, or 0.75 mcg/kg intravenously or subcutaneously every two weeks; for patients with CKD not on dialysis 0.45 mcg/kg intravenously or subcutaneously every four weeks; and for oncology patients 2.25 mcg/kg subcutaneous weekly or 500 mcg subcutaneously every three weeks. Doses are subsequently adjusted every four weeks according to response. The intravenous route is preferred for patients on hemodialysis.

#### Methoxy polyethylene glycol-epoetin beta (Micera)

Endogenous erythropoietin as discussed above is highly glycosylated and exists in several forms with identical amino acid sequence but different glycosylation patterns.<sup>23</sup> Epoetin alfa and epoetin beta have exactly the same amino acid sequence, but the latter has a higher molecular weight and lower number of sialic acid residues. Epoetin beta is commonly used outside the United States. Methoxy polyethylene glycol-epoetin beta was created by coupling polyethylene glycol to the N-terminal amino acid of epoetin beta thereby creating a molecule with a much longer circulating half-life, comparable to that of darbepoetin alfa. This 60 kD protein has a reduced affinity for the erythropoietin receptor, but given its long half-life of 130 hours (darbepoetin has a half-life of 20–70 hours and epoetin alfa has a half-life of about 8 hours) it has a prolonged biological effect.<sup>24,25</sup> In multiple studies compared with epoetin alfa, epoetin beta or darbepoetin alfa, methoxy polyethylene glycol-epoetin beta demonstrated response rates of up to 97.5% when given intravenously or subcutaneously every two weeks with the comparator response rates up to 96.3%.<sup>25,26</sup> In patients being switched from other erythropoiesis-stimulating agents (ESA), stable hemoglobin levels were maintained with administration every 2–4 weeks. The drug was well tolerated with side effects comparable to those of other ESAs. Note that pure red cell aplasia has been reported with this drug but is rare.

Methoxy polyethylene glycol-epoetin beta is supplied as a solution of 30–360 mcg in prefilled syringes for intravenous or subcutaneous administration. It is not a treatment for anemia due to cancer chemotherapy. It is only FDA approved for:

- The treatment of anemia associated with chronic kidney disease (CKD) in adult patients on dialysis

The initial dose of 0.6 mcg/kg body weight is administered every two weeks by subcutaneous or intravenous injection. Subsequent doses are based upon the hematocrit response. It is important to maintain adequate iron stores.

#### Effects and adverse effects of erythropoietin administration

After the administration of epoetin alfa, epoetin beta, methoxy polyethylene glycol-epoetin beta, or darbepoetin alfa to healthy individuals, the reticulocyte count starts to rise on Day 3 and peaks on Day 10 while the hematocrit starts to rise on Day 8 and peaks at Days 20–25.<sup>27</sup> Importantly, the iron saturation and transferrin begin to fall by Day 4 and drop 74% by Day 16.<sup>27</sup>

The major effect of treatment with any of these ESAs is to increase the basal hemoglobin and reduce the need for transfusions in the disorders for which they are indicated. In most disease states, response rates and hemoglobin rises occur in well over 90% as long as adequate iron stores are provided. Although none of the FDA approvals for oncology patients supported an improvement in quality of life, multiple studies dispute that statement.<sup>28–30</sup> The quality of life benefits of erythropoietin administration for CKD patients have been amply documented in many studies.<sup>31</sup>

The major side effects of erythropoietin administration are listed in Table 52.1. Of these, thrombosis and potential adverse outcomes of cancer patients deserve further detail.

**Table 52.1** Adverse Effects of Erythroid Growth Factors

Hypertension—renal failure patients
Seizures—renal failure patients
Allergic reactions—rare
Antibody formation leading to pure red cell aplasia—European formulation; very rare USA
Thrombosis—twofold increase in all patients
Increased cardiovascular complications and death at Hgb >11—renal failure patients
Decreased survival if target Hgb >12—cancer patients (breast, head-neck, cervical, and lymphoma) on chemotherapy; not indicated for cancer chemotherapy patients when anticipated outcome is cure
Decreased survival—cancer patients not receiving chemotherapy

## Thrombosis

There is an approximate twofold increased rate of thromboembolism in renal failure and cancer patients receiving these agents. A meta-analysis of 91 trials with 20,102 cancer patients showed that the risk ratio (RR) of thromboembolic complications was increased in patients receiving these drugs compared to controls (RR 1.52; 95% CI, 1.34–1.74; 57 trials, N = 15,498).<sup>32</sup> In a meta-analysis of 27 trials (10,452 patients), chronic kidney disease patients at a higher hemoglobin target (median 13.0 g/dL, IQR, 12.0–14.0 g/dL) had higher risks for stroke (RR 1.51; 95% CI, 1.03–2.21), hypertension (RR 1.67; 95% CI, 1.31–2.12), and vascular access thrombosis (RR 1.33; 95% CI, 1.16–1.53) compared with those at a lower hemoglobin target (median 10.1 g/dL, IQR, 9.2–11.0 g/dL). However, there were no statistically significant differences in the risks for mortality (RR 1.09; 95% CI, 0.99–1.20), serious cardiovascular events (RR 1.15; 95% CI, 0.98–1.33), or end-stage kidney disease (RR 1.08; 95% CI, 0.97–1.20).<sup>33</sup>

## Tumor progression/cancer mortality

The second major concern with the erythroid growth factors is that they may increase tumor progression and mortality in some cancer patients. In one study, head and neck cancer patients treated with erythropoietin to a target hemoglobin of 14–15 had worse progression-free survival (RR 1.62; 95% CI, 1.22–2.14; p = 0.0008), loco-regional control (RR 1.69; 95% CI, 1.16–2.47; p = 0.007), and overall survival (RR 1.39; 95% CI, 1.05–1.84; p = 0.02) than placebo.<sup>34</sup> However, this study and others reporting similar worsened outcomes<sup>35</sup> were not sufficiently structured or powered to assess cancer progression or survival. In the absence of adequate prospective clinical trials, a massive meta-analysis has been conducted of 21,102 cancer patients in 91 trials treated with epoetin alfa or darbepoetin alfa.<sup>32</sup> These erythroid growth factors significantly reduced the relative risk of red cell transfusions (RR 0.65; 95% CI, 0.62–0.68; 70 trials, N = 16,093). On average, patients in the erythropoietin arms received one unit less blood than the control group with a suggestion of increased quality of life. However, erythroid growth factors increased mortality during active study (HR 1.17; 95% CI, 1.06–1.29; 70 trials, N = 15,935) and might have decreased overall survival (HR 1.05; 95% CI, 1.00–1.11; 78 trials, N = 19,003). There was no evidence that ESAs affected tumor response (RR 1.02; 95% CI, 0.98–1.06; 15 trials, N = 5012).

## Clinical use of erythroid growth factors

The erythroid growth factors have been used in a wide range of medical conditions ranging from renal failure to hemochromatosis (Table 52.2).

### Chronic renal failure

In patients with chronic renal failure, the use of erythroid growth factors has been shown to increase hematocrit, decrease transfusion needs, and increase quality of life. For most such patients, the hemoglobin target is no higher than 11.5 g/dL.<sup>36</sup> This is based upon clinical studies that showed that patients experienced greater risk for death and serious cardiovascular events with a target hemoglobin of >13 g/dL; those with a target of 11.5–13 g/dL had no better or worse outcomes, but possibly improved quality of life compared to those with a lower hemoglobin level.<sup>33,36</sup>

### Cancer patients

The use of erythroid growth factors has been shown to decrease the need for red cell transfusions in most populations studied;<sup>19</sup> the exceptions being anemic cancer patients who are not receiving chemotherapy<sup>37</sup> and those with EPO levels >500 IU.<sup>38</sup> Given the concerns over

**Table 52.2** Clinical Uses of Erythroid Growth Factors

Chronic renal failure in patients on dialysis or not on dialysis
HIV infection being treated with zidovudine
Cancer chemotherapy
Preoperative anemia (hemoglobin 10–13) for elective, noncardiac, and nonvascular surgery
(Myelodysplastic syndromes, primary myelofibrosis, postpolycythemia myelofibrosis, and postessential thrombocythemia myelofibrosis)
(Anemia in rheumatoid arthritis)
(Anemia of chronic disease)
(Anemia in congestive heart failure)
(Anemia in epidermolysis bullosa)
(Anemia due to porphyria cutanea tarda)
( $\beta$ thalassemia)
(Treatment of anemia in patients whose religious beliefs forbid transfusion)
(Anemia due to hepatitis C treatment)
(Mobilization of iron in hemochromatosis)
(Improved harvesting of autologous blood)
(Mobilize peripheral blood progenitor cells)
(Neuroprotection—studies in progress)

Uses in parentheses denote lack of FDA approval but for which compendial data exist. See prescribing information for each individual erythroid growth factor.

tumor progression and survival, current guidelines<sup>39–41</sup> encourage a thorough discussion of the risks and benefits of these agents before their use in all patients receiving myelosuppressive chemotherapy. Furthermore, these agents are not to be used in cancer patients not receiving myelosuppressive chemotherapy or in those for whom cancer treatment is not expected to be curative, and when the hemoglobin is <10 g/dL with only the lowest dose being used to avoid transfusions; treatment is discontinued when chemotherapy ends. It should be remembered that improvement in quality of life reaches a plateau once the hemoglobin rises above 11 g/dL (range, 11–13 g/dL).<sup>28,29,42,43</sup>

### HIV infection

Clinical benefit has been demonstrated in anemic (Hct <30) HIV-infected patients with endogenous EPO levels ≤500 IU/mL undergoing treatment with zidovudine at doses ≤4200 mg/week. In a pooled analysis of four trials,<sup>44</sup> 297 AIDS patients treated with zidovudine received either epoetin alfa or placebo for 12 weeks. No benefit was seen in those with EPO levels >500 IU, but in those with lower levels epoetin alfa therapy decreased the mean number of units of blood transfused per patient compared with placebo (3.2 units vs. 5.3 units, respectively; p = 0.003) and increased the mean hematocrit from the baseline level (4.6 vs. 0.5 percentage points, respectively; p <0.001). Overall quality of life improved in patients on epoetin alfa but was not statistically significant (p = 0.13).

### Preoperative anemia

In patients who are not candidates for autologous blood transfusion, epoetin alfa has been shown to increase the hemoglobin and decrease the need for subsequent allogeneic transfusions when given to patients with hemoglobin between 10 and 13 prior to therapy. In two studies of 461 patients undergoing major orthopedic surgery, 358 received epoetin alfa (100–300 IU/kg sq daily for 15 days [10 days preoperative] or 600 IU/kg weekly for four weeks). The use of ESAs significantly reduced (p < 0.001) the need for allogeneic transfusions.<sup>45</sup>

### An important issue associated with the use of erythroid growth factors

The response to erythroid growth factors depends on the availability of adequate reserves of bone marrow and iron. Since the ferritin

falls dramatically after administration of EPO,<sup>27,46,47</sup> iron supplementation has been shown to be critical in amplifying the hemoglobin response to erythroid growth factors in chronic kidney disease patients<sup>48</sup> and in cancer chemotherapy patients.<sup>49</sup> In most studies, intravenous iron was superior to oral iron repletion.

### Implications for transfusion medicine

The impact of the erythroid growth factors on blood resource utilization has received little attention. With the recognition of adverse events especially thrombosis and possibly cancer progression, their use in oncology has dropped dramatically<sup>50</sup> with a prediction that RBC transfusions would increase 1% nationally.<sup>51</sup> However, the decreased use in cancer seems to have resulted in the acceptance of a lower hemoglobin target in this patient population.<sup>52</sup> Similar regulatory changes have also reduced the use of these agents in chronic kidney disease and have been accompanied by slightly increased rates of transfusion.<sup>53</sup> These changes have minimally impacted the use of red cell transfusions, although exact numbers are difficult to obtain.<sup>54</sup>

The erythroid growth factors remain the only option for many patients who cannot receive RBC transfusions for religious or medical reasons. Administration of 140 IU/kg epoetin alfa three times a week for three weeks along with oral iron three times a day to 45 Jehovah's Witness patients increased the hemoglobin and allowed all patients to undergo major cardiac procedures without blood transfusion.<sup>55</sup>

Finally, the use of erythroid growth factors has been demonstrated to increase the preoperative collection of RBCs, a practice that is falling out of favor currently. In a randomized study of 47 patients scheduled for orthopedic surgery, patients received 600 IU/kg or placebo twice a week for 21 days (along with oral iron) during which time up to six units of RBC were scheduled to be collected. Epoetin alfa treated patients collected a mean ( $\pm$ SD) of  $5.4 \pm 0.2$  units compared with  $4.1 \pm 0.2$  for the placebo group.<sup>56</sup>

Overall, it is hard to find any major impact of the use of erythroid growth factors on demand for red blood cell transfusions. Rather recent clinical studies have eschewed the prior acceptance of the need for higher hemoglobin in many clinical situations

### Novel erythropoiesis-stimulating agents

Increased understanding of erythropoiesis has led to the development of a number of novel stimulators of red cell production. These include agents that affect the HIF pathway and molecules that shorten the time to produce a red cell.

### Modulators of the HIF pathway

As described previously, HIF affects erythropoietin production. The HIF $\alpha$  subunit is inactivated by prolyl hydroxylase in normoxia, and this prevents transcription of the erythropoietin gene. Inhibitors of prolyl hydroxylase have been generated to maintain full HIF activity and thereby allow continued erythropoietin production unregulated by the "oxygen sensor." One of these, vadadustat, an oral prolyl hydroxylase inhibitor, has undergone extensive evaluation in anemic CKD patients on dialysis and prior to dialysis.<sup>57</sup> In two phase III, noninferiority studies in CKD patients not on dialysis (one in patients with no prior exposure to ESA and the second in patients with ongoing ESA treatment) and a hemoglobin less than 10 g/dL, vadadustat was compared with darbepoetin. While there was no difference in hemoglobin response between the two drugs, vadadustat did not meet the prespecified noninferiority margin for cardiovascular safety.<sup>58</sup> However, in CKD patients

who are on hemodialysis, two phase III, noninferiority studies of vadadustat were conducted (one in patients with minimal prior ESA exposure and one in patients previously on ESA), and there was no difference in the incidence of cardiovascular events and no difference in the hemoglobin responses.<sup>59</sup>

### Activin receptor ligand traps

Although no ESA acts sufficiently rapidly to replace RBC transfusions in acute settings, a newly described family of novel recombinant proteins comprising the modified extracellular domain of the human activin type II receptor linked to the Fc region of human IgG1 might accelerate erythropoiesis. It appears that late stage erythropoiesis is normally highly damped down by a number of members of the transforming growth factor beta (TGF- $\beta$ ) family, particularly growth differential factor (GDF)11, which are produced by nonerythroid cells.<sup>60</sup> These new activin receptor molecules act as "ligand traps" for these TGF- $\beta$  superfamily (including activin) negative regulators of late-stage erythropoiesis, and thereby accelerate late-stage erythropoiesis and rapidly increase the hemoglobin. One of these, sotatercept (ACE-011), is a recombinant human fusion protein containing the extracellular domain of the human activin receptor IIa that binds to and inhibits activin, growth differentiation factor (GDF11), and other members of the TGF- $\beta$  superfamily.<sup>61</sup> Administration of sotatercept to healthy volunteers led to a rapid and sustained increase in hematocrit in phase I clinical trials; at the highest dose tested, a mean (range) increase of 1.21 g/dL (0.4–2.0) after eight days and an increase of 1.75 g/dL (0.9–3.1) after 15 days were observed.<sup>62,63</sup>

Luspatercept (ACE-536) is the second novel activin receptor ligand trap and contains a modified extracellular domain of the human activin type IIb receptor and binds with high affinity to select TGF- $\beta$  family ligands such as GDF11 and GDF8. It is distinct from sotatercept in that it binds minimally to activin A and does not increase bone density. In healthy volunteers, a dose-dependent increase in hemoglobin was observed after one week with over 80% eventually achieving a 1.0 g/dL increase.<sup>64</sup> Luspatercept-aamt (Reblozyl) is currently FDA approved for the treatment of anemia:

- In adult patients with beta thalassemia who require regular RBC transfusions.
- In adult patients with very low- to intermediate-risk myelodysplastic syndromes with ring sideroblasts (MDS-RS) or with myelodysplastic/myeloproliferative neoplasm with ring sideroblasts and thrombocytosis (MDS/MPN-RS-T) failing an erythropoiesis-stimulating agent and requiring two or more RBC units over eight weeks.

However, given the current cost for luspatercept, some have felt it is not cost effective when compared to transfusion.<sup>65</sup>

### Myeloid growth factors

While deficiencies in RBCs and platelets can be readily treated with transfusion, neutropenia may be a severe, life-threatening disorder that is not readily amenable to transfusion. Although neutrophil transfusions may be arranged, they are neither readily available nor have they demonstrated benefit.<sup>66</sup> With the FDA approval of both G-CSF (filgrastim) and GM-CSF (sargramostim) in early 1991, treatment and prevention of neutropenia became a reality.

### Structure, function, and physiology

Granulocyte colony-stimulating factor (G-CSF) is a 174 amino acid glycosylated protein (MW = 25,000 Da) and is encoded by a single

gene (17q21.1). G-CSF is synthesized by macrophages, monocytes, endothelial cells, and fibroblasts, and its production can be vastly increased by other inflammatory molecules (TNF $\alpha$ , IL-1, IL-3, interferon  $\gamma$ , IL-4). It is made without a storage form and immediately released into the circulation where it is cleared by neutrophils via their G-CSF receptor ( $K_d = 65 \text{ pM}$ ) and less so by the kidney. In the noninfected individual, production is constant and circulating levels are inversely proportional to the absolute neutrophil count (ANC) by way of neutrophil clearance.<sup>9,67</sup> With infection, production is stimulated by inflammatory cytokines.<sup>10</sup> Normal G-CSF levels are  $<39 \text{ ng/L}$ , but rise to a different extent depending on the type of infection: bacterial,  $799 \pm 1501 \text{ ng/L}$ ; viral,  $58 \pm 34 \text{ ng/L}$ ; mycoplasma,  $60 \pm 33 \text{ ng/L}$ .

G-CSF has many biological effects (Table 52.3). For existing neutrophils, it promotes demargination and release from bone marrow reserves as well as increases neutrophil survival. Neutrophil production is markedly increased with the maturation time reduced from six days to three days, an increase in “left shift,” and often the appearance of Dohle bodies and toxic granulation. Neutrophil motility is altered. Neutrophil function (phagocytosis, O $_2^-$  generation, endomitosis, ADCC, and Fc $\gamma$ RI receptors) is increased, an underappreciated effect. Finally, G-CSF mobilizes peripheral progenitor cells. In animals lacking G-CSF,<sup>68</sup> neutrophils are 20–30% of normal, neutrophil precursors 50% of normal, and there is decreased neutrophil mobilization into the circulation.

Although G-CSF is necessary for the normal production and maturation of myeloid precursors into neutrophils, GM-CSF has no physiologic importance for hematopoiesis. In GM-CSF-deficient mice, the number of neutrophils, macrophages, and their precursors are normal but the mice develop pulmonary problems.<sup>69</sup>

GM-CSF is a 127 amino acid (23,000 Da) glycosylated protein made from a single gene (5q31.1). It is made by T cells, macrophages, monocytes, endothelial cells, and fibroblasts. Significant levels are not detected in the circulation, and amounts of this protein do not vary inversely to the absolute neutrophil count. GM-CSF does not increase in amount during infection, but its production by bone marrow stromal cells maybe decreased by interferon 1 $\beta$ . It is normally cleared by the GM-CSF receptor on neutrophils and monocytes with less than 40% being renally cleared.

The biologic effects of GM-CSF on neutrophil survival, production, and mobility are comparable to those of G-CSF but are extended to eosinophils and monocytes (Table 52.3). In addition to increasing neutrophil function like G-CSF, it also increases the destruction of *T. cruzi*, *mycobacterium avium*, *influenza A*, and *candida*. Unlike G-CSF, GM-CSF can increase the production of TNF $\alpha$  and IL-1, which may explain some of its clinical side effects.

### Clinically relevant myeloid growth factors

#### Filgrastim (Neupogen)

Filgrastim is a 175 amino acid protein (MW = 18,800 Da) identical to the native molecule except for an added N-terminal methionine (r-met huG-CSF) and a lack of glycosylation since it is made in *E. coli*. Vials and prefilled syringes of filgrastim are available in doses of 300 and 480 mcg. For most uses, the dose is 5 mcg/kg/day (~230 mcg/m $^2$ /day). Remembering the log-linear dose-response curve for hematopoietic growth factors, one rounds down to the nearest vial or syringe size. While doses for stem cell mobilization are usually higher, much lower doses (0.4 mcg/kg/day) are often adequate in neutropenic patients with HIV, idiopathic neutropenia, or drug-induced neutropenia. Filgrastim has a T $1/2$  of 3.5 hours

**Table 52.3** Biological Effects of G-CSF and GM-CSF

↑ Demargination of neutrophils
↑ Release of neutrophils, monocytes, and eosinophils from marrow reserves
↑ Neutrophil, monocyte, and eosinophil survival
↑ Production of neutrophils, monocytes, and eosinophils
↓ Motility of phagocytes, ↓ skin window migration
↑ Neutrophil function (↑ of both normal and abnormal neutrophils)
↑ phagocytosis
↑ endocytosis
↑ destruction of <i>T. cruzi</i> , <i>Mycobacterium avium</i> , <i>Influenza A</i> , <i>Candida</i>
↑ O $_2^-$ -generation
↑ Mobilization of peripheral blood progenitor cells

Italics denote effects seen only with GM-CSF.

and is metabolized mostly by mature neutrophils and less so by the kidney. Like most hematopoietic growth factors, subcutaneous administration gives a better response than intravenous administration. In chemotherapy patients, filgrastim is usually started 24 hours after the end of chemotherapy and stopped at least 24 hours before the next chemotherapy dose. In chemotherapy patients, prescribing information suggests stopping once the ANC is  $>10,000$ , but it is often stopped once the ANC is  $>2000$ .

Filgrastim is FDA approved to:

- Decrease the incidence of infection, as manifested by febrile neutropenia, in patients with nonmyeloid malignancies receiving myelosuppressive anticancer drugs associated with a significant incidence of severe neutropenia with fever.
- Reduce the time to neutrophil recovery and the duration of fever, following induction or consolidation chemotherapy treatment of patients with acute myeloid leukemia (AML).
- Reduce the duration of neutropenia and neutropenia-related clinical sequelae, e.g., febrile neutropenia, in patients with nonmyeloid malignancies undergoing myeloablative chemotherapy followed by bone marrow transplantation (BMT).
- Mobilize autologous hematopoietic progenitor cells into the peripheral blood for collection by leukapheresis.
- Reduce the incidence and duration of sequelae of severe neutropenia (e.g., fever, infections, and oropharyngeal ulcers) in symptomatic patients with congenital neutropenia, cyclic neutropenia, or idiopathic neutropenia.
- Increase survival in patients acutely exposed to myelosuppressive doses of radiation (Hematopoietic Subsyndrome of Acute Radiation Syndrome).

#### Filgrastim biosimilars (filgrastim-sndz [Zarxio] and filgrastim-aafi [Nivestym])

Since the expiration of the patent on Neupogen on December 2013, at least 25 biosimilar filgrastims have been evaluated by regulatory agencies around the world. As of writing this chapter, three have been approved by the FDA and several are under consideration. All of these molecules have the same protein structure as filgrastim and have gone through the rigorous biosimilar development process wherein their structural identity, pharmacokinetics, and pharmacodynamics are compared with filgrastim. All of these biosimilars have been tested in some but not all of the clinical settings in which the initial filgrastim is approved, but with approval all biosimilars receive the same regulatory approval for all the uses in which the original filgrastim is indicated.

It is beyond the scope of this review to describe the development of each filgrastim biosimilar but an example is provided for one. Filgrastim-sndz has exactly the same peptide structure on HPLC as filgrastim.<sup>70</sup> In healthy humans, it displays identical pharmacokinetic properties and elevates the neutrophil count to exactly same degree as filgrastim.<sup>70</sup> In breast cancer patients undergoing TAC chemotherapy, the neutrophil response over several chemotherapy cycles was identical for filgrastim-sndz compared with filgrastim.<sup>71</sup>

The biosimilar filgrastims described above are available in the same doses as filgrastim and are used in identical fashion. The current FDA labeling for their use is the same as for filgrastim except for their lack of approval for use in Acute Radiation Syndrome.

### Tbo-filgrastim (Granix)

Tbo-filgrastim is a protein identical to filgrastim, but with slightly different concentrations of excipients. Its pharmacokinetics and neutrophil response are identical to filgrastim in clinical trials in chemotherapy, stem cell mobilization, and stem cell transplantation. Although it has been regarded as a “biosimilar,”<sup>72</sup> it has gone through the full FDA approval process just like filgrastim. It is available as 300 and 480 mcg prefilled syringes and used just like filgrastim. It is FDA approved:

- In adult and pediatric patients one month and older for reduction in the duration of severe neutropenia in patients with nonmyeloid malignancies receiving myelosuppressive anticancer drugs associated with a clinically significant incidence of febrile neutropenia.

### Pegfilgrastim (Neulasta)

Pegfilgrastim is a longer acting form of filgrastim produced by adding a 20,000 Da polyethylene glycol (PEG) moiety to the amino terminus of the filgrastim protein. It has a MW of 39,000 Da and is available as a 6 mg prefilled syringe that is usually administered every two weeks. Its action is prolonged because it is cleared mostly by neutrophils, not the kidney. Its T<sub>1/2</sub> of 33 hours (varies from 15 to 80 hours depending upon the ANC) is at least 10 times longer than filgrastim. It is usually given one day after the end of the chemotherapy cycle and is not to be given in the period 14 days before to 24 hours after chemotherapy. It may be administered as a manual injection or used with an on-body injection system (Onpro®). Its major attribute is that of convenience; one 6 mg pegfilgrastim dose every two weeks is equivalent to 10–14 daily doses of filgrastim.<sup>73</sup> For the treatment of Acute Radiation Syndrome, it is given as soon as possible after myelosuppressive doses of radiation and the second dose given one week later. It is FDA approved to:

- Decrease the incidence of infection, as manifested by febrile neutropenia, in patients with nonmyeloid malignancies receiving myelosuppressive anticancer drugs associated with a clinically significant incidence of febrile neutropenia.
- Increase survival in patients acutely exposed to myelosuppressive doses of radiation (Hematopoietic Subsyndrome of Acute Radiation Syndrome).

### Pegfilgrastim biosimilars (pegfilgratim-jmdb [Fulphilia], pegfilgrastim-cbqv [Udenyca], pegfilgrastim-apgf [Nyvepria], and pegfilgrastim-bmez [Ziectenzo])

With the expiration of the patent for PEG filgrastim in the United States in October 2015, 19 biosimilar pegfilgrastims have undergone development and 4 are available in the United States. They have undergone the same biosimilar development process as described briefly above for the filgrastim biosimilars. They all have the same FDA labeling indications except the lack the indication for

the treatment of Acute Radiation Syndrome. They are available at the same dosing sizes as pegfilgrastim with the same dosing instructions. However, none has an on-body injection system to allow delayed automated drug administration.

### Sargramostim (Leukine)

Sargramostim has a structure identical to native GM-CSF except that it has a leucine at amino acid 123. This glycosylated protein is prepared in yeast and has three different molecular forms of 19,500, 16,800, and 15,500 Da. It is available as a solution in multidose vials of 500 mcg/mL and as individual vials containing 250 mcg of lyophilized drug. Doses of 250 mcg/m<sup>2</sup>/day are given intravenously or subcutaneously over different periods of time (2–24 h) for the various indications; for the treatment of Acute Radiation Syndrome, the adult subcutaneous dose is 7 mcg/kg. It is more effective when given subcutaneously than intravenously and its major use has been in the treatment of patients undergoing induction chemotherapy for acute myeloid leukemia and stem cell transplantation. It is FDA approved:

- To shorten time to neutrophil recovery and to reduce the incidence of severe and life-threatening infections and infections resulting in death following induction chemotherapy in adult patients 55 years and older with acute myeloid leukemia (AML).
- For the mobilization of hematopoietic progenitor cells into peripheral blood for collection by leukapheresis and autologous transplantation in adult patients.
- For the acceleration of myeloid reconstitution following autologous bone marrow or peripheral blood progenitor cell transplantation in adult and pediatric patients two years of age and older.
- For the acceleration of myeloid reconstitution following allogeneic bone marrow transplantation in adult and pediatric patients two years of age and older.
- For the treatment of delayed neutrophil recovery or graft failure after autologous or allogeneic bone marrow transplantation in adult and pediatric patients two years of age and older.
- To increase survival in adult and pediatric patients from birth to 17 years of age acutely exposed to myelosuppressive doses of radiation (Hematopoietic Subsyndrome of Acute Radiation Syndrome).

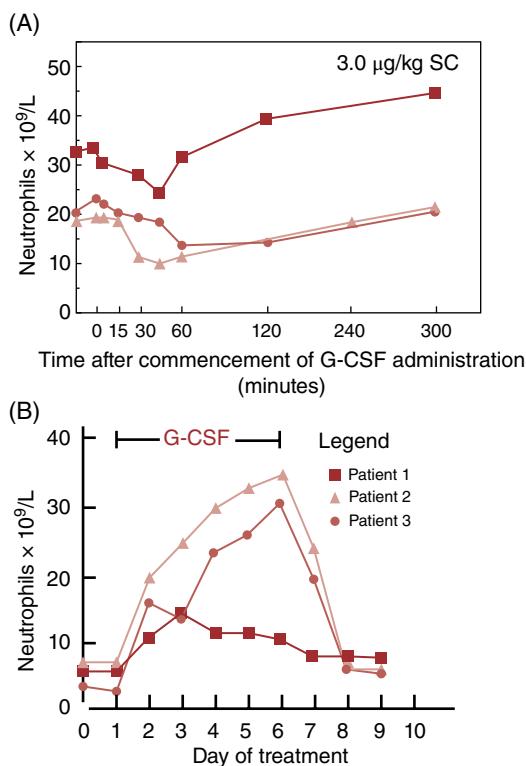
### Effects and adverse effects of G-CSF and GM-CSF administration

The neutrophil response to the filgrastims, pegfilgrastims, and sargramostim in healthy volunteers after a single injection is comparable in some ways (Figure 52.4):

- 15–30 minutes—neutrophils decrease modestly and then return to baseline, probably due to transient sequestration/margination.
- 1–36 hours—the neutrophils gradually rise due to demargination and release from bone marrow stores.
- 36 hours—increased production of neutrophils.

But in other ways, the filgrastims/pegfilgrastims and sargramostim differ greatly with much of the WBC response of the latter coming from increases in eosinophils. Most of the GM-CSF effect on neutrophils is to increase their survival<sup>74</sup> not their production (Table 52.4).

The side effects of the myeloid growth factors are listed in Table 52.5. The filgrastims/pegfilgrastims are comparable in terms of their side effects, but GM-CSF has a somewhat expanded repertoire. These commonly include myalgias, but capillary leak syndrome and a “first dose” phenomenon of hypotension, tachycardia, and dyspnea may be seen at high doses in the transplant setting. If myeloid growth



**Figure 52.4** Time course of the effect of G-CSF on neutrophil counts in human subjects. (A) G-CSF was administered subcutaneously to three subjects at a dose of 3 mcg/kg and the neutrophil count assessed over the next 300 minutes. (B) G-CSF was administered to three human subjects by continuous subcutaneous infusion at 10 mcg/kg/day for five days and the neutrophil response assessed. Figure modified and redrawn from data in Morstyn.<sup>13</sup>

**Table 52.4** Effects of G-CSF and GM-CSF on Neutrophil Kinetics

Neutrophil production	Normal	GM-CSF	G-CSF
Maximum count ( $\times 10^9/\mu\text{L}$ )	5.2	17.0	35.0
Appearance in peripheral blood (days)	4–7	4.5–6.5	1–2
Peripheral T1/2 (hours)	8	48	7.6
Amplification factor	1	1.5	9.4
Extra divisions	0	0.6	3.2

Adapted from data in Lord *et al.* (1992).<sup>74</sup>

factors are administered concurrent with chemotherapy or radiation therapy, subsequent neutropenia may be worsened.

The use of myeloid growth factors in chemotherapy patients may be associated with a small increased risk of treatment-related acute myeloid leukemia or myelodysplasia.<sup>75,76</sup>

### Clinical uses of myeloid growth factors

Table 52.6 lists the uses of myeloid growth factors. Most of these have been studied only with filgrastim and pegfilgrastim.<sup>77</sup>

### Chemotherapy-induced neutropenia

The filgrastims, tbo-filgrastim, and pegfilgrastims have all shown marked ability to stimulate neutrophil production and to mitigate chemotherapy-induced neutropenia. In small cell lung cancer patients undergoing chemotherapy,<sup>78</sup> neutropenic fever for all chemotherapy cycles was decreased from 77% with placebo to 40%

**Table 52.5** Adverse Effects of Myeloid Growth Factors

Bone pain (15–39% on filgrastim vs. 0–21% on placebo)
Exacerbation of preexisting inflammatory conditions (eczema, psoriasis, and vasculitis)
Allergic reactions at injection sites (rare)
Sweet's syndrome (acute and febrile neutrophilic dermatosis)
Antibody formation (none are neutralizing)
Splenic rupture (rare)
Adult respiratory distress syndrome (rare)
Precipitate sickle cell crisis (rare)
↑ Risk MDS and AML in patients receiving chemotherapy or with congenital neutropenia
↑ LDH, uric acid, and LAP; ↓ cholesterol
<i>Myalgias and fever</i>
<i>"First dose phenomenon"—rare; hypotension, tachycardia, and dyspnea due to transient pulmonary leukocyte sequestration (very high doses)</i>

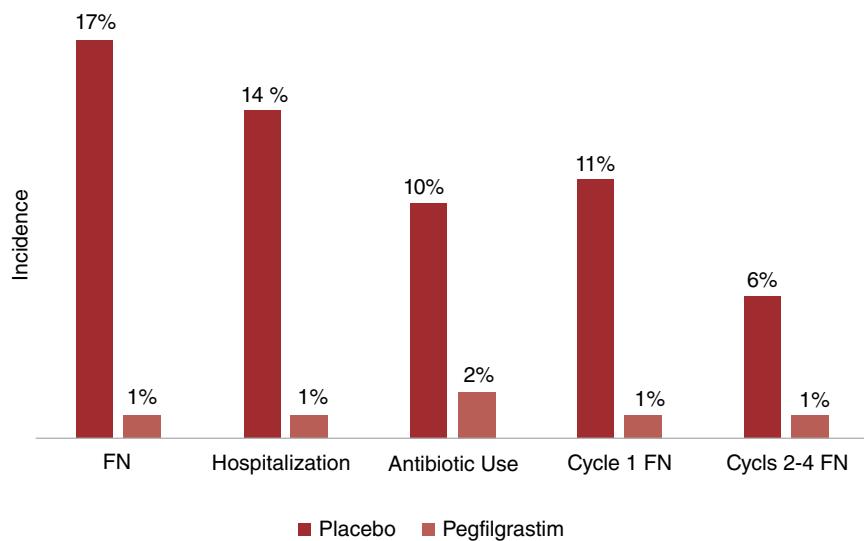
Italics denote effects seen only with GM-CSF.

**Table 52.6** Clinical Uses of Myeloid Growth Factors

Primary prophylaxis of FN if incidence >20%
Primary prophylaxis considered if FN incidence >10–20% (usually "high-risk" patients)
Primary prophylaxis if FN incidence <10% (rarely)
Secondary prophylaxis of FN to keep dose intensity
All high-risk chemotherapy patients admitted with FN
After allogeneic or autologous stem cell transplantation
Peripheral blood progenitor cell mobilization
AML induction/consolidation chemotherapy
Severe chronic neutropenia
Hematopoietic Subsyndrome of Acute Radiation Syndrome (MDS patients with neutropenia and recurrent infection)
(MDS patients with symptomatic anemia)
(Drug-induced neutropenia—low dose [0.4–5 mcg/kg])
(Aplastic anemia)
(HIV neutropenia)
(Neutropenia due to renal transplantation)
(Hairy cell leukemia)
(Acute lymphoblastic leukemia chemotherapy)
(Agranulocytosis)

FN: febrile neutropenia; MDS: myelodysplastic syndrome; AML: acute myeloid leukemia. Uses in parentheses denote lack of FDA approval but for which compendial data exist. See prescribing information for each individual myeloid growth factor.

( $p < 0.001$ ) with filgrastim; hospital days were shortened from 4.2 to 2.3 for all cycles. Antibiotic use and days with an ANC <500 were also markedly decreased. In breast cancer patients undergoing chemotherapy with docetaxel, pegfilgrastim markedly decreased the incidence of febrile neutropenia, hospitalization, and antibiotic use.<sup>79</sup> (Figure 52.5). NCCN guidelines<sup>77</sup> suggest using myeloid growth factors for primary prophylaxis of febrile neutropenia when the expected incidence of such is greater than 20%. Routine chemotherapy regimens for breast cancer, non-small cell lung cancer, ovarian cancer, and non-Hodgkin's lymphoma have respective rates of febrile neutropenia of 15%, 28%, 24%, and 20%.<sup>77</sup> Additionally, myeloid growth factors can be considered for primary prophylaxis for patients receiving chemotherapy where febrile neutropenia risk is 10–20% if patients have one or more risk factors (age  $> 65$ , prior chemotherapy or radiation therapy, recent surgery and/or open wounds, renal dysfunction [creatinine clearance  $< 50$ ], or liver dysfunction [bilirubin  $> 2.0$ ]). Prophylactic myeloid growth factors are rarely considered for chemotherapy patients where the rate of febrile neutropenia is expected to be <10%.



**Figure 52.5** The effect of pegfilgrastim or placebo in breast cancer patients receiving docetaxel every three weeks. Figure prepared from data in Vogel.<sup>79</sup>

In chemotherapy patients experiencing febrile neutropenia in a chemotherapy cycle not supported by myeloid growth factor, subsequent use of myeloid growth factor (secondary prophylaxis) is recommended to maintain dose intensity.

Unless considered high risk, myeloid growth factor treatment is not indicated for chemotherapy patients (not on myeloid growth factor therapy) at the time they develop severe neutropenia without fever or are currently hospitalized with febrile neutropenia.

### Neutropenia due to induction/consolidation therapy for acute myeloid leukemia

Myelosuppression and neutropenia with consequent infection are major complications of the induction and consolidation treatment for acute myeloid leukemia (AML). In a study of 521 AML patients undergoing induction and consolidation chemotherapy, patients were randomized to filgrastim (5 mcg/kg daily) or placebo. There was no effect upon remission rate or survival but patients receiving filgrastim during induction had a five-day earlier neutrophil recovery ( $p < 0.0001$ ), fewer days of fever (7 days vs. 8.5 days;  $p = 0.009$ ), less parenteral antibiotic use (15 days vs. 18.5 days;  $p = 0.0001$ ), and shorter hospitalization (20 days vs. 25 days;  $p = 0.0001$ ) than those receiving placebo.<sup>80</sup> Subsequent follow-up of these patients showed no long-term detriment from exposure to filgrastim.<sup>81</sup> In another study of patients with AML receiving diaziquone and mitoxantrone, those receiving 5 mcg/kg daily filgrastim had no greater response or survival than prior cohorts not so treated, but the duration of neutropenia was markedly reduced.<sup>82</sup> While there has been considerable discussion whether this form of therapy is cost effective, a recent analysis showed a reduction in total costs of around \$5000 for those receiving filgrastim after induction chemotherapy, driven primarily by a shorter median hospital stay (24 days vs. 26 days,  $p < 0.01$ ), faster median neutrophil count recovery (23 days vs. 25 days,  $p = 0.03$ ), and fewer mean days of antibiotics (18.5 days vs. 21.4 days,  $p = 0.01$ ).<sup>83</sup>

### Neutropenia with stem cell transplantation

As with other myelosuppressive therapies, neutropenia and infection are a common complication of stem cell transplantation. In a randomized study of patients with lymphoma undergoing autologous stem cell transplantation receiving either filgrastim or placebo,

filgrastim reduced the time to obtain an absolute neutrophil count (ANC) over 500 (12 days vs. 20 days,  $p = 0.0004$ ), the duration (11 days vs. 27 days,  $p = 0.0001$ ) of neutropenia (ANC < 500), and the number of febrile neutropenic days (5 days vs. 10 days,  $p = 0.036$ ).<sup>84</sup> In a phase III randomized study of filgrastim versus placebo in patients undergoing autologous bone marrow transplantation for hematological malignancy, filgrastim significantly shortened the median (range) time to neutrophil engraftment (ANC > 500) from 19.0 days (15.0–28.0) to 15.0 days (1.0–22.0) ( $p < 0.0001$ ).<sup>85</sup> There was no effect on the rate of survival or GVHD. In general, filgrastims and pegfilgrastins decrease the time to ANC recover, reduce febrile neutropenic days, reduce hospital days, and may reduce overall costs. In a retrospective analysis of autologous stem cell transplantation patients receiving pegfilgrastim or filgrastim, there was an improved reduction in time to neutrophil engraftment (9.6 days vs. 10.9 days,  $p < 0.001$ ), lower incidence of febrile neutropenia (59% vs. 78%,  $p = 0.015$ ), shorter hospital stay, fewer days on antibiotics (6.3 days vs. 9.6 days,  $p = 0.006$ ), and fewer radiographic tests, decreasing costs by about \$8000.<sup>86</sup> The biosimilar filgrastims have shown identical effects on neutrophil engraftment and febrile days but were not associated with reduced overall transplant costs.<sup>87</sup>

### Mobilization of peripheral blood progenitor cells

Four to six days after administration of a myeloid growth factor, peripheral blood progenitor cells increase as much as 100-fold. This has allowed for the marked improvement of collection of such cells in patients or donors for stem cell transplant. The basic physiologic principles governing mobilization of PBSC probably involve breakage of molecular bonds between the adhesive marrow elements, i.e., CXCR-4 on the progenitors and stromal-cell-derived factor 1 (SDF-1) on the marrow stromal cells.<sup>88</sup> Administration of G-CSF expands myeloid and progenitor mass, and there is good evidence that the proteases released from neutrophils disrupt key adhesive bonds holding the progenitors in the marrow and allow their mobilization from marrow spaces.<sup>89</sup>

### Severe chronic neutropenia

The clinical benefit of long-term myeloid growth factor administration for patients with severe congenital neutropenia (including Kostmann syndrome), severe idiopathic neutropenia, or cyclic

neutropenia has been well established.<sup>90–92</sup> In a large randomized clinical trial of patients with severe chronic neutropenia, the occurrence of fever, oropharyngeal ulcers, infections, hospitalizations, and antibiotic use were all significantly reduced with filgrastim treatment. The quality of life and activity profiles of patients also improved. The only complication of therapy was the increased risk of developing myelodysplasia and acute leukemia in patients with severe congenital neutropenia.<sup>93,94</sup> This risk appears to be disease specific with substantially lower or no risk for patients with cyclic or idiopathic neutropenia. Those with severe congenital neutropenia need to be monitored with regular white counts, clinical observation, and possibly bone marrow examination.

### Treatment of Hematopoietic Subsyndrome of Acute Radiation Syndrome

Acute exposure to high levels of radiation leads to significant myelosuppression for which there is no standard current therapy. Studies in mice and nonhuman primates have shown that filgrastim can reduce the extensive myelosuppression and improve survival.<sup>95–99</sup> Given the ethical constraints of doing such studies in humans, approval for the use of filgrastim and pegfilgrastim (but not their biosimilar variants) was granted based upon nonhuman primate studies and bioequivalence modeling studies in humans.<sup>100</sup> These studies showed that for adult and pediatric populations exposed to a wide range of radiation doses, 10 mcg/kg filgrastim initiated 1–10 days after exposure and given daily for up to three weeks provided a 50% increase in survival.

### Implications for transfusion medicine

The use of myeloid growth factors to mobilize peripheral blood progenitor cells is a standard transfusion medicine practice. They can also be used to ameliorate drug-induced neutropenia such as that occurring after the administration of IVIG. Widespread use of myeloid growth factors in oncology may reduce the need for hospitalization and antibiotics. Unfortunately, their use to provide neutrophils for transfusion has not met with much success. Indeed, a recent heroic effort was unable to show a significant effect of neutrophil transfusion on the outcomes of infected, neutropenic patients.<sup>66</sup>

### Thrombopoietic growth factors

Although James Homer Wright described how bone marrow megakaryocytes produced platelets in 1902,<sup>101</sup> it was not until 1958 that Kelemen proposed that a “thrombopoietin” regulated this process.<sup>102</sup> This last of the major hematopoietic growth factors was not purified until 1994.<sup>103</sup> Initial clinical studies with two types of recombinant thrombopoietin (recombinant human TPO [rhTPO] and pegylated human megakaryocyte growth and development factor [PEG-rhMGDF]) showed they were quite effective in raising the platelet count in healthy subjects<sup>104</sup> and increasing platelet apheresis yields.<sup>105,106</sup> They raised the platelet count nadirs and decreased platelet transfusions after nonmyeloablative chemotherapy, but had minimal effect on the recovery of platelets to 20,000 or need for platelet transfusions in patients undergoing leukemia induction chemotherapy or stem cell transplantation.<sup>104</sup> There was a modest platelet count increase when given to MDS patients. Unfortunately, these studies were terminated in most countries when antibodies developed against one of these recombinant molecules (PEG-rhMGDF) that crossreacted with native thrombopoietin and produced thrombocytopenia.<sup>107,108</sup> rhTPO has been developed and is approved in China for the treatment of ITP and chemotherapy-induced thrombocytopenia.

Subsequently, a number of thrombopoietin growth factors, now referred to as thrombopoietin receptor agonists (TPO-RA), have been developed that are nonantigenic, strongly stimulate platelet production, and some are orally active.<sup>109</sup> Romiplostim is a recombinant protein TPO-RA while eltrombopag, avatrombopag, lusutrombopag, and hetrombopag are all orally available small molecule TPO-RA.

### Thrombopoietin structure, function, and physiology

Thrombopoietin (TPO) is encoded by a single gene (3q27.1) that produces a 332 amino acid (MW = 95,000 Da) glycoprotein of which the first 153 amino acids are 23% homologous with EPO, and probably 50% similar if conservative amino acid substitutions are considered.<sup>110</sup> This region also contains four cysteine residues like those in EPO and is responsible for binding to the TPO receptor. But despite these similarities, TPO does not bind the EPO receptor and EPO does not bind the TPO receptor. The rest of the molecule is rich in carbohydrates which increase its half-life

Thrombopoietin is the key regulator of platelet production. In mice deficient in both genes for either TPO or the TPO receptor, the platelet count, bone marrow megakaryocytes, and megakaryocyte precursors are 10–15% of normal.<sup>111</sup> These animals also have reduced levels of erythroid and myeloid precursors but with no anemia or neutropenia.<sup>112</sup>

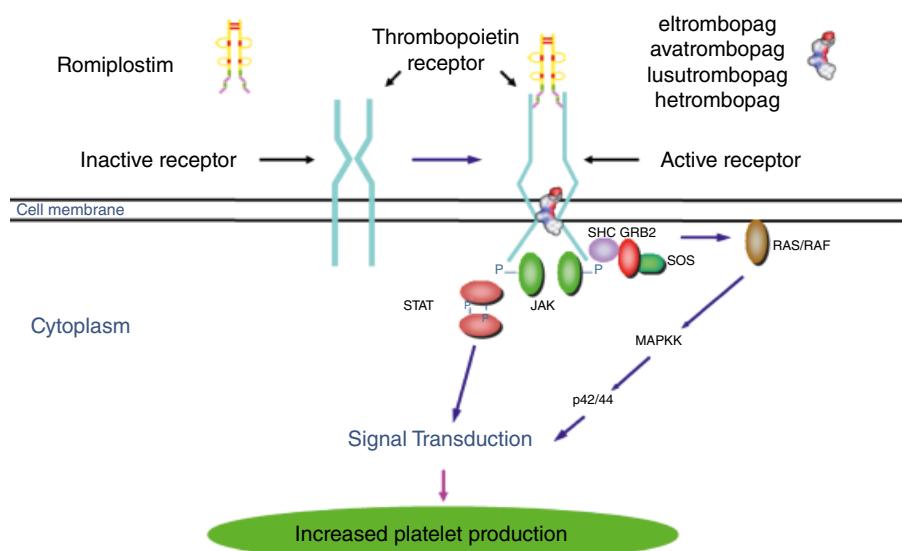
Thrombopoietin is primarily produced in the liver at a constant basal rate. Multiple studies of thrombocytopenic animals with ITP or after chemotherapy have shown no increase in the hepatic TPO mRNA levels.<sup>113</sup> Thrombopoietin levels decrease in liver disease and return to normal upon liver transplantation.<sup>11</sup> Only interleukin-6 (IL-6) seems capable of increasing basal thrombopoietin production.<sup>114</sup>

Thrombopoietin levels are inversely related to the rate of platelet production. Once in the circulation, TPO is bound to and cleared by high affinity platelet TPO receptors leaving a small basal amount of TPO.<sup>8</sup> This primitive normal feedback loop is shared with other hematopoietic growth factors such as M-CSF<sup>7</sup> and G-CSF;<sup>9</sup> there appears to be no specific sensor of the platelet count in the body. In aplastic anemia patients, TPO levels are >2000 pg/mL (normal: 7–99 pg/mL).<sup>115</sup> In animals or humans transfused to platelet counts above normal, TPO is suppressed below basal levels. In patients with chronic liver disease (CLD), TPO production is reduced.<sup>116</sup>

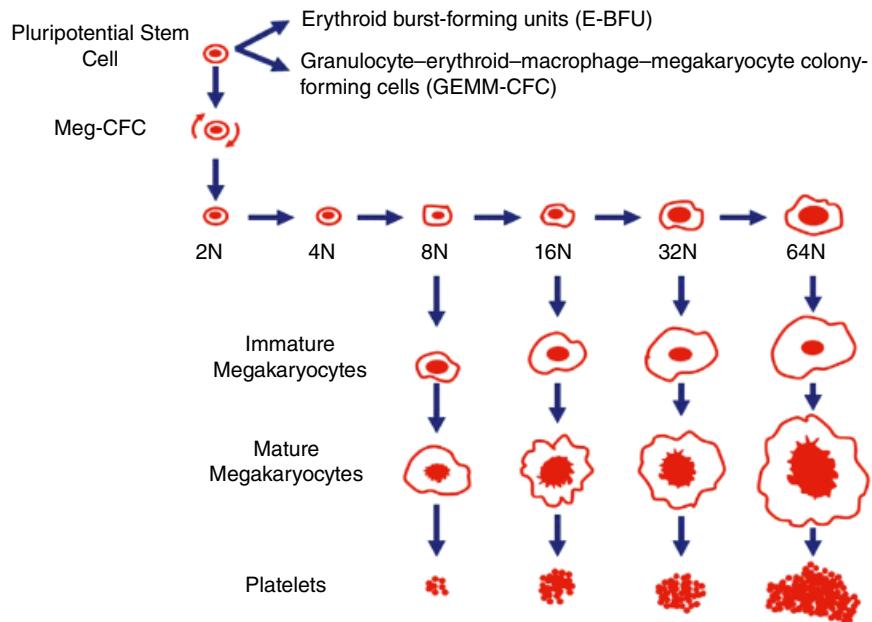
TPO has no carrier molecule and works by binding to TPO receptors on target hematopoietic cells leading to a number of downstream signal transduction events (Figure 52.6). TPO is necessary for the viability of stem cells; humans born without it are thrombocytopenic at birth and eventually become pancytopenic. TPO is necessary for the viability of precursors of all lineages (Figure 52.1) but only amplifies the megakaryocyte lineage by promoting the mitosis of megakaryocyte colony-forming cells, increasing the rate of megakaryocyte endomitosis and maturation, thereby increasing platelet production (Figure 52.7).

### Clinically available thrombopoietin receptor agonists Romiplostim (Nplate)

Romiplostim is a TPO receptor agonist composed of an IgG1 heavy chain carrier molecule into which four identical 14 amino acid peptides have been inserted which activate the TPO receptor.<sup>117</sup> This peptide (IEGPTLRLRWLAARA) has no sequence homology with TPO but was found to bind and activate the TPO receptor. If dimerized, it was as potent as thrombopoietin in vitro but given its short half-life had minimal activity in vivo. By inserting this peptide into



**Figure 52.6** Mechanism of action of the various TPO-RA on the thrombopoietin (TPO) receptor. The TPO-RA bind the TPO receptor and initiate a number of signal transduction pathways that stimulate megakaryocyte growth and increase platelet production. STAT: signal transducer and activator of transcription; JAK: Janus kinase; GRB2: growth factor receptor bound protein 2; SHC: Src homology 2 domain containing; SOS: Son of Sevenless kinase; RAF: rapidly accelerated fibrosarcoma kinase; RAS: rat sarcoma kinase; MAPK: mitogen-activated protein kinase. Figure courtesy of Prof. David Kuter.



**Figure 52.7** Scheme of megakaryocyte maturation from stem cell to mature platelet. Stem cells and megakaryocyte colony-forming cells (M-CFC) undergo mitosis, but at some stage they stop mitosis and undergo endomitosis during which nuclear and cytoplasmic division do not occur giving rise to polyploid early megakaryocytes that contain 2–8 times the normal diploid (2N) amount of DNA. The early megakaryocytes then stop endomitosis and mature into morphologically identifiable megakaryocytes and then shed platelets. TPO plays a major role in all but the last process. Figure courtesy of Prof. David Kuter.

the IgG carrier construct, romiplostim has a T<sub>1/2</sub> of ~120 h, three times longer than native TPO.<sup>118</sup> Although romiplostim binds to the TPO receptor with an affinity 25% of that of TPO, it is a very potent activator of platelet production. Romiplostim is available in vials containing 125, 250, or 500 mcg of lyophilized drug. It is administered as a subcutaneous injection of 1–10 mcg/kg once a week for the treatment of ITP. There is no effect upon white cell or red cell

production. Romiplostim is currently FDA approved for the treatment of thrombocytopenia in:

- Adult patients with ITP who have had an insufficient response to corticosteroids, immunoglobulins, or splenectomy.
- Pediatric patients one year of age and older with ITP for at least six months who have had an insufficient response to corticosteroids, immunoglobulins, or splenectomy.

In Japan, romiplostim is also approved for the treatment of aplastic anemia.<sup>119,120</sup>

### Eltrombopag (Promacta, Revolade)

Efforts to identify small molecules that bound and activated the TPO receptor successfully identified a number of compounds. Eltrombopag was the first of these compounds identified and subsequently modified to enhance its pharmacological and biological properties.<sup>121</sup> Eltrombopag binds the TPO receptor at a transmembrane site distant from where TPO binds (Figure 52.6). It thereby activates the TPO receptor differently than TPO or romiplostim: there is less phosphorylation of JAK and STAT and no effect upon the AKT pathway.<sup>122</sup> Nonetheless, eltrombopag increases megakaryocyte growth and maturation to increase platelet production.

Eltrombopag is available as 12.5, 25, 50, or 75 mg tablets and an oral suspension of 12.5 and 25 mg. Eltrombopag is primarily metabolized by the liver, and dose adjustments are necessary for patients with reduced metabolism of this drug due to East Asian ancestry or liver dysfunction. It is usually taken orally once a day and distant from calcium-containing compounds or food stuffs that would neutralize its activity if coadministered. Eltrombopag is currently FDA approved for:

- The treatment of thrombocytopenia in adult and pediatric patients one year and older with persistent or chronic ITP who have had an insufficient response to corticosteroids, immunoglobulins, or splenectomy. Eltrombopag should be used only in patients with ITP whose degree of thrombocytopenia and clinical condition increase the risk for bleeding.
- The treatment of thrombocytopenia in patients with chronic hepatitis C to allow the initiation and maintenance of interferon-based therapy. Eltrombopag should be used only in patients with chronic hepatitis C whose degree of thrombocytopenia prevents the initiation of interferon-based therapy or limits the ability to maintain interferon-based therapy.
- In combination with standard immunosuppressive therapy for the first-line treatment of adult and pediatric patients two years and older with severe aplastic anemia.
- For the treatment of patients with severe aplastic anemia who have had an insufficient response to immunosuppressive therapy.

### Avatrombopag (Doptelet)

Avatrombopag is structurally different from eltrombopag but also binds to the transmembrane region of the thrombopoietin receptor (Figure 52.6) which it activates in a similar manner. Like eltrombopag, it is orally available but differs in that it is better absorbed when administered with food, lacks any adverse effects on liver function tests, and does not chelate iron.<sup>123</sup> It is available in 20 mg tablets. For ITP, daily dosing starts at 20 mg/day and is subsequently altered based on platelet count with dosing ranging from 20 mg weekly to 40 mg daily. For CLD patients needing procedures the daily dose is based upon platelet count prior to procedure, given for 5 days beginning 10–13 days before procedure. For platelet count less than 40,000, the dose is 60 mg (three tablets) once daily; for platelet counts 40,000–<50,000 the dose is 40 mg (two tablets) once daily.

Avatrombopag is FDA approved for the treatment of:

- Thrombocytopenia in adult patients with chronic liver disease who are scheduled to undergo a procedure.
- Thrombocytopenia in adult patients with chronic immune thrombocytopenia who have had an insufficient response to a previous treatment.

### Lusutrombopag (Mulpleta)

Lusutrombopag is another orally available small molecule TPO-RA that binds the transmembrane region of the TPO receptor (Figure 52.6). It is available as a 3 mg tablet. For the treatment of CLD patients, a dose of 3 mg orally (with or without food) is administered daily for 7 days in a period 8–14 days prior to a scheduled procedure. It is currently FDA approved for:

- The treatment of thrombocytopenia in adult patients with chronic liver disease who are scheduled to undergo a procedure.

### Effects and adverse effects thrombopoietin administration

After giving a single dose of romiplostim<sup>118</sup> or avatrombopag<sup>123</sup> or 10 daily doses of eltrombopag<sup>124</sup> to healthy volunteers, platelet counts begin to rise by Day 5, peak at Days 10–15, and return to baseline by Day 28. These three TPO RA differ in their relative potency in healthy volunteers when tested at their maximal FDA-approved doses for ITP.<sup>125</sup> At 75 mg/d for 10 days, eltrombopag increased the platelet count by about 119,000 (1.4-fold) above baseline;<sup>126</sup> after a single 10 mcg/kg dose of romiplostim, the platelet count increased 1,300,000 (6-fold) over baseline;<sup>118</sup> and after 10–14 days of 20 mg avatrombopag, the platelet count rose by 372,000 (~3-fold) over baseline.<sup>123</sup> Nonetheless, these differences in potency have not translated into any different response rate for these agents in ITP clinical trials. With continued administration of either molecule, peak platelet counts can be maintained indefinitely with no effect on the RBC or WBC. The platelets so produced have normal function.

The TPO receptor agonists have not demonstrated any tachyphylaxis or significant long-term complications.<sup>127,128</sup> The potential and real adverse effects are listed in Table 52.7. A few deserve additional comment.

### Thrombosis

Although the TPO-RA can significantly increase the platelet count, thrombosis has not been clearly associated with TPO-RA in placebo-controlled ITP studies. Rather, what has been uncovered is that ITP itself is a prothrombotic disorder<sup>129</sup> whose rate of thrombosis does not appear to be exacerbated by TPO-RA. Compared with placebo treatment, thrombosis was not increased by recombinant TPO treatment in earlier trials in cancer patients. Platelet function studies have shown no increase in platelet activation with any TPO-RA<sup>130</sup> despite the finding that romiplostim (but not the small molecule TPO-RAs) reduces the activation threshold for other agonists by about 50%.

### Tumor progression/cancer mortality

Unlike the controversy over erythroid growth factors, solid tumor cells lack the TPO receptor.<sup>131</sup> Furthermore, in cancer chemotherapy studies with recombinant thrombopoietins in the 1990s, there was no effect on progression or survival for patients with myeloid leukemia, MDS, or solid tumors.<sup>104</sup> In the oncology studies so far completed

**Table 52.7** Adverse Effects of Thrombopoietic Growth Factors

Thrombocytosis	Autoantibody formation (romiplostim only)
Thrombosis	Liver function abnormalities (eltrombopag only)
Increased bone marrow reticulin	Reduction in threshold for platelet activation (romiplostim only)
Stimulation of leukemic blasts	Rebound worsening of thrombocytopenia upon discontinuation

with TPO-RAs, there was no apparent increased rate of progression of myeloid or nonmyeloid malignancies.<sup>132</sup> The one early exception was in one romiplostim clinical trial with MDS patients that was stopped due to monitoring committee concern about progression to leukemia; upon final analysis this had not occurred.<sup>133</sup>

### Antibody formation

Although antibody formation ended the development of PEG-rhMGDF,<sup>134</sup> no clinically relevant antibodies have developed against the TPO-RA. There have been no reports of any antibody formation against the small molecule TPO-RA. Antibody formation against romiplostim has been extensively analyzed.<sup>135</sup> Out of 961 patients in 13 clinical trials, 60 patients developed antibodies against romiplostim of which only four were neutralizing; none of these four patients lost their therapeutic effect and none cross-reacted with endogenous TPO.

### Hepatic toxicity

Eltrombopag is metabolized by the liver and in one ITP study 11% of patients developed abnormal liver function tests.<sup>136</sup> In general, these were mild, reversible, and did not often require cessation of medication. Intermittent monitoring of liver function tests is recommended for eltrombopag.

### Rebound thrombocytopenia

The major concern with TPO receptor agonists occurs in ITP patients in whom the drug is abruptly stopped. Current prescribing information is unhelpful and states that TPO-RA be stopped when the platelet count exceeds 400,000. Unfortunately in 10–20% of such situations, stopping the drug results in a rebound thrombocytopenia with the platelet count rapidly dropping below prior baseline values 7–10 days later and a markedly increased bleeding risk.<sup>109</sup> Such patients are better treated by a gradual dose reduction over several weeks.<sup>137–139</sup>

### Bone marrow reticulin and collagen fibrosis

Reticulin is a normal component of the bone marrow and may increase in patients with ITP<sup>140</sup> and in those receiving TPO receptor agonists.<sup>141</sup> This reticulin deposition is generally mild, reversible, clinically silent, and does not portend progression to the disease myelofibrosis. Prospective bone marrow studies of ITP patients receiving TPO receptor agonists have been performed. In one study, romiplostim-treated patients showed an ~5% incidence of increased bone marrow reticulin (reticulin stain) that was reversible upon discontinuation of the drug, was not associated with abnormal blood counts, and often did not require drug cessation.<sup>142</sup> Only two patients demonstrated increased collagen fibrosis (trichrome stain) that was reversible. In another two-year study with eltrombopag, normal (MF-0) reticulin staining was seen in 94% at baseline and 89% after two years. None had MF-2 or MF3. Collagen staining was rare, and none of the patients had blood count abnormalities.<sup>143</sup> Routine bone marrow assessment is not required for patients on TPO-RA.

### Clinical use of thrombopoietin receptor agonists

Table 52.8 provides a complete list of conditions that have been treated with TPO-RA.

### Immune thrombocytopenia (ITP)

ITP is a disease of increased platelet destruction and inappropriately low platelet production.<sup>144</sup> Although standard therapies such as immunosuppression, rituximab, and splenectomy decrease platelet destruction, TPO-RA increase platelet production thereby miti-

**Table 52.8** Clinical Uses of Thrombopoietic Growth Factors

Immune thrombocytopenia (ITP)—all stages	(Liver failure)
Hepatitis C-related thrombocytopenia being treated with interferon	(Myosin heavy chain 9-related disease)
Aplastic anemia	(Peripheral blood progenitor cell mobilization)
Thrombocytopenia of chronic liver disease patients needing procedure (ITP initial therapy)	(Stem cell transplant, failed engraftment)
(Chemotherapy-induced thrombocytopenia)	(Acute leukemia)
(Presurgical thrombocytopenia)	(Platelet apheresis)
	(Myelodysplastic syndromes)

Uses in parentheses denote lack of FDA approval but for which compendial data exist. See prescribing information for each individual thrombopoietic growth factor.

gating thrombocytopenia. All approved TPO-RA increase the platelet count >50,000 in more than 85% of patients accompanied by reduced bleeding and need for other therapies.<sup>136,145,146</sup> Long-term use of TPO-RA is successful<sup>127,128</sup> and may be associated with increased numbers of T regulatory lymphocytes with increased disease remission.<sup>147,148</sup> The TPO-RA have now been incorporated into most ITP guidelines as the major second-line therapy.<sup>149–152</sup> They are as effective in early ITP (months 0–12) as they are in chronic ITP (over 12 months).<sup>137,153</sup>

### Hepatitis C-related thrombocytopenia

Hepatitis C is associated with two forms of thrombocytopenia that may be difficult to distinguish in any one patient. One is an ITP-like condition that responds to ITP therapies; the second is TPO deficiency due to hepatic injury. Consequently, in patients with platelet counts <70,000, older forms of antiviral therapy with ribavirin and interferon may be contraindicated. When such patients with a mean baseline platelet count of 55,000 were treated with eltrombopag 75 mg/day for four weeks, the mean platelet count rose to 209,000 compared with 54,000 in those receiving placebo.<sup>154</sup> With continued eltrombopag support, 65% of patients could finish antiviral treatment compared with 6% receiving placebo. In a large phase III study of such hepatitis C patients with platelet counts under 100,000, 95% increased their platelet count to over 100,000 by week 9 and were then randomized to receive eltrombopag or placebo during the next 24–48 weeks of antiviral treatment.<sup>155</sup> Sustained virologic response was seen in 19–23% of eltrombopag patients compared with 13–15% on placebo ( $p < 0.02$ ) with more patients maintaining a platelet count >50,000 (69–81% vs. 15–23%, respectively). However, the newer treatments for hepatitis C have markedly reduced the need for this form of supportive care.

### Aplastic anemia

Although TPO levels are markedly elevated (>2000 pg/mL) in most aplastic anemia patients, eltrombopag produced a platelet count raise in 9/25 patients with a trilineage response in 6/25 patients.<sup>156</sup> In responders, marrow cellularity increased and response was maintained for long term with some coming off treatment.<sup>157</sup> Subsequent studies assessed the addition of various regimens of eltrombopag to antithymocyte globulin in the initial treatment of patients with severe aplastic anemia.<sup>158</sup> At six months, 26–50% of the different eltrombopag groups had a complete response compared with a historical cohort in which the rate was 10%; overall response rates at six months ranged from 80% to 94% compared to 66% in historical cohorts. The rate of clonal evolution was similar to

the historical cohort. These striking results were not seen in prior studies with recombinant TPO<sup>104</sup> but studies with romiplostim reported comparable outcomes.<sup>159</sup> One mechanism for the success of eltrombopag might be that its binding to the TPO receptor may not be impaired by the high levels of interferon present in patients with aplastic anemia; it has been suggested that interferon- $\gamma$  forms a heterodimer with TPO and prevents it from binding to the receptor. Since eltrombopag binds to a different location on the TPO receptor and is not bound by interferon- $\gamma$ , it bypasses this inhibitory mechanism.<sup>160</sup>

### Presurgical treatment of thrombocytopenic patients

Thrombocytopenia complicates many illnesses, and such patients are often given aggressive platelet transfusions or even refused needed surgical procedures. Several studies have shown that romiplostim and eltrombopag can increase platelet counts in such patients, reduce bleeding, and allow procedures to be performed. In one study of 51 thrombocytopenic patients treated with romiplostim, all were able to raise their platelet count (from a median of  $47 \times 10^9/L$  at baseline to  $164 \times 10^9/L$  at the time of surgery) and undergo surgery with no excessive bleeding and minimal need for transfusion.<sup>161,162</sup> In a second study of thrombocytopenic cirrhotic patients being prepped for liver biopsy, platelet transfusion was avoided in 72% of patients receiving eltrombopag compared with 19% of those receiving placebo ( $p < 0.001$ ); there was no difference in bleeding (17% vs. 19%, respectively).<sup>163</sup> However, this study was terminated early because 6/145 eltrombopag patients developed portal vein thrombosis versus 1/147 on placebo. The study was weakened by the absence of standardized assessment for portal vein clot. In the only randomized, prospective study of TPO-RA in the treatment of thrombocytopenic ITP patients undergoing surgery, eltrombopag was felt to be comparable to IVIG.<sup>164,165</sup>

### Chemotherapy-induced thrombocytopenia

Chemotherapy-induced thrombocytopenia (CIT) remains a major challenge in identifying a strategy for both studying this patient population and crafting an effective therapy. Early studies in patients receiving nonmyeloablative chemotherapy for ovarian cancer<sup>166</sup> or lung cancer<sup>167</sup> showed that treatment with the first generation of thrombopoietin growth factors, rhTPO and PEG-rhMGDF, increased the nadir platelet count, decreased the need for platelet transfusions, and allowed for chemotherapy to be given on time.<sup>104</sup> However, neither recombinant TPO showed benefit in patients receiving myeloablative chemotherapy for acute leukemia induction or for stem cell transplantation.

In a very large retrospective study of 173 patients receiving chemotherapy for solid tumors ( $n = 153$ ) and lymphoma/myeloma ( $n = 20$ ), romiplostim was highly effective: 71% had a response, 79% avoid chemotherapy dose reduction/treatment delays, and 89% avoided platelet transfusions. Median platelet counts rose from  $60 \times 10^9/L$  to  $116 \times 10^9/L$ , and treatment was most effective in those who lacked bone marrow tumor invasion, prior pelvic radiation, prior temozolamide, or hematologic malignancy.<sup>168</sup> In a small randomized, prospective trial of chemotherapy patients with platelet counts under 100,000 for at least four weeks, 93% of patients received romiplostim but only 12.5% of control patients restored their platelet count to over 100,000 within three weeks. Continuation of romiplostim allowed maintenance of adequate dose intensity with subsequent chemotherapy.<sup>132</sup> Current NCCN guidelines for hematopoietic growth factors list TPO-RA as alternatives to platelet transfusions during the COVID-19 pandemic.<sup>169</sup>

### Myelodysplastic syndrome (MDS)

Thrombocytopenia commonly complicates the care of patients with MDS. In a study of thrombocytopenic (<20,000 or history of bleeding and platelets  $\geq 20,000$ ) low-risk/intermediate-1-risk MDS patients, romiplostim or placebo were administered for 58 weeks.<sup>133</sup> Overall, clinically important bleeding events were not significantly reduced with romiplostim (HR 0.83; 95% CI, 0.66–1.05;  $p = 0.13$ ), but in those with platelet counts  $\geq 20,000$  significant reductions were seen (HR, 0.34; 95% CI, 0.20–0.58;  $p < 0.0001$ ). Romiplostim reduced bleeding events (RR, 0.92) and platelet transfusions (RR, 0.77) and increased platelet response (OR, 15.6). This study was stopped at an interim analysis because of a perceived increase in the AML rate (HR, 2.51) with romiplostim, but final analysis showed AML rates of 6% with romiplostim and 4.9% placebo (HR, 1.20; 95% CI, 0.38–3.84) and similar survival rates. Current clinical practice is supportive of using TPO-RA to treat thrombocytopenia and bleeding in MDS patients who are not adequately controlled by platelet transfusions.

### Implications for transfusion medicine

Since 2008, the TPO-RA have played an increasing role in the treatment of a number of thrombocytopenic disorders. TPO receptor agonists clearly decrease the use of IVIG and platelet transfusions in ITP patients.<sup>145</sup> They can also reduce or eliminate the need for transfusions in thrombocytopenic patients undergoing major surgery. They have helped a number of Jehovah's Witness patients or those with severe platelet alloimmunization to undergo surgery without platelet transfusion.<sup>161,162</sup>

The bigger issue is whether TPO-RA can decrease the thrombocytopenia associated with nonmyeloablative chemotherapy and reduce the need for platelet transfusions. Early data showed that rhTPO reduced the rate of platelet transfusion from 75% to 25% in women being treated for ovarian cancer.<sup>166</sup> It is possible that studies with the current TPO-RA will show similar benefit in nonmyeloablative chemotherapy.<sup>170</sup> A number of CIT studies are currently underway and may clarify the benefit of TPO-RA treatment.

Patients undergoing myeloablative chemotherapy for the treatment of leukemia or for stem cell transplantation often require extensive platelet transfusion support. Early studies with recombinant thrombopoietin agents showed no significant reduction in platelet transfusions or time to platelet recovery. The underlying biology of bone marrow recovery may limit the potential benefit of TPO-RA in this patient population.<sup>171,172</sup>

Whether TPO receptor agonists will ever play a role in enhancing platelet apheresis yields remains unclear. Prior studies showed that PEG-rhMGDF increased the median platelet count from 248,000 to 602,000 with an increase in median (range) apheresis yield from  $3.8 \times 10^{11}$  ( $1.3 \times 10^{11}$  to  $7.9 \times 10^{11}$ ) to  $11.0 \times 10^{11}$  ( $7.1 \times 10^{11}$  to  $18.3 \times 10^{11}$ ) platelets.<sup>105</sup> Once transfused, these products were hemostatically active and provided a dose-dependent rise in platelet count and increased transfusion-free interval.<sup>173</sup> rhTPO-mobilized platelets have also been frozen and later transfused with benefit into alloimmunized chemotherapy patients.<sup>106</sup>

TPO may be helpful in improving hematopoietic recovery after radiation injury.<sup>174–177</sup> Early intervention mitigates multilineage suppression and improves survival after radiation of nonhuman primates;<sup>178</sup> cross-species modeling studies might suggest human benefit as was done for filgrastim and pegfilgrastim in this setting.<sup>100</sup>

### Development of newer TPO receptor agonists

Hetrombopag (5-[2-hydroxy-3-[[5-methyl-3-oxo-2-(5,6,7,8-tetrahydronaphthalen-2-yl)-1H-pyrazol-4-yl]diazenyl]phenyl]furan-2-

carboxylic acid) is another oral TPO-RA small molecule (580.6 Da) that is being developed in China for ITP and chemotherapy-induced thrombocytopenia. Like the other small molecule TPO-RAs, it too binds to the transmembrane region of the TPO receptor (Figure 52.6). In some in vitro experiments, it has been shown to be 10-fold more potent than eltrombopag.<sup>179–181</sup> In a large phase III trial, 424 ITP patients (with platelet counts <30,000) were randomized to two different doses of hetrombopag or placebo for 10 weeks.<sup>182</sup> After eight weeks, platelet counts >50,000 were seen in 58.9% and 64.3% of those receiving either 2.5 or 5.0 mg daily, respectively, versus 5.9% in the placebo group. Bleeding and rescue medication use was reduced in those on hetrombopag. No new safety signals were observed. There were no adverse effects on liver function tests.

## General conclusions

The hematopoietic growth factors have markedly impacted the practice of medicine over the past 30 years. Beginning with erythropoietin and then extended with myeloid growth factors and now with thrombopoietic growth factors, there is an ability to increase specific blood cell production in patients who have anemia, neutropenia, or thrombocytopenia. These molecules are generally safe, effective, and with adequate time will raise the blood count. Unfortunately, none is immediately active and most take days for the onset of activity. None of these will replace the need for transfusion in acute situations of anemia or thrombocytopenia. However, all three offer the opportunity to prevent subsequent RBC transfusion, infection, or bleeding. Although expensive, in some settings they have been demonstrated to reduce medical care costs.

## Acknowledgments

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## CHAPTER 53

# Hematopoietic stems cells and transplantation

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### Stem cells

The human being is composed of trillions of native cells, the majority of which resides in the vascular space.<sup>1</sup> Despite stem cells representing just 1 in every 10–20,000 cells in the bone marrow (Figure 53.1), they possess a remarkable ability to both differentiate into the varying cellular components of blood, but also self-replicate to sustain a lifetime of hematopoiesis (Table 53.1).

Hematopoietic stem cells (HSCs) and hematopoietic progenitor cells (HPCs) have been utilized in the treatment of a range of diseases, both for neoplastic conditions, as well as non-neoplastic disorders, with a diverse set of reported outcomes. Since the 1950s, with the initial successful transplantation of bone marrow,<sup>4</sup> health-care providers have expanded the use of HSCs for the treatment of three broad categories.

Allogeneic stem cell transplant allows for reconstitution of hematopoiesis in patients with either a genetic disorder affecting blood production/function or for the treatment of a bone marrow failure syndromes. Additionally, allogeneic or autologous stem cell products can be used following irradiation and/or systemic chemotherapy to afford the patient the opportunity for bone marrow reconstitution following eradication of the malignant process. Lastly, genetic manipulation of HSCs can be undertaken to insert normal genomic material into defective stem cells with a primary goal of establishing sufficient levels of the gene product to result in clinical improvement.

### Hematopoietic stem cells

For clinical use, there are three primary sources of hematopoietic stem cells (HSCs): bone marrow harvested by aspiration from the cavity of the ilium (pelvis), peripheral blood obtained through leukapheresis, and umbilical cord blood (UCB) collected from the placenta after childbirth (Table 53.2).<sup>5</sup>

Transplant providers must balance the allogeneic risk of graft-versus-host disease (GVHD) and graft-versus-tumor effect that can impact both patient satisfaction and clinical outcome.<sup>6–8</sup> Transplant providers seek to control the progression of the malignancy early following stem cell transplant while preventing GVHD. If no viable donor can be identified for either marrow harvest or peripheral

blood stem cell collection, providers may also be confronted with the prospect of utilizing umbilical cord blood (UCB) as a stem cell source.<sup>9</sup>

Umbilical cord blood (UCB) stem cells have been successfully used for hematopoietic cell transplant (HCT) since the first report in 1989 and to date have been successfully utilized in over 40,000 transplants worldwide.<sup>10–12</sup> The biologic properties of UCB units can be safely cryopreserved for more than 20 years under appropriate conditions, with efficient recovery of functional HSCs and HPCs.<sup>13</sup>

### Hematopoietic microenvironment

The bone marrow microenvironment is composed of adipose, hematopoietic cells, and vascular sinuses.

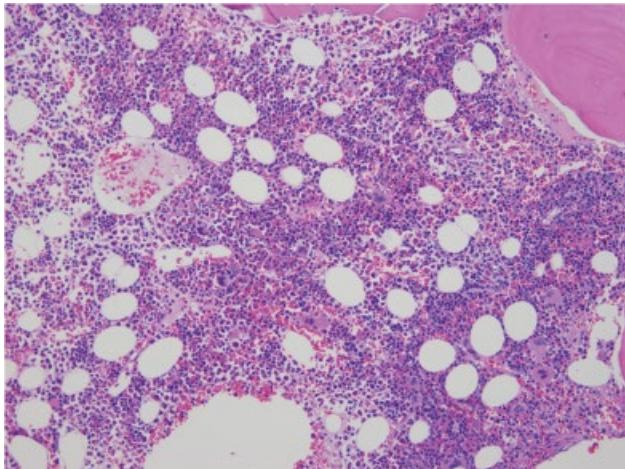
Bones such as vertebrae, sternum, pelvic bones, and metaphyses of long bones remain active sites of hematopoiesis throughout life, but longer weight bearing bones, facial bones, and distal upper extremity bones are slowly switched off and the marrow cavity is replaced by fat.<sup>14,15</sup> The central cavity of the active hematopoietic sites consists of hematopoietic tissue islands and adipose cells surrounded by vascular sinuses interspersed within a meshwork of trabecular bone, collectively forming the bone marrow niche.<sup>16</sup>

The bone marrow niche is not singular in nature as various microenvironments rely on differing endocrine, paracrine, and direct cell-to-cell interactions for progression of maturation. The arrangement of HSCs is in a nonrandom nature, locating close to endosteal bone allowing for differentiated cells to slowly migrate to central bone marrow regions around the perivascular compartment.<sup>17,18</sup> The current understanding of early multipotent stem cells indicates that in the steady state, stem cells are found as single cells adjacent to perivascular cells and within 30 µm from sinusoids.<sup>19</sup> The primary contributor of the endosteal niche is the osteoblast, which secretes multiple cytokines and growth factors that directly regulate HSCs.

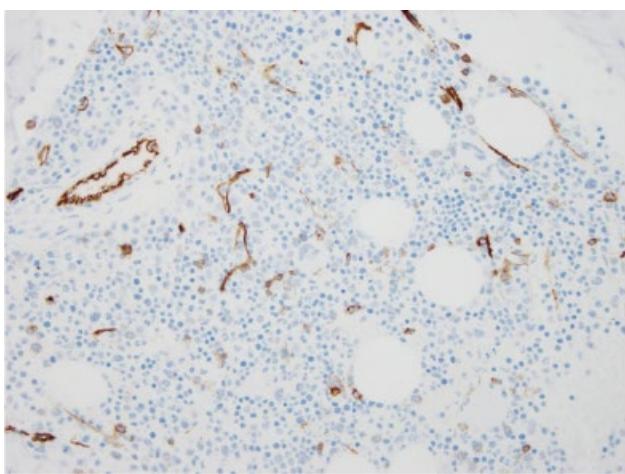
Osteoblast production of CXC-chemokine ligand 12 (CXCL12), stem-cell factor (SCF), osteopontin (OPN), granulocyte colony-stimulating factor (G-CSF), annexin 2 (ANXA2), angiopoietin 1 (ANG1), or thrombopoietin (TPO) allow for the control of homing, mobilization, and quiescence of HSCs.<sup>20–23</sup>

In contrast to the bone forming osteoblasts, osteoclasts are responsible for bone digestion. Mouse models lacking osteoclasts

(A)



(B)



**Figure 53.1** (A) Bone marrow biopsy demonstrating normal trilineage hematopoiesis (hematoxylin and eosin stain at 40 $\times$  magnification; photo credit Garrett Booth). (B) Immunohistochemical stain for CD34+ shows membranous staining of adult hematopoietic stem cells with normal vascular endothelium as a positive internal control (CD34+ IHC stain at 40 $\times$  magnification; photo credit Garrett Booth).

**Table 53.1** Immunophenotype of Hematopoiesis<sup>1–3</sup>

HSC	CD34+, CD38+
	Thy-1 (CD90)+ Lin-, CD45RA-, Rho low
<b>Lymph Precursors</b>	
Common lymph precursor	IL7R+/Lin /Thy /Sca <sup>lo</sup> /kit <sup>lo</sup>
Bipotent progenitor	CD3+/Thy <sup>+</sup>
B cell progenitors	CD3 /NK1.1 <sup>+</sup>
ProB cell	CD34+/CD10+/CD38+/CD19+/CD20+
PreB cell	CD34 /CD10+/CD38 /CD19+/CD20+
Immature B cells	CD10+/CD19+/CD20+
<b>Myeloid Precursors</b>	
<b>Erythroid Precursors</b>	
Proerythroblasts and mature RBCs	CD45-/GPA-/Band 3+/CD49d-
All lineages	IL7Ra/Lin /cKit+/Sca1 /CD34+/FcR $\gamma$ <sup>lo</sup> CFU-MIX, BFU-E, CFU-Meg, MEP, CFU-GM, CFU-G, CFU-M IL7Ra /Lin /cKit+/Sca-1 /CD34 /FcR $\gamma$ <sup>lo</sup>

**Table 53.2** Sources and Cellular Characteristics of Stem Cells

	Bone Marrow	Peripheral Blood	Umbilical Cord Blood
Stem cell content	Adequate	Good	Low
Progenitor cell content	Adequate	High	Low
T-cell content	Low	High	Low
GVHD	Low	Moderate–high	Low

show reduced bone marrow activity,<sup>24,25</sup> but the overall role in human bone marrow activity remains elusive. Observations of osteoclast poor bone marrow impairing adequate stem cell mobilization have been reported, but conflicting studies have shown the opposite.<sup>26,27</sup>

### Different cells in BM niche

Over decades in axial bones, hematopoietic sites are progressively replaced by fatty yellow marrow with reduced hematopoietic activity.<sup>28</sup> Mice with abundant fat stores within the bone marrow appear to contain fewer HSCs; however, newer medications that expand adipocyte stores within the marrow (triglitazone) demonstrate that adipose has virtually no impact in bone marrow regulation.<sup>29,30</sup>

The sympathetic nervous system regulates the daily circadian rhythm of HSC mobilization through the  $\beta 3$  adrenergic receptor, downregulating CXCL12 expression by stromal cells and assisting in hematopoiesis recovery following chemotherapy.<sup>31,32</sup> Adrenergic cellular signaling between neural cells can influence early progenitor cells with corresponding receptors, allowing for migration and engraftment.<sup>33</sup> Additionally, aberrations of the neural network within the bone marrow via intragenic ablation from chemotherapy or irradiation has also been shown to influence the bone marrow microenvironment by anomalous expansion of mesenchymal cells resulting in bone marrow interference.<sup>32,34</sup>

Megakaryocytes ensure hematopoietic regulation by secreting CXCL4, TGF Beta, and CLEC2.<sup>35–37</sup> These signals and their associated feedback loops have been shown to regulate bone marrow quiescence.<sup>35,37</sup>

Bone marrow CD169+ macrophages have been identified as a regulator of bone marrow HSC migration owing to modulation of osteoblast and osteoclast activity.<sup>38,39</sup> A small subset of bone marrow macrophages have been identified as expressing the Duffy antigen receptor for chemokines (DARC) that may also enable bone marrow quiescence regulation.<sup>40</sup>

The discovery and description of neutrophils dates back to the 1800s with the Nobel Prize winning work of Metchnikoff.<sup>41</sup> Although not thought to be the primary driver of maturation of immature HSCs to differentiated blood elements, the daily steady-state production of  $\sim 1 \times 10^{10}$  neutrophils suggests that the sheer mass of neutrophils could influence the background inflammatory state and circadian rhythm of the bone marrow microenvironment.<sup>42,43</sup> In emergency situations, the production of reactive oxygen species from neutrophils can stimulate hematopoiesis.<sup>44</sup> Bone marrow neutrophils can also assist in the regeneration of sinusoid following transplantation, enabling re-establishment of the vascular framework needed for the maintenance of marrow activities.<sup>45,46</sup>

### Circulation and vasculature

The bone marrow niche is enabled by the vast network of blood vessels and their endothelial cells. These cells serve to transfer oxygen, as well as cell-to-cell signaling for tissue maintenance and

maturity. The overall flow of blood inside the bone marrow is from a small artery entering from cortical bone to branching thin-walled arterioles traveling close to the endosteum, giving way to the venous sinusoidal network that ultimately drains into a central vein.<sup>47,48</sup> Both endothelial cells and perivascular stromal cells provide abundant cellular signaling within the bone marrow niche, including CXCL12 and SCF.<sup>49,50</sup>

The current model of definitive hematopoiesis relies on the idea of two functionally different HSC populations: the long-term HSCs that give rise to new or additional HSC and the short-term HSCs. Long-term HSCs have lifelong self-renewing potential, whereas the short-term HSCs—that show more restricted self-renewing capacity—producing common myeloid progenitors and common lymphoid progenitors.<sup>51</sup>

Successful hematopoietic recovery following stem cell transplantation depends on efficient homing and subsequent engraftment of HSCs into specific bone marrow niches. Various adhesion molecules, chemokines, glycoproteins, and integrins—present both on the surface of stem cells and sinusoidal endothelium—play critical roles in transvascular migration.<sup>52</sup>

Integrins are heterodimeric transmembrane molecules consisting of  $\alpha$  and  $\beta$  subunits that are expressed on the surface of HSCs.<sup>53</sup> Together with other intercellular adhesion molecule-1/leukocyte function-associated antigen-1, vascular cell adhesion molecule-1/very late antigen-4, and few junctional adhesion molecules, stem cells remain closely adherent to endosteal bone and perivascular regions.

### Mobilization of HSCs

G-CSF is a recombinant hematopoietic growth factor that is produced by multiple bone marrow cells resulting in decreases in SDF-1 production and degradation of the SDF-1 molecule.<sup>54</sup> G-CSF is a cytokine that promotes granulocyte proliferation and differentiation and enhances mobilization through direct and indirect protease activation.<sup>55</sup> Neutrophils releases cathepsin, elastase, and matrix metalloproteinases, and peptidases. These molecules enable cleavage of the adhesion molecules on HPCs; in addition, G-CSF indirectly inhibits osteoblast activity and reduces SDF-1 expression that enhances HPC mobilization.<sup>56</sup> Further mobilization pathways following G-CSF administration include disruption of VCAM-1/VLA-4 interaction and cleavage of CXCR4 and CD44 on HSCs. Following these changes, HSCs are more prone to release from the periosteum, and subsequent migration to the peripheral blood where they may be collected by apheresis.

Administration of G-CSF is utilized in both autologous and allogeneic mobilization, resulting in high cell doses collected enabling downstream increases in engraftment rates; however, the exact mobilization regimen for autologous and allogeneic donations are variable.

G-CSF-mobilized peripheral blood stem cells are the preferred graft source for virtually all autologous and an increasing majority of allogeneic HSCs owing to its generally higher stem cell content, reduced rates of graft failure, and better overall survival as compared to the BM.<sup>57,58</sup> After 4–6 days of subcutaneous administration of G-CSF, HSCs can increase nearly 100 fold,<sup>59</sup> allowing for safe mobilization schemes in both adult and pediatric patient populations.<sup>60,61</sup> Despite well-established safety guidelines for the use of G-CSF, several limitations due exist, namely, the slow onset of effect (generally 4–5 days of therapy are needed before a donor can proceed with collection), bone pain, and mobilization failure.<sup>62–64</sup> Adverse effects of G-CSF are generally well tolerated, but caution

should be exercised in donors with known coronary artery disease or sickle cell disease, given reports of ischemic complications or provocation of a sickle cell crisis, respectively.<sup>65–67</sup> Enlargement of the spleen by 10–15% has also been shown to occur with G-CSF injections with rare but potentially fatal splenic rupture occurring in a single case.<sup>68</sup>

Optimal dosing can be influenced by donor and recipient body weight, and does not appear to be influenced by location of administration.<sup>69,70</sup> Dose escalation alone cannot overcome poor mobilization. In healthy allogeneic donors, a typical five-day course of G-CSF raises the WBC count to about  $40 \times 10^9/L$ , although rare reports of WBC expansion to over  $100 \times 10^9/L$  have been reported.<sup>71</sup> The rise in WBC mirrors an increase in CD34+ expansion, with allogeneic donors typically showing a 50–100-fold increase.<sup>72</sup>

Recently, biosimilar versions of filgrastim have been approved by the US FDA as alternate versions of biologic products that have demonstrated the same clinical safety and efficacy.<sup>73</sup>

Donor characteristics, including advanced age, diabetes mellitus, history of radiotherapy, and multiple cycles of chemotherapy, have all been identified as increasing the risk of low CD34+ mobilization.<sup>74–76</sup> Chemotherapeutic agents that are directly toxic to the bone marrow niche, including melphalan, fludarabine, or intensive regimes like hyper CVAD, can result in significant HSC damage and mobilization failure. Furthermore, the review of premobilization laboratory testing results can aid in the identification of donors with a reduced or low steady state of bone marrow production, including a below normal preprocedure platelet count.<sup>64</sup> For patients that fail to mobilize with G-CSF alone, newer mobilizing agents like plerixafor can be used in combination or alone (Table 53.3).

Plerixafor (AMD3100; Mozobil; Sanofi-Aventis, Paris, France) is the antagonist of CXCR4, preventing CXCR4 on HSCs from interacting with SDF-1 on bone marrow stroma.<sup>77,78</sup> AMD3100 administration not only blocks CXCR4, but also induces activation of MMP-9 and serine protease urokinase-type plasminogen activator.<sup>79</sup> Since 2008, plerixafor has been clinically approved by the US FDA to use alone or in combination with G-CSF to mobilize HSPC in heavily pretreated lymphoma and myeloma patients.<sup>80</sup> Although plerixafor is successful in increasing optimal CD34+ yield, decreasing mobilization failure, and reducing the number of apheresis sessions, its universal use is limited by its substantial cost.<sup>81</sup> Plerixafor is usually given as a single subcutaneous dose as part of a combined regimen in which G-CSF is given for five days, with plerixafor added in the evening on Day 4 or the morning on Day 5, followed by the apheresis collection.<sup>82</sup>

GM-CSF (sargramostim, Leukine, Bayer Healthcare Pharmaceuticals, Seattle, WA, USA) plays a limited role in HSC mobilization today owing to significantly fewer CD34+ cells than

**Table 53.3** Comparison Between HPC Mobilizing Agents

Mobilizing Agent		
	G-CSF	Plerixafor
Mechanism of action	Cleavage of adhesion molecules	CXCR4 antagonist
Half-life	3–5 hours	3–5 hours
Time to maximum mobilization	4–6 days	6–9 hours
Common side effects	Bone pain and headache	Diarrhea and edema at injection site
Dosing	10–15 mcg/kg/day	0.24 mg/kg/day

G-CSF in healthy subjects.<sup>83</sup> Moreover, in patients with NHL, mobilization with GM-CSF resulted in a lower CD34+ cell yield than mobilization with G-CSF.<sup>84</sup>

Chemotherapy-induced stem cell mobilization most commonly occurs with cyclophosphamide (CY), which induces the release of stress signals that cause inflammation, thereby activating the host immune system increasing HSC mobilization. The balance between toxicity and cell dose yields, along with logistical challenges in determining the ideal mobilization and collection day, have resulted in shifting utilization patterns for CY in the treatment of adults.<sup>85-87</sup>

The current modeling of donor stem cell mobilization utilizes five general strategies: G-CSF alone, G-CSF plus plerixafor, plexafor alone, chemotherapy alone, or chemotherapy plus G-CSF. These varying mobilization strategies have differing success and debate related to the cost-effectiveness of using newer more expensive mobilizing agents remains.<sup>88,89</sup> Plerixafor has gained considerable attention as a rescue agent for donors with suboptimal mobilization following G-CSF alone, and off-label use of plerixafor as a single mobilizing agent in allogeneic donors continues to be explored, especially in situations when a full five days of G-CSF is not practical.<sup>90-94</sup>

## Collection of HSCs for transplantation

### Bone marrow

Bone marrow harvests are invasive medical procedures and are commonly performed in an operating room under general anesthesia.

Specialized stainless-steel beveled needles are used to gain access to the marrow cavity, wherein the product can be safely siphoned off in heparinized syringes into a disposable collection bag. Large filters within the tubing sets help to reduce unwanted bone chips, fat, clots, and fibrin stands. The product is then drained to a collection bag wherein anticoagulant solutions are available or can be safely supplemented.<sup>95,96</sup>

Special consideration should be given to pediatric bone marrow donors as these patients may often be siblings of the intended recipient and the collection process is not devoid of risk or ethical entanglements.<sup>97</sup>

### Peripheral blood/PBSC collection

The acquisition of stem cells via peripheral blood collection is highly disparate when compared to bone marrow harvesting. Aggregate data from Center for International Blood and Marrow Transplant Research (CIBMTR) showed that almost all (>99%) of autologous HCT and 69% of allogenic HCT were supported by peripheral blood progenitor cells (<https://www.cibmtr.org>).

Due to the high flow rates of blood utilized during an apheresis procedure (some centers use flow rates in excess of 100 mL/min), adequate venous access must be maintained; this can be achieved via two large bore needles in peripheral veins most commonly in the antecubital fossa or placement of a central venous catheter (CVC). Given documented adverse reactions to in well donors, the use of CVC should be utilized sparingly in well donors.<sup>71,98</sup> Throughout the 3–6 hour duration of the apheresis collection, an anticoagulant must be used to prevent clotting in the device; this is most frequently achieved with the use of citrate. Given the known complication of hypocalcemia during the collection process, some centers elect to concurrently administer oral or intravenous calcium supplementation to prevent citrate toxicity.<sup>99</sup>

The apheresis collection of stem cells is accomplished by separating cells via centrifugation; however, this separation technique is

not perfect and can result in significant platelet reductions. Among unrelated donors, the average peripheral platelet count decreased 30% following apheresis collection, but thankfully the bleeding risk remains very low.<sup>98</sup> Review of hematologic indices is advisable given the concurrent loss in platelets secondary to the CD34+ collection.

In unrelated allogeneic stem cell donors who have received five days of mobilization with G-CSF, the average apheresis yield is  $3.5 \times 10^{10}$  MNCs, of which approximately 1% are CD34+ cells.<sup>100</sup> Autologous collections are more likely to require either consecutive day apheresis collections or infrequently the use of several collection time periods.<sup>101</sup> In aggregate, the stem cell collection efficiency achieved via apheresis ranges from 40% to 60%.<sup>102,103</sup> Comparisons of the two primary apheresis devices available, Spectra Optia (Terumo BCT) and Amicus (Fresenius Kabi), illustrate comparable cell yields and collection efficiencies.<sup>104</sup>

Comparison of bone marrow and PBSC has demonstrated higher rates of serious adverse events in peripheral blood stem cell donors.<sup>105</sup> Both BM and PB donation procedures may be burdened by the same psychologic effects, such as fatigue and reduced energy, after the procedure. More severe pain at the donation site, greater incidence of hemorrhage, anemia, and hypotension, and a tendency to have more days of restricted activity and hospitalization may occur related to BM donation, but for patient-related outcomes of overall or disease-free survival there is no discernable difference.<sup>106,107</sup> Peripheral blood stem cell products do however appear to confer a faster engraftment of neutrophils and platelets in both autologous and allogeneic hematopoietic transplants.<sup>108</sup>

### ABO and HSCs and antibodies

HLA and ABO antigens are independently inherited; thus, allogeneic hematopoietic stem cell transplantation is routinely (up to 50%) performed across the ABO blood group barrier.<sup>109</sup> Three groups of ABO mismatch can be distinguished in the course of a stem cell transplant: minor, major, and bidirectional ABO incompatibility.<sup>110</sup>

The discrepancies between ABO and HLA typing call for a sequential intervention beginning at the time of pretransplant evaluation and extending through post-transplant transfusion support management (Table 53.4).

In clinical situations that necessitate the use of stem cell donor that has a major or bidirectional ABO mismatch, providers should be aware of pure red blood cell aplasia (PRCA), which can be seen in 10% of ABO-incompatible stem cell transplants.<sup>114</sup> This immunologic insult from residual recipient plasma cells secreting offending isoantibodies, anti-A or anti-B, does not significantly impact overall survival<sup>115,116</sup> but can prolong the period of transfusion dependence. Indeed, hemolysis can be impressive in some cases and may lead to prolonged red blood cell transfusion dependence in the post-transplant period. In these (relatively) rare cases, serial assessment of incompatible isoantibody titer (usually anti-A) can be informative. Emerging evidence supports the use of daratumumab as a favorable treatment option for PRCA<sup>117,118</sup> but requires close communication with the blood bank prior to initiation of therapy to ensure adequate serologic testing has been completed.<sup>119</sup>

### Cryopreservation

HSCs, once harvested, are only viable for several hours to a few days, limiting their geographical reach. Freshly frozen cells can be transported from the site of processing to a clinical site, extending both the geographical reach of viable cells and the genetic diversity

**Table 53.4** ABO-Incompatible Stem Cell Transplant Interventions\*

	Clinical Intervention	Apheresis	Stem Cell Laboratory
Pretransplant evaluation	-Two independent ABO/Rh typing and antibody screen	-Consideration for predonation plasma exchange**	-Confirmation of ABO incompatibility status
Stem cell acquisition	-Communication with blood bank regarding specialty blood products needed, including matching for minor RBC antigens as appropriate	-Minimize incompatible RBC collection, adjust cell collection dose given product modification consideration	-Major mismatch: RBC depletion/ sedimentation of product -Minor mismatch: plasma depletion of product
Peri-Transplant Management	-Selection of blood products to ensure transplant compatibility -Major mismatch: monitor for acute RBC hemolysis -Minor mismatch: monitor for acute/chronic hemolysis and PLS	-Optimization of stem cell mobilizing agent for donors with sickle cell disease†	-Slow rate of infusion with concurrent monitoring for signs of hemolysis

\* Booth *et al.* (2013).<sup>111</sup>\*\* Sheppard *et al.* (2013).<sup>112</sup>† Boulad *et al* (2018).<sup>113</sup>

of cells available to patients. Despite these benefits, the cryopreservation of HSCs poses several challenges, most notably a decline in cell viability after freeze/thaw cycling and adverse reactions in recipients during infusion due to cryoprotectants used.

The predominant cryoprotectant for HSCs is dimethyl sulfoxide (DMSO), but owing to the adverse event rate seen at time of infusion, the dominant line of research in HSC cryopreservation focuses on reducing or removing DMSO from cryoprotectant solutions to allow for a safer infusion.

Cryopreservation of HSCs is generally carried out using slow cooling rates in specialized controlled rate freezers (approx. 1 °C/min), yet comparisons between slow cooling (2 °C/min) and rapid cooling (vitrification) of human UCB cells found that cell viability and CD34+ enumeration after rapid cooling was significantly higher than that after slow cooling. These results suggest that rapid cooling is a promising cryopreservation method for UCB.<sup>120</sup>

Delays in cryopreservation after collection may adversely affect cell viability,<sup>121</sup> but may be unavoidable if the collection site and cell processing laboratory are significantly geographically separated or constrained by staffing issues or if a logistical problem or weather event prevents timely delivery of the product to the laboratory. Avoiding room temperature storage and delayed cryopreservation provide better post-thaw potency in hematopoietic progenitor cell grafts.<sup>122</sup> Cell doses that are very high can lead the collected product to clump after thawing or rarely seizures.<sup>123</sup>

### Cord blood

Since 1989, umbilical cord blood (UCB) stem cells have been successfully used for hematopoietic stem cell transplantation.<sup>10</sup> To date, more than 40,000 umbilical cord blood stem cell transplants have been performed with overall comparable clinical outcomes when compared to bone marrow or peripheral blood stem cell transplants.<sup>11,124,125</sup> UCB offers a multitude of pragmatic advantages over bone marrow or peripheral blood stem cells.

Two main types of UCB banks exist: public and private (Table 53.5).

Collectively, there are several hundred thousand UCB products available worldwide.<sup>126</sup> Some hospitals have in-house processing capabilities, whereas others rely on collection at site of delivery followed by shipment to a remote site for processing.<sup>127</sup> Several states, including California and Connecticut, have adopted an extensive public cord blood banking practice.<sup>12,28,129</sup>

The advantages of public cord blood banks over private cord blood banks include no financial burden to the donor, mandatory

**Table 53.5** Comparison of Public and Private Cord Blood Banks

Cord Blood Bank		
	Public	Private
Donor	Altruistic donor	Paying families
Ownership	General public	Paying families
Recipient	Unrelated persons requiring stem cell transplant	Member of paying family
Financial burden	Taxpayer	Paying families

international standards, searchable registries of products, broader availability to society, and higher probability of utilization.<sup>130–132</sup> The disadvantages of public cord blood banks over private cord blood banks are high discard rate of products that fail strict collection and storage criteria and reliance of governmental or grant funding that can shift abruptly. Given the inherent uncertainty of use of privately banked UCB products and their associated financial costs, hybrid models that employ cost sharing and transition opportunities for products to reach at need patients have evolved.

If a donor is deemed to be initially acceptable, a complete medical history and laboratory screening can be undertaken. Maternal infectious disease testing for hepatitis B, hepatitis C, HIV-1 and HIV-2, HIV, cytomegalovirus, syphilis, and in some programs human T-lymphotropic virus (HTLV)-I/II, malaria, Chagas disease, and West Nile virus needs to be performed.<sup>133,134</sup> In some regions, consent is obtained after successful collection of umbilical cord blood to ensure adequate collection has been achieved, reducing the burden and costs associated with screening in the prenatal or delivery time period.<sup>135</sup>

Especially given the relative scarcity of non-Caucasian donors for unmatched stem cell transplants, there remains an ever-present need to utilize UCB for transplants.<sup>136</sup> Notwithstanding the needs to maintain sufficient numbers of cord blood products, further outreach and funding are essential to recruit, optimize, and sustain an adequate umbilical cord blood inventory for diverse patient population such as the United States.<sup>137</sup>

### Stem cell thawing

Cryopreserved HPCs are less prone to injury if thawed expeditiously; however, just as with freezing, the thawing process has critical impact on sample quality and downstream utility. It is advisable to perform the thawing process at the bedside with close collaboration between the cell processing laboratory staff and the clinical

staff to ensure proper processing and handoff for a safe infusion. If excess DMSO is to be avoided or if overall volume reduction is required, washing of cryopreserved products can be undertaken, although this is rarely done.<sup>138,139</sup>

A warm water bath (37 °C) is used to thaw frozen products (vials, bags, or other products). The products themselves should have overlapping bags to ensure for visual checks of leaks or bag tears. Bags should be thawed one at a time and immediately administered via center venous catheter or intravenous access site once the product has reached 15 °C. High volumes of platelets and plasma proteins can lead to clumping of HPCs following the thaw processes.<sup>140</sup>

### Stem cell infusion

Whether a patient receives an autologous peripheral blood stem cell product, a matched unrelated allogeneic hematopoietic stem cell product, bone marrow, or umbilical cord blood product, there will always be clinical and product factors to consider at time of infusion.

Adverse reactions to HSC infusion are common and range in severity from mild to potentially life-threatening.<sup>141</sup> Common reactions include gastrointestinal manifestations such as nausea or vomiting, flushing, fever, malodor, hypertension and cough and more rarely respiratory compromise, seizures, or cardiac arrest.<sup>142–144</sup>

Frozen HSCs/HPCs must be suspended in a cryoprotective agent, typically dimethyl sulfoxide (DMSO), which can lead to histamine release, and nervous system abnormalities.<sup>145</sup> Though infused products generally contain less than 15% DMSO, further efforts have been made in certain conditions to remove DMSO prior to infusion, though this manipulation must be balanced against potential for cell loss.

Even in the absence of DMSO, preserved HSC infusion products can contain lysed cells, debris, metabolites, and electrolytes which may induce symptoms associated with infusion.<sup>146</sup> Symptoms associated with infusion are likely multifactorial and related not only to DMSO or substances within the product but clinical circumstances in individual patients as well.

Numerous steps are taken to ensure sterility of HSCs, and regulations, specifically by the US FDA, require microbiological cultures be performed on HSCs, despite this, contamination rates have been reported to occur between 0.2% and 26.3%.<sup>9–11</sup> The suspected etiology of this microbiologic contamination may be secondary to collection or transfer bag leaks or microfractures, inadvertent collection of skin plugs and associated skin commensal contaminants, subclinical donor bacteremia, or other etiologies.<sup>147,148</sup> Common organisms are Gram-positive skin commensal bacteria or rarely Gram-negative or fungal pathogens.

Infusion of products containing known infectious pathogens (usually bacteria) is occasionally necessitated due to clinical circumstance surrounding individual patients. Patients generally receive products with positive cultures under preparative regimen schedules and with appropriate antimicrobial therapy show no significant difference in outcomes.<sup>149</sup> Patients with viral illnesses such as hepatitis B or C and HIV require monitoring as well as prophylactic therapy for optimal outcomes, likewise special considerations must be undertaken for processing and storage of these products.<sup>110,111,150</sup>

### Stem cell collection and transfusion considerations in the COVID-19 pandemic

This ongoing catastrophic pandemic event has resulted in significant barriers to the collection and transport of donor cells. In an early report, case fatality rates in nonsevere and severe

COVID-19 infections were 0.1% and 8.1%, respectively.<sup>151</sup> Additionally, ongoing immunosuppression, mucositis, malnutrition, and/or graft-versus-host disease may increase the risk of complications in HCT patients with COVID-19. As such, a comprehensive high frequency inspection and evaluation for individual patients, donor, staff members, and programs remains essential in the pandemic era.

From a programmatic perspective, there are a number of expert consensus opinions related to stem cell transplant programs responsibilities in the COVID-19 era.<sup>152</sup> For low-risk patients with a confirmed COVID-19 diagnosis, these patients should be deferred for three months; however, for high-risk patients, serial weekly PCR testing resulting until negative times three and asymptomatic is believed to be sufficient. For patients without a known diagnosis of COVID-19 but have symptoms of an upper respiratory infection consider PCR testing and deferral, as well as adherence to local guidelines. Individuals with close contacts to a confirmed COVID-19 should also be tested and potentially deferred based on clinical status.

For stem cell donors in the COVID-19 era, any confirmed diagnosis of COVID-19 should almost always result in exclusion from donation. Additionally, recent travel to high-risk areas or close contacts with confirmed COVID-19 cases can result in donor deferral for at least 28 days with close monitoring.<sup>153</sup>

The American Society for Transplantation and Cellular Therapy (ASTCT) and the National Marrow Donor Program/Be The Match (NMDP) have also issued recommendations that unrelated donor products should be delivered and cryopreserved at transplantation centers before the initiation of patient conditioning. The NMDP now requires that grafts be delivered and cryopreserved at the transplantation center before the initiation of a transplantation conditioning regimen for any patient scheduled for unrelated donor hematopoietic cell transplantation (HCT) in the absence of unique considerations.<sup>154,155</sup> The balance for flexibility and patient/donor safety remains a challenge for transplant program. Clinical vigilance surveilling stem cell transplants for any increase in early graft failure or delays in platelet or neutrophil engraftment with newly found cryopreserved allogeneic donor products is essential.<sup>156</sup>

Further, the maintenance of the blood supply necessary for transplant patients has been under significant strain.<sup>157</sup> Clinical team members should remain vigilant in their blood use and continue to transfuse only when necessary. Evidence-based guidelines for transfusion practices in the stem cell transplant population include recommendations from the AABB to restrict red cell transfusion practice with a hemoglobin threshold of 7 g/dL in a majority of patients.<sup>158</sup> Randomized controlled trials in oncology patients comparing liberal versus restricted transfusion strategies have identified that lower hemoglobin thresholds of 7–9 g/dL are no worse than liberal transfusion triggers of 10 g/dL or higher.<sup>159</sup>

Prophylactic transfusion of platelets should occur at a threshold of 10,000/µL for all hospitalized patients. The TOPPS trial demonstrated reduced bleeding with prophylactic transfusion in patients below this level and similar trial compared prophylactic transfusion at 10,000/µL versus platelet transfusion for symptomatic bleeding among acute myeloid leukemia and autologous stem cell transplant patients.<sup>160,161</sup>

Joint statements from the AABB, America's Blood Centers, and the American Red Cross underscore the importance of judicious use of these limited biologic products for transfusion, as well as encouraging the donation of blood if individuals are willing and able.<sup>162</sup> One area of ongoing debate is whether outpatient stem cell

recipients should have the same transfusion triggers as inpatients. Theoretically, inpatients can be more conservatively managed, given that they remain at the hospital and can be transfused if the need arises. Further study is needed in this area to optimally manage scarce resources.

## Regulation

The US Food and Drug Administration's Center for Biologics Evaluation and Research (CBER) is responsible for the regulation of cellular and tissue-based therapies. Additionally, the Center for Devices and Radiological Health (CDRH) is tasked with regulatory oversight of medical devices and safe-radiation-emitting products.

For any product that does not meet the FDA standards set forth under 21 CFR 12710(a), these products are regulated under the Federal Food, Drug, and Cosmetic Act (FDCA) and Section 351 of the PHS Act.

To comply with the myriad of legal requirements set forth by the US FDA, several national and international nonprofit organizations and professional bodies have developed comprehensive programs for stem cell transplant clinical programs, apheresis/bone marrow

collection facilities, and cellular therapy laboratories. The overarching goals of these organizations are to ensure maintenance of current good tissue practice, donor screening, and donor testing throughout the life of a cellular product from initiation of donor selection, processing, storage, release, and administration to the end patient.

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## CHAPTER 54

# HLA antigens, alleles, and antibodies

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In 1958, Professors Jean Dausset, Jon van Rood, and Rose Payne detected and reported on what were the first HLA antibodies.<sup>1–3</sup> Dausset's discovery was with serum from a patient who had received multiple blood transfusions, while van Rood and Payne's observations were in sera obtained from multiparous women. Originally thought to be autoantibodies responsible for nonhemolytic transfusion reactions, subsequent studies determined them to be alloantibodies<sup>6</sup> with their target antigens expressed on the cells of most tissues.<sup>4</sup> Due to inconsistent results with the assay used to identify HLA antibodies (leukoagglutination), further advances were understandably limited. This changed with the introduction of the microlymphocytotoxicity assay (also known as the complement-dependent cytotoxicity [CDC] assay) developed by Terasaki and McClelland.<sup>5,6</sup> The CDC assay became the gold standard for both HLA antibody testing and HLA antigen testing, remaining so until the worldwide adoption of solid-phase antibody detection assays and nucleic-acid-based technology to HLA antigens/alleles.<sup>7,8</sup>

In 1964, Dr. Bernard Amos organized the first International Histocompatibility where 23 pioneering investigators participated in a “wet workshop” to study HLA antigens and antibodies.<sup>9</sup> There have since been 16 additional workshops (Table 54.1), all designed to (1) confirm scientific observations; (2) develop unifying concepts regarding the major histocompatibility complex (MHC), and (3) update HLA nomenclature. In the third workshop (1967), it was established that HLA antigens all belonged to the same genetic system.<sup>10</sup> The term HL-A (a combination of the Hu leukocyte system and the LA system named by Dausset *et al.*<sup>11</sup> and Payne *et al.*,<sup>12</sup> respectively) was assigned to identify this system. The “-” was dropped when it became apparent that there were at least two genes (HLA-A and HLA-B) that encoded the antigens.<sup>13</sup> HLA-C antigens and antibodies were then identified.<sup>14</sup> Skin grafting studies in human subjects involving HLA-identical siblings then helped determine that these antigens were important in organ transplantation.<sup>15</sup> An assay known as the mixed lymphocyte culture identified a fourth HLA region (HLA-D),<sup>16,17</sup> which is now known to be composed of three separate loci (HLA-DR, HLA-DQ, and HLA-DP).<sup>18</sup> Other aspects of the HLA system were also recognized. By 1973, the association between HLA and a variety of diseases was recognized.<sup>19,20</sup> Then, between 1980 and 1987, advances in biochemistry

and molecular biology plus the development of monoclonal antibodies to HLA antigens and understanding of the biology of the MHC dramatically expanded.

## Major histocompatibility complex

The Class I, II, and III genes of the MHC are distributed over a 4-megabase region on the short arm of chromosome 6.<sup>21</sup> The telomeric end of the MHC contains 2000 kb and >200 polymorphic genes, including those encoding the Class I antigens (Figure 54.1). The classical Class I HLA-A, HLA-B, and HLA-C genes reside there as do the nonclassical MHC-Ib genes HLA-E, HLA-F, and HLA-G. The MHC Class I chain-related genes MICA and MICB are located between the HLA-B locus and the tumor necrosis factor loci. These genes encode proteins that interact with the activating NKG2D receptor on T cells and NK cells.<sup>22</sup>

Centromeric to the HLA-B locus is the 1-megabase segment Class III region (Figure 54.1).<sup>21</sup> This region has a dense and diverse array of genes which encode proteins associated with innate immunity and inflammation such as complement components C2, C4, and Bf (factor B protein of the alternate pathway of complement activation).

Centromeric to the Class III region and containing ~1 megabase of DNA are the Class II gene loci (Figure 54.1).<sup>21,22</sup> Within this region there are at least 18 closely linked loci that encode the  $\alpha$  and  $\beta$  chains of the Class II antigenic proteins. Proceeding from the telomeric to the centromeric boundaries of the region, the first gene cluster is composed of several  $\beta$ -chain loci and one  $\alpha$ -chain locus that encode the HLA-DR antigens. The DRB1 locus encodes for the DR1 to DR18 specificities, while the DRB3, DRB4, and DRB5 loci encode for the DR52, DR53, and DR51 antigens, respectively. Centromeric to the DRB genes are the DQ (DQA and DQB) and DP (DPA and DPB) genes which encode for HLA-DQ and HLA-DP antigen specificities (Figure 54.1). Other genes with important accessory functions to Class I and II antigen presentation are contained in the Class II region and include LMP2 and LMP7 genes, TAP1 and TAP2 genes, and tapasin. These genes encode proteins that participate in protein degradation and peptide transport and loading in the Class I system.<sup>23</sup> HLA-DM and HLA-DO loci encode proteins involved in peptide loading in the Class II system.

## Class I and II antigens and their function

The structure, tissue distribution, and function of the expressed proteins of Class I and Class II genes differ considerably. Both Class I and Class II antigens are heterodimeric, having three to four extra-

**Table 54.1** Historical Overview of International Histocompatibility Workshops

International Workshops	Year	Major Theme
I	1964	Comparison of serologic techniques
II	1965	Standardized techniques and nomenclature
III	1967	One major genetic system named HL-A
IV	1970	International antisera analysis
V	1972	Population differences for HLA
VI	1975	Identification of D(DR) locus by cellular techniques; HLA-C locus confirmed
VII	1977	Serologic detection of DR antigens
VIII	1980	Compendium of HLA antigen frequencies
IX	1984	Introduction of RFLP molecular techniques; DQ/DP loci confirmed
X	1987	Standardization and application of RFLP; introduction of PCR/SSOPH typing
XI	1991	Standardization and application of PCR/SSOPH HLA Class II typing to transplantation, disease association, and population genetics
XII	1996	Analysis of Class I and Class II alleles by means of DNA sequencing
XIII	2002	Definition of extent of Class I and II gene variation and application to typing of volunteer donors
XIV	2005	Genomics and immune responses
XV	2008	Applications in clinical medicine, anthropology, etc.; new HLA nomenclature
XVI	2012	Global distribution of KIR genes and ligands; MICA antibodies, HLA IgA antibodies; next-generation sequencing
XVII	2017	HLA and KIR genomics, define full length MHC sequences, map serologic epitopes, and design and develop robust informatics for HLA and KIR research.

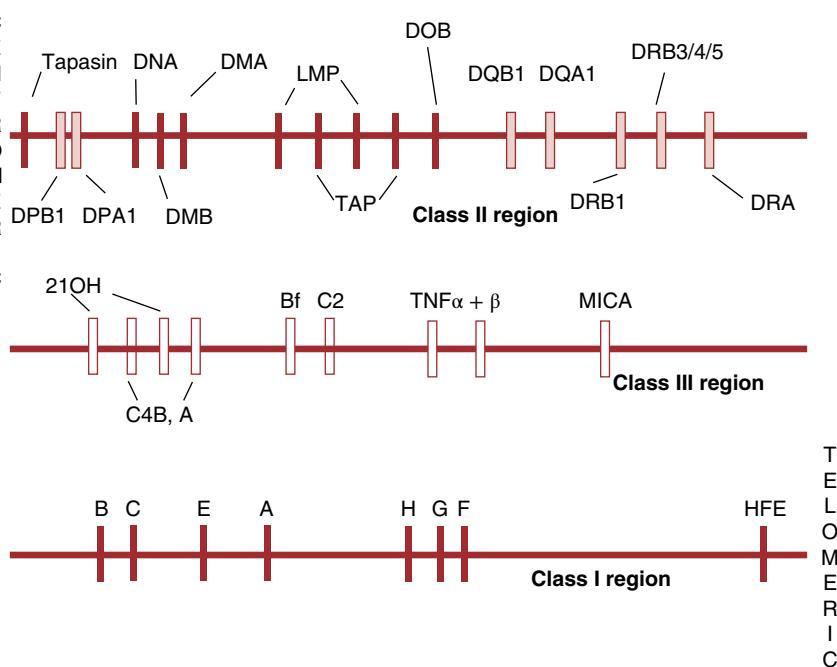
RFLP: restriction fragment length polymorphism; PCR: polymerase chain reaction; SSOPH: sequence-specific oligonucleotide probe hybridization.

cellular domains in addition to transmembrane and cytoplasmic regions (Figure 54.2).<sup>21</sup> Class I antigens are composed of a 45,000-D  $\alpha$  chain encoded by the HLA-A, HLA-B, and HLA-C gene loci. This heavy chain is noncovalently bound to a nonpolymorphic 12,000-D light chain,  $\beta_2$ -microglobulin, encoded on chromosome 15.  $\beta_2$ -Microglobulin stabilizes the Class I complex; however, studies have shown that  $\beta_2$ -microglobulin-free, Class I heavy chains can be expressed and may present peptides.<sup>24,25</sup> The  $\alpha_1$  and  $\alpha_2$  domains contain variable amino acid sequences and thus provide the antigenic polymorphisms that serologically define individual HLA antigens. The  $\alpha_3$  domain may also contain polymorphic residues, but it is believed that these do not contribute significantly to the antigenic nature of Class I molecules.

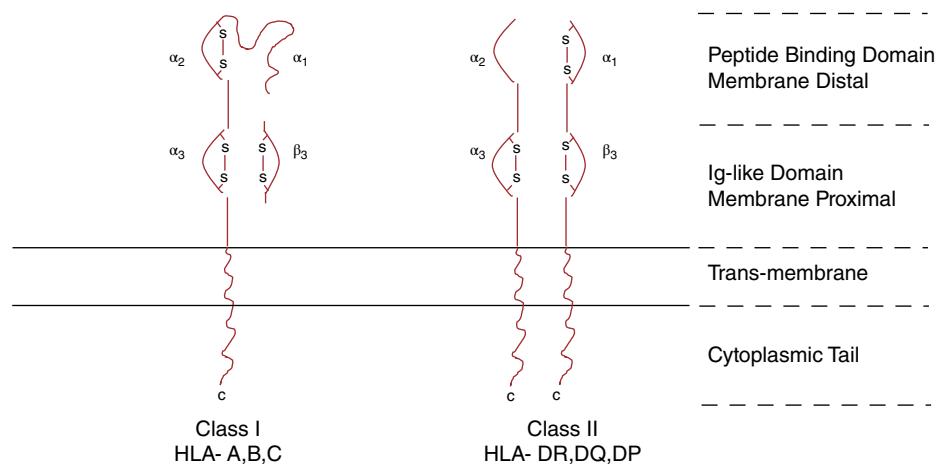
The  $\alpha_3$  domain of the heavy chain and  $\beta_2$ -microglobulin are noncovalently associated with each other near the cell membrane. The  $\alpha_1$  and  $\alpha_2$  domains sit on top of them and form a peptide-binding groove. This pocket has a floor of eight flat  $\beta$  sheets bounded by two long  $\alpha$  helices, as illustrated in Figure 54.3B. Most of the polymorphic amino acid changes of the Class I histocompatibility antigen differences are associated with the floor or sides of this pocket, although some map to other domains.<sup>26,27</sup>

Class II antigens are composed of a 33,000-D  $\alpha$  chain noncovalently associated with a 28,000-D  $\beta$  chain (Figure 54.2). The  $\alpha$  chain is encoded by the A gene loci and the  $\beta$  chain by the B gene loci in the HLA-D region of the MHC. For example, the DR antigen  $\alpha$  and  $\beta$  chains are encoded by the DRA1 and DRB1 genes, respectively. The  $\alpha_1$  and  $\beta_1$  domains form an antigen-binding groove similar to that described earlier.<sup>28</sup>

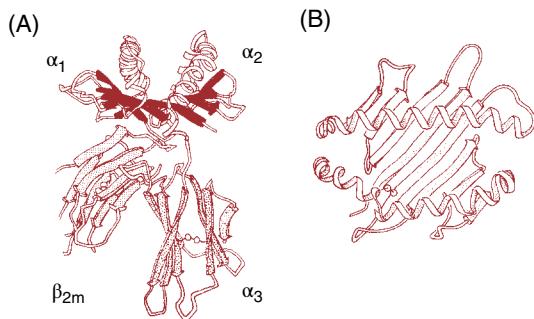
Class I antigens are expressed on the surface of all nucleated cells and platelets. Though some studies have demonstrated low expression of HLA-C antigens on platelets,<sup>29</sup> other studies suggest that this low expression is insufficient to clear platelets in the face of corresponding HLA-C locus antibodies.<sup>30</sup> Class II antigens have a limited tissue distribution primarily on antigen presenting cells, such as B lymphocytes, macrophages, and dendritic cells, although they can be induced in several cell types, including activated T cells and endothelial cells.



**Figure 54.1** Selected genes in the major histocompatibility region on human chromosome 6 in the Class I, II, and III regions. Distances between loci are not drawn to scale.



**Figure 54.2** Simplified illustration shows the biochemical structure of Class I and II antigens.



**Figure 54.3** (A) Side view of Class I antigen molecule. (B) View of the antigen-binding pocket looking down toward the cell membrane. Source: Adapted from Bjorkman *et al.* (1987).<sup>26</sup>

Class I and II molecules can acquire both self- and non-self-peptides in their binding cleft. This peptide-MHC complex is responsible for the presentation of peptides to T-cells via the T-cell antigen receptor.<sup>31</sup> During the selection process, T cells that bind with high affinity to self-peptides in the context of an individual's Class I and II molecules are either deleted or suppressed by a variety of mechanisms to prevent autoimmune disorders. However, peptides derived from viruses, bacteria, and some parasites that are presented by the individual's Class I and/or Class II molecules to T-cells can evoke an immune response. Class I molecules primarily present peptides of approximately 8–10 amino acids that have been generated via the degradation of cytoplas-

mic proteins in the proteasome system. Class I antigens function as MHC restriction elements in the destruction of virus-infected target cells and present peptides to CD8 cytotoxic T cells. Class II molecules bind peptides of approximately 13–25 amino-acids, derived from exogenous and endogenous origins, that have been degraded within the endosomal system. Class II antigens function as MHC restriction elements and present peptides to CD4 helper T cells. Activation of CD8 and CD4 T cells then results in the cellular expansion and differentiation into the cellular and humoral immune responses.<sup>31</sup>

### Nomenclature and polymorphism of the HLA system

Table 54.2 illustrates the HLA gene loci, the variable polypeptide chains, the number of related serologic specificities, and the number of alleles within each specificity. In the Class I region, the HLA-A, HLA-B, and HLA-C loci are all highly polymorphic with more than 20,000 known alleles.<sup>32,33</sup> Multiple alleles are present within most of the known serotypes. For example, the A2 serotype has more than 900 alleles. In the Class II region, the HLA-DRA locus is almost monomorphic, whereas DRB1 is highly polymorphic, with nearly 3000 known alleles and ~2000 expressed proteins. The DQA1 and DQB1 genes and the DPA1 and DPB1 loci demonstrate similar but less extensive polymorphism.

DPA1 and DPB1 allelic variation can be detected with DNA typing while a significantly lesser number can be distinguished with serologic testing.<sup>34</sup> It is important to note that the serological reactivity of many HLA alleles, not just DPB1, has not been tested. One

**Table 54.2** HLA Nomenclature

Genetic Locus	Encoded Polypeptide	Antigenic Specificity	Alleles	Number of Known Alleles/Proteins
HLA-A	α	A1 to A80	A*01:01 to *80:07	6425/3929
HLA-B	α	B5 to B82	B*07:02 to *83:01	7754/4885
HLA-C	α	Cw1 to Cw10	C*01:02 to *18:14	6329/3719
DRA	α		DRA*01:01 to *01:02	7/2
DRB1	β1	DR1 to DR18	DRB1* 01:01 to *16:68	3621/2504
DQA1	α		DQA1* 01:01 to *6:02	279/124
DQB1	β1	DQ1 to DQ9	DQB1* 02:01 to *06:385	1968/1288
DPA1	α1		DPA1*01:03 to *04:02	233/92
DPB1	β1	DPW1 to DPW6	DPB1*01:01 to *1167:01	1674/1081

The number of polypeptides known for each locus reflects unique amino acid sequences encoded by a larger number of alleles that may differ only by silent polymorphisms. Above data can be found at <https://www.ebi.ac.uk/ipd/>, release 3.43.0, January 2021.

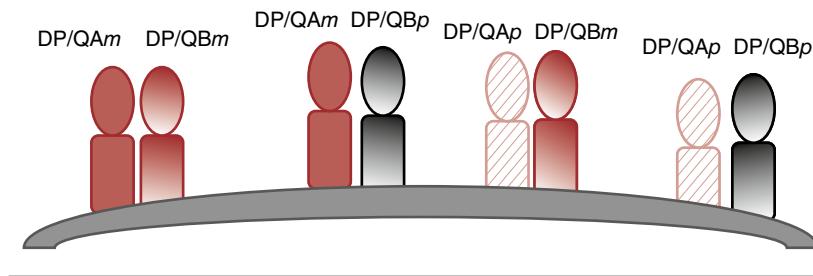
additional aspect that adds to the polymorphic diversity of both DP and DQ alleles is their ability to form heterodimeric isoforms. For certain DQA/DQB combinations, an individual heterozygous for both alpha and beta chains may produce surface heterodimers formed from both cis- and trans-linked alleles (Figure 54.4). Thus, rather than expressing two DQA/DQB heterodimers, one from each parental haplotype, a cell may express up to four different heterodimers. Note that not all DQA/DQB heterodimers are even possible as there are physical constraints as to which dimers can be formed.<sup>35</sup>

The HLA system is the most polymorphic genetic system known to exist in humans. The number of different phenotypes possible from all combinations of HLA alleles is greater than the global population. New alleles appear to emerge at a fairly constant rate and become fixed in populations. New alleles are generated by means of point mutation, recombination, and gene conversion-like events.<sup>34,36</sup> It is clear though that the HLA system is unique in that it appears to embrace or, at the very least, be tolerant of new mutations. When a new mutation does arise in an HLA gene, unless the mutation prevents transcription, translation, or expression, the new HLA molecule may merely bind a unique set of peptides. Whether these new alleles confer a survival advantage is unknown.

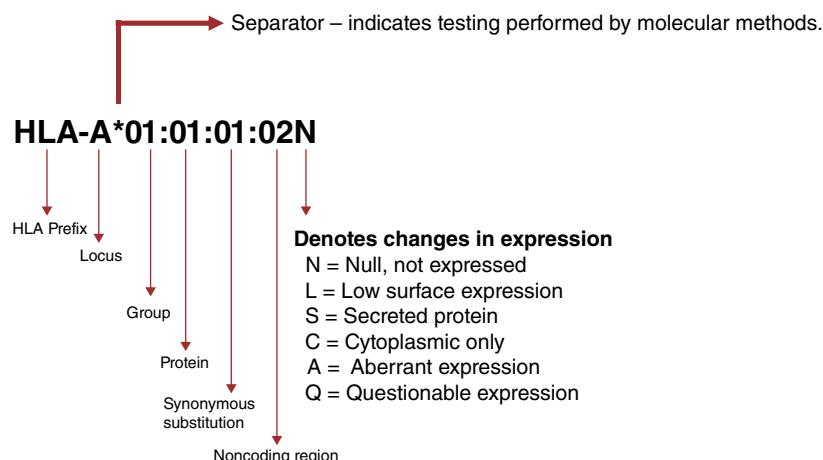
The nomenclature of the HLA system is established by an international committee sponsored by the World Health Organization and is updated frequently.<sup>37,38</sup> Figure 54.5 shows the general scheme for designating HLA antigens and alleles. Each serologic specificity is prefixed by the genetic system designation HLA-, followed by a

letter denoting the encoded antigen (e.g., A, B, C, DR, or DQ). This letter is followed by digits, arranged in distinct fields, indicating the exact allele specificity.<sup>37</sup>

The nomenclature of the HLA system was first established with serologic data. Molecular data were introduced later according to the precise DNA sequences of each allele. The existing nomenclature had to be modified to accommodate this information. For Class I alleles, because only the  $\alpha$  chain is variable, the molecular designation is the system (HLA-), the locus (A, B, or C), an asterisk (\*), followed by up to four fields of digits separated by colons. All alleles receive at least a four-digit name, which corresponds to the first two fields; longer names are assigned when necessary (see Figure 54.5). The first field corresponds to the serologic specificity while the second field denotes the expressed protein. For example, HLA-A\*03:01 is the first molecular allele of the HLA-A3 serologic specificity, and HLA-A\*03:02 is the second. In the Class II region, because both the  $\alpha$  and  $\beta$  chains can be variable, the locus designation must include the polypeptide chain responsible for the allele. For example, Class II, HLA-DRB1\*15:01 is interpreted as HLA system, DR locus,  $\beta 1$  polypeptide chain (B1), DR15 serologic specificity, first molecular allele (01). For molecular alleles that have no serologic equivalents, alleles are sequentially numbered, for example, HLA-DQA1\*01:01 is the first molecular allele of the  $\alpha_1$  polypeptide chain of DQ. The third and fourth fields are employed to designate alleles with silent polymorphisms and with variation occurring outside exons, respectively (Figure 54.5).



**Figure 54.4** Possible expression isoforms for DPB/A and DQB/A that could arise in a fully heterozygous individual. DP/Q = either DPB/A or DQB/A. *m*: maternal haplotypes; *p*: paternal haplotypes.



**Figure 54.5** Schematic of HLA nomenclature.

**Table 54.3** Detection of HLA Antigens and Alleles

- |   |   |
|---|---|
| Serologic methods*  | <ul style="list-style-type: none"> <li>• Microlymphocytotoxicity test, modified microlymphocytotoxicity test, B cell-enriched lymphocytes</li> </ul>  |
| Cellular methods*   | <ul style="list-style-type: none"> <li>• One-way mixed lymphocyte reaction (MLR)</li> </ul>   |
| Nucleic-acid-based methods for Class I and II allele identification | <ul style="list-style-type: none"> <li>• Sequence-specific oligonucleotide probe (SSOP) hybridization analysis</li> <li>• Sequence-specific primer polymerase chain reaction (SSP and real-time PCR)</li> <li>• Sequence-based typing (Sanger method)*</li> <li>• Next-generation sequencing (NGS)</li> </ul> |

\* Historic, no longer commonly used in clinical practice

### Identification of HLA antigens and alleles

Three methods have been used for the detection of HLA antigens and alleles (Table 54.3). Serologic detection of HLA antigens by the microlymphocytotoxicity assay utilizes the addition of rabbit serum to HLA antibodies fixed to the membrane of peripheral blood lymphocytes to cause cell death. Vital dyes that enable identification of dead cells can then be used to deduce the presence of particular HLA antigens. Modifications of this test, primarily an increase in incubation time and isolation of B lymphocytes from peripheral blood, allowed for typing for the HLA-DR and -DQ antigens.<sup>39</sup> Variations of the mixed leukocyte reaction with homozygous testing cells, primed lymphocytes, or T-cell clones were historically used for detecting HLA-DR, HLA-DQ, and HLA-DP antigens.<sup>40–42</sup>

Over the last decade, serologic and cellular based approaches have been replaced by sequencing-based methods. The application of DNA-based techniques to the field of histocompatibility has led to an unprecedented expansion in knowledge of the MHC at the molecular level.<sup>37</sup> More specifically, sequence-based HLA typing methods have produced the following advances:

- They have revealed the underlying DNA sequence variations among individuals (and, therefore, amino acid variations) responsible for the antigenic differences in HLA molecules detectable with traditional serologic and cellular HLA testing.
- They have made apparent the fact that results of serologic HLA tests define only broad groups of Class I and Class II alleles. Numerous subgroups of alleles distinguishable by sequence differences are present within these broad serologic groups. The result is a very large number of known alleles.<sup>43</sup>
- Sequence data have allowed detailed definition of the genetic basis of the HLA-mediated disease associations described later in this chapter.

The success and precision of these methods have rendered serologic and cellular methods essentially obsolete for the purposes of clinical transplantation testing. Several sequenced-based methods have been developed and are in widespread use. Sequence-based genotyping methods focus on the antigen-binding regions of HLA Class I (exon 2 and exon 3) and Class II (exon 2), respectively, for the identification of HLA alleles (with the exception of NGS, as discussed below).

### Sequence-specific oligonucleotide probe hybridization (SSOP)

Relevant regions of the Class I and Class II genes are amplified from genomic DNA by means of the polymerase chain reaction (PCR) with two oligonucleotide primers that anneal to 5' and 3' flanking regions that are conserved (are identical) among individuals. These primers are locus-specific (e.g., amplify HLA-A but not the related HLA-B locus), can amplify all known alleles at a locus, and result in

roughly equal amplification of the two alleles in a heterozygous individual. After the PCR, the amplified DNA is denatured and labeled with biotin, and hybridized to a bead array containing a range of individual allele-specific DNA probes. Following the addition of streptavidin linked to a fluorescent probe, the identity of the HLA alleles can be deduced using the combination of positive and negative signals in the hybridized bead array.

### Sequence-specific primer polymerase chain reaction (SSP and real-time PCR)

Sequence-specific primer assays have been adapted to HLA genotyping in end-point PCR and real-time PCR formats. In both formats, multiple separate reactions are conducted for Class I and Class II genes in order to assess the HLA genotype at intermediate resolution. In endpoint analysis, PCR primers are designed with 5' ends that bind to conserved sequences and 3' ends that are complementary to HLA gene sequences of particular alleles.<sup>44</sup> Thus, only DNA sequences complementary to the allele-specific 3' end will result in DNA amplification, and other alleles will not amplify. Gel electrophoresis can then be used to detect the presence or absence of PCR products of the appropriate size, and hybridization assays are avoided. Sequence-specific PCR is most useful for intermediate-resolution HLA typing and for initial quick identification of broad antigen groups, such as DR52. Real-time PCR approaches utilize 100 or more combination of sequence-specific primers, and commonly used fluorescent real-time, such as Sybr Green. The combination of positive and negative reactions can quickly be interpreted by computer algorithms in order to provide HLA genotyping that is typically at the level of allele groups.

### Next-generation sequencing (NGS)

Next-generation sequencing has recently been adapted to HLA genotyping, and its use in clinical practice has grown exponentially in the last several years. The major advantage to NGS for HLA genotyping is the generation of full-length gene sequence data, which allows for unambiguous high-resolution HLA genotyping and phasing of HLA genotypes such that haplotypes can be determined. NGS also allows sequencing beyond the antigen-binding site, and thus has also led to the identification of many novel alleles. Limitations to NGS are the extensive hands-on time required to complete NGS assays, the length of time required to complete the genotyping and produce results, and the large amount of data generated.<sup>45</sup> However, all of these limitations are rapidly being addressed as NGS is adopted more widely. Currently, there are several vendors that offer NGS-based HLA genotyping platforms, and the methods and analysis software are updated frequently. HLA genotyping by NGS has been conducted using third-generation sequencing platforms with workflows that range from 2 to four days. However, newer methods in development, such as the Oxford Nanopore platform, offer the potential for NGS-based typing in only a few hours.<sup>46</sup>

### Choice of HLA typing method

Several factors influence the decision to use one HLA typing method over another, and often multiple methods are used in combination in a given laboratory. Sequence-specific real-time PCR can be performed in a relatively short time (3–5 hours) that accommodates clinical situations such HLA typing of deceased donors in the setting of solid organ transplantation. SSP, SSOP, and NGS are used in less time-sensitive situations, such as solid organ transplant

candidate genotyping and stem cell transplantation. In these cases, allele-level HLA matching of donors and recipients is desired and turnaround times of a few days to a week are acceptable. In regard to test volume, SSP platforms are generally optimized to run one patient sample at a time, whereas SSOP and NGS permit batching of patient samples to be run simultaneously.

### Genotypes, phenotypes, and haplotypes

The HLA locus has several defining molecular genetic features. First, HLA genes are expressed codominantly such that both copies of each HLA gene are expressed. This property is one that made antigen-level HLA typing important (and complicated) before the advent of sequencing-based techniques. Second, HLA genes are closely linked on chromosome 6, and they are inherited together as haplotypes. Thus, each child inherits two complete HLA haplotypes, one from each parent.

Figure 54.6 illustrates the segregation of haplotypes in a family of seven, focusing on the A, B, Cw, and DR serologic specificities. HLA genotyping of a single individual does not allow definitive assignment of the HLA haplotypes. For example, if sibling 3 was genotyped in isolation, one cannot be certain whether the DR7 is present on the chromosome carrying B8 or B13. However, the analysis of the typing data for the entire family and the transmission of specificities to the children make it possible to phase each of the pairs of parental chromosomes and to determine haplotypes. Each child in this family inherits a paternal [a] or [b] haplotype and a maternal [c] or [d] haplotype, and also differs from each parent by one HLA haplotype. Because there can be only four parental haplotypes, the chances are 1 in 4 that the siblings will have the same paternal–maternal haplotypes (i.e., HLA-identical), 1 in 2 that siblings will differ by one haplotype, and 1 in 4 that they will differ by two haplotypes. For example, siblings 4 and 5 are HLA-identical, and sibling 4 shares one haplotype with siblings 2 and 3 but no haplotypes with sibling 1. There are infrequent exceptions to haplotype inheritance due to recombination within the HLA locus during meiosis.

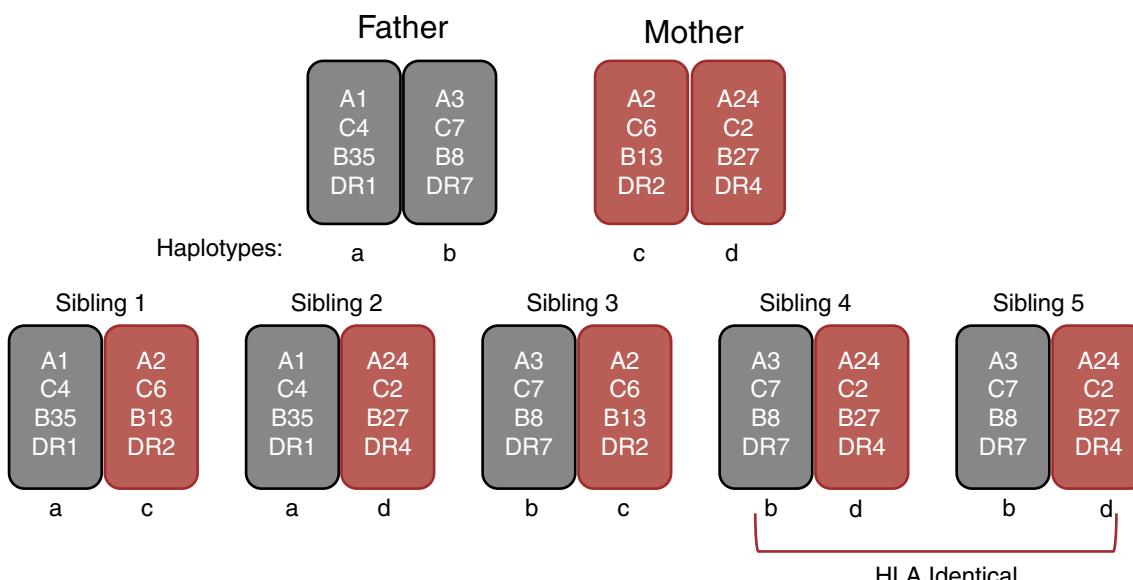
Finally, on a population level, there is strong linkage disequilibrium within the HLA loci such that certain populations have higher frequencies of particular HLA alleles based on geography and ethnicity. Linkage disequilibrium means that in randomly mating populations, the haplotype frequency for two or more linked gene loci is significantly higher than would be expected by chance alone. Some HLA alleles and haplotypes are widely distributed around the globe, and others are almost exclusively within a particular ethnic group. The expected frequency is obtained from the product of the gene frequencies of the involved genes. For example, among persons of European ancestry, the observed A1, B8 haplotype frequency (7.0%) is approximately 4.4 times greater than the expected haplotype frequency (1.6%). These excess haplotype frequencies may exist for a variety of reasons, including high prevalence in the founders of a population and selective pressure from infectious organisms.<sup>47</sup>

### Medical and biologic significance of HLA

The HLA system plays a role in several areas of biomedical significance, including the following:

- Antigen presentation
- Association with certain diseases
- Population genetics and anthropology
- Transfusion Medicine
  - Management of immune mediated platelet refractoriness (see Chapter 16)
  - Febrile nonhemolytic transfusion reactions (see Chapter 48)
  - Transfusion-related acute lung injury (see Chapter 49)
  - Transfusion-associated graft-versus-host disease (see Chapter 50)
- Solid organ and stem cell transplantation

For the purposes of this chapter, we focus on the role of HLA in transplantation and disease association, as the role of HLA in Transfusion Medicine is covered in more detail in the chapters noted above.



**Figure 54.6** HLA-A, HLA-B, HLA-C, and HLA-DR antigens segregating as haplotypes in a family. The data reflect the genotypes of each family member as well as the parental haplotypes.

## Transplantation

HLA and ABO are inherited independently from each other on different genes and separate chromosomes, and both need to be considered in the setting of transplantation. In regard to histocompatibility, the extreme polymorphism of the HLA loci makes it unlikely that an unrelated donor of cells, tissues, or organs will be matched at an allele level or at a serologic level with a recipient without a concerted effort to identify matched donors and recipients. One solution is to identify an HLA-identical sibling, but many potential transplant recipients do not have access to these donors. For solid organ transplantation, powerful immunosuppressant drugs have made transplantation feasible with mismatched living and cadaveric donors and recipients. However, better HLA-A, HLA-B, and HLA-DR matching at a serologic level generally increases the half-life of the transplanted organ and decreases overall morbidity.<sup>48</sup> Thus, zero HLA-A, HLA-B, and HLA-DR mismatched deceased donor kidney transplants are given high ranking priority in the kidney allocation scheme.<sup>49</sup>

Finding well-matched donors of hematopoietic stem cells if there are no HLA-identical siblings is facilitated by resources such as the National Marrow Donor Program, which has enrolled several million potential donors with known HLA types.<sup>50</sup> The goal for unrelated stem cell donation is increasingly allele-level matches for HLA-A, HLA-B, HLA-C, HLA-DRB1, HLA-DQB1, and HLA-DPB1, although the level of mismatching permissible is incompletely understood. Subtle allelic mismatches between stem cell donors and recipients appear to increase the risk of severe graft-versus-host disease and decrease overall survival.<sup>51,52</sup> The use of umbilical cord blood stem cells and the depletion of T cells from transplanted cells may allow greater donor-recipient mismatches.<sup>53,54</sup> Retrospective studies are continuing to assess in a definitive manner the level of matching necessary for successful stem cell transplantation.<sup>55</sup> Hematopoietic stem cell transplantation is discussed in Chapter 53.

Two major mechanisms by which Class I and II donor-recipient mismatches may adversely affect transplantation are cell-mediated rejection and production of HLA antibodies. Donor HLA antigens mismatched with recipient HLA antigens can elicit a strong cell-mediated immune response. Recipient T cells are able to recognize and respond to donor mismatched HLA antigen.<sup>56</sup> These responses contribute to acute rejection in organ transplantation and graft-versus-host disease in stem cell transplantation. HLA matching and immunosuppressive therapy help to mitigate this problem. Transplant recipients become sensitized or produce antibodies directed against Class I and Class II specificities through several routes.<sup>57</sup> Exposure to fetal HLA molecules encoded by paternal haplotypes during pregnancy, especially multiple pregnancies, can lead to the presence of long-lasting, high-titer HLA antibodies. Similarly, mismatched HLA antigens from previous organ donors and donors of transfused blood components can lead to sensitization. The presence of recipient preformed HLA antibodies may lead to hyperacute rejection of a donor organ bearing the relevant HLA specificities.<sup>58</sup> Therefore, laboratories maintain serum-screening programs to detect and identify HLA antibodies in waiting recipients so that inappropriate donors can be avoided. Some transplant centers have developed desensitization protocols that employ plasmapheresis and a variety of immunosuppressive/immunomodulatory agents in attempts to reduce and decrease production of HLA antibodies, respectively, thereby facilitating transplantation either by removing donor-specific HLA antibodies or widening the pool of compatible donors.<sup>59,60</sup>

## Identification of HLA antibodies

Detection of HLA-specific antibodies is critical in both solid organ and stem cell transplantation. Pretransplant, HLA antibodies can limit access to transplantation and may cause early allograft rejection or failed stem cell engraftment. Post-transplant, donor-specific HLA antibodies (DSAs) can lead to untoward consequences, such as antibody-mediated rejection and/or graft loss in renal, heart, lung, and liver transplants.<sup>61-65</sup> The clinical impact of HLA antibodies helped propel antibody testing forward from historical serologic tests to current solid-phase methods.<sup>66</sup> While the importance of HLA antibodies in solid organ transplant has been recognized for many years, the clinical relevance of HLA antibodies in hematopoietic stem cell transplantation has recently gained greater recognition with the increasing use of haploidentical transplantations.<sup>67</sup>

### Serological assays

Historically, HLA antibody testing was performed utilizing a complement-dependent cytotoxic assay (CDC) for both antibody identification and crossmatching. The panel-reactive antibody (PRA) test is another serological assay used to assess the breadth of HLA sensitization. The CDC assay is employed to test recipient serum against panel of cells from donors with varied HLA types. The number of positive reactions corresponds to the degree of HLA alloimmunization. For example, if 15 of 30 panel cells showed positive reactions, then the PRA would be 50%. However, these serological assays were complicated, required extensive quality control, and had a number of drawbacks.<sup>68</sup> More recently, the flow cytometric crossmatch was developed permitting direct identification of HLA antibody with fluorescently conjugated secondary antibodies independent of complement engagement. However, even with these advancements, there were drawbacks such as the need for viable cells and limited specificity. Such limitations could produce false-positive reactions precluding truly compatible transplantation.<sup>69</sup> Fortunately, the advent of solid-phase HLA antibody testing helped alleviate many such shortcomings.

### Solid-phase testing

Solid-phase testing further honed HLA antibody identification to the point where unique HLA antibody specificities could be determined. In lieu of viable cells, solid-phase testing utilized HLA antigens adhered to plastic matrices (e.g., microtiter plates or microparticle spheres). HLA proteins could be either purified native HLA antigens or single recombinant HLA Class I or II molecules.<sup>70</sup> Solid-phase platforms include ELISA, flow cytometry, and Luminex<sup>TM</sup> technologies. Currently, the Luminex platform is the most widely used technology in modern HLA laboratories. Recipient serum is incubated with hundreds of microparticle beads, each coated with a distinct HLA antigen.<sup>71</sup> Fluorescently labeled secondary antibody is then added prior to running the sample on a high throughput flow cytometer. This method permits the simultaneous detection of multiple HLA antibodies and their respective specificities, which is particularly useful in highly sensitized patients with complex antibody profiles, including allele-specific antibodies.<sup>72</sup> The HLA solid-phase Luminex assays used in most clinical laboratories report mean fluorescence intensity (MFI), which is at best a semiquantitative value that is not necessarily a reflection of the antibody titer and/or strength.

### The calculated PRA and virtual crossmatch

Solid-phase testing has led to the development of two major concepts in modern-day transplantation: the calculated PRA (CPRA)

and the virtual crossmatch (VXM). Whereas the aforementioned serologic PRA test relies on HLA types among a panel of cells, CPRA considers the antigen frequency in the general donor population.<sup>73</sup> Antigens to which recipients have corresponding antibody will be listed as unacceptable antigens. The CPRA is calculated as the collective frequency of the total number of these unacceptable antigens in a population. Therefore, the CPRA provides a more accurate assessment of the probability of finding a compatible donor compared to the conventional PRA.<sup>74</sup> For example, the overall frequency of HLA-A\*02 is approximately 48% in the US donor population; as such, a recipient with an A\*02 antibody would have a CPRA of 48%. Consequently, that individual would be expected to be compatible with approximately 52% of the donor population.

Akin to the electronic crossmatch in the blood bank, the VXM is an *in silico* assessment of recipient:donor histocompatibility. In lieu of performing a physical crossmatch, the VXM is performed by comparing the recipient's HLA antibodies to the donor's HLA antigens to determine the presence/absence of DSAs. If the donor expresses HLA-B\*27 and the recipient has B\*27 antibodies, then the VXM is considered positive. In this manner, the VXM has been shown to be effective in predicting the physical crossmatch.<sup>75</sup> Given that the VXM can be performed in a matter of minutes versus hours it takes to perform a physical crossmatch, the VXM can shorten time to transplant, decrease cold-ischemia time of allograft, and increase organ sharing between regions.<sup>76–78</sup> The VXM is becoming heavily relied upon as a predictor of compatibility pretransplantation.<sup>79–81</sup> Despite the advantages of the VXM, it is important to note that it is only as reliable as the quality of recipient antibody testing and accuracy of donor HLA typing upon which it is based. As with all assays, solid-phase antibody testing and molecular HLA typing have shortcomings that can preclude accurate VXM interpretation. Hence, there are still instances when the physical crossmatch has clinical utility.

### Epitopes

In recent years, focus in the field of histocompatibility has shifted from the antigens and alleles to epitopes and eplets (these terms are often used interchangeably although not exactly equivalent). If the antigen or allele was a puzzle, the epitopes would be the pieces that comprise the puzzle. Structurally, eplets are considered the antigenic components of the protein to which antibody can bind (i.e., "functional epitopes"). Eplets can be linear amino acid sequences or noncontiguous three-dimensional areas created as a result of protein folding. In solid organ transplant literature, eplet analysis has focused on quantifying donor-recipient pair eplet mismatches in relation to risk of poor transplant outcomes. Studies have shown that the number of eplet mismatches, particularly for HLA-DR and HLA-DQ loci, have correlated with de novo DSA formation and graft rejection.<sup>82,83</sup> Other studies have identified potential immunogenic eplets that may predispose to de novo DSA formation.<sup>84</sup> Lists of clinically relevant epitopes/eplets (i.e., antibody confirmed) and theoretical epitopes/eplets (i.e., antibody nonconfirmed) can be found at the HLA Epitope Registry website (<https://www.epregistry.com.br/>). In relation to transfusion medicine, recent publications have investigated the use of epitope matching using HLA Matchmaker software (<http://www.epitopes.net/>) for obtaining matched platelets in alloimmunized patients experiencing HLA-mediated platelet refractoriness.<sup>85</sup>

### Disease association

The association of certain HLA alleles and their encoded antigens with particular diseases, especially autoimmune disorders, has been

**Table 54.4** Selected Disease and Pharmacologic Associations with HLA Alleles

Disorder	HLA Linkage
Ankylosing spondylitis	B*27 alleles
Narcolepsy	DQB1*06:02
Celiac disease	DQ2.5, DQ2.2, and DQ8
Birdshot chorioretinopathy	A29
Behçet's disease	B51
Type 1 diabetes mellitus	DRB1*03/*04 (often inherited with DQB1*02 and *03 alleles)
Rheumatoid arthritis	DRB1*04
Abacavir hypersensitivity	B* 57:01
Allopurinol hypersensitivity	B*58:01
Carbamazepine hypersensitivity	B*15:02

known for some time. In more than 100 disorders, there is significant deviation in the frequency of HLA antigens from that of healthy controls (Table 54.4).<sup>86</sup> In general, HLA-associated diseases have certain common features. They are known or suspected to have an inherited component, usually have autoimmune features, and display a clinical course often featuring repeated acute relapses followed by remission. For most HLA-associated diseases, the etiologic factor and pathophysiological mechanism are incompletely understood. Possible mechanisms proposed include the following: presentation of pathogenic autoantigens or hybrid antigens (alternatively spliced self-peptides) to T-cell receptor, molecular mimicry whereby pathogenic peptides induce target T-cell reactivity with self-peptides, and linkage disequilibrium of HLA gene with the disease susceptibility gene.<sup>87</sup>

Two of the strongest HLA associations are the DQB1\*06:02 allele with narcolepsy<sup>88</sup> and B\*27 alleles with ankylosing spondylitis.<sup>89</sup> Almost all patients with narcolepsy have the associated alleles. Similarly, approximately 90% of patients with ankylosing spondylitis have the associated alleles. In populations without these disorders, the frequencies of DQB1\*06:02 (25–30%) and of the B\*27 group (5–10%) are substantial but do not approach the frequencies among persons with these disorders. Thus, the antigens involved are not unique to narcolepsy and ankylosing spondylitis but are over-represented among affected persons. Approximately 3% of persons with a B\*27 allele have ankylosing spondylitis, a risk approximately 100-fold greater than that among persons without B\*27. Results of HLA testing that show the absence of DQB1\*06:02 or B\*27 alleles are useful to help rule out a diagnosis of narcolepsy or ankylosing spondylitis for a patient. However, results that show the presence of these alleles are less useful because of the prevalence of these alleles in the general population.

Celiac disease<sup>90,91</sup> occurs almost exclusively in individuals that express HLA-DQ2.5 (encoded by DQA\*05:01 and DQB1\*02:01), HLA-DQ2.2 (encoded by DQA1\*02:01 and DQB1\*02:02), or HLA-DQ8 (encoded by DQA1\*03 and DQB1\*03:02). Ninety percent of the patients express the DQ2.5 heterodimer, 5% percent of the patients express DQ2.2, and 5% express DQ8. The  $\alpha$  and  $\beta$  chains of DQ2.5 may be encoded by the same chromosome (*cis* configuration) or different chromosomes (*trans* configuration).<sup>92</sup> Most commonly, it is encoded in *cis* by the DR3–DQ2 (DQB1\*02:01–DQA1\*05:01–DRB1\*03:01) haplotype, but may also be encoded in *trans* by the DR5–DQ7 (DQB1\*03:01–DQA1\*05:05–DRB1\*11/12) and DR7–DQ2 (DQB1\*02:02–DQA1\*02:01–DRB1\*07) haplotypes.<sup>93</sup> Though the HLA association is with DQ2.5, DQ2.2, and DQ8, these haplotypes demonstrate the close linkage disequilibrium between the DR and DQ loci. Of note, these haplotypes can make it difficult to determine the associated disease-related allele.

The association of HLA antigens with most other autoimmune disorders usually does not carry the high relative risk that narcolepsy, ankylosing spondylitis, and celiac disease do. Although these HLA associations are generally less useful in diagnosis, they provide important insights into the pathophysiologic mechanism of these diseases and may help assess prognosis. For example, approximately 95% of patients with type 1 diabetes mellitus have either the DRB1\*03 or the DRB1\*04 allele or both.<sup>94</sup> Patients heterozygous for DRB1\*03/\*04 have a relative risk for type 1 diabetes mellitus three to six times greater than that of patients with DRB1\*03 or DRB1\*04 alone or in combination with another DRB1 allele. Susceptibility to type 1 diabetes mellitus appears to be linked to haplotypes containing DRB1\*03 or DRB1\*04 with DQB1\*02 and DQB1\*03 alleles. Many persons with rheumatoid arthritis (RA) have inherited DRB1\*04 alleles, which may bind better as a result of the peptide citrullination found in patients with RA.<sup>87</sup> Heterozygosity and homozygosity for alleles encoding this epitope may be predictive of a more severe course of arthritis. Thus, allele identification may be helpful in assessing prognosis.<sup>95</sup>

Of note, not all HLA disease associations confer risk of disease. In fact, some HLA alleles are thought to be protective against some diseases. For example, in addition to risk alleles, there are some alleles—DQB1\*06:03, DQB1\*05:01, DQB1\*06:09, and DQB1\*02—that appear to be protective against narcolepsy.<sup>96</sup> Some have also implicated DQA1 in disease susceptibility, demonstrating that certain DQA1 alleles form heterodimers with DQB1 subunits that do not bind to implicated peptides/autoantigens as readily, and are, thus, more protective.<sup>97</sup> Also, certain DRB1\*13 alleles have been shown to be protective against some autoimmune diseases such as systemic lupus erythematosus, Sjogren's syndrome, and rheumatoid arthritis.<sup>98</sup> Different mechanisms have been proposed, including conserved amino acid sequences within the DRB1\*13 protein that may function in tolerance.

Because the MHC contains genes with no obvious role in immune system function, some diseases caused by point mutation or deletion of genes in this region cause disorders not directly related to immunity. However, because of linkage disequilibrium, over-representation of specific Class I or Class II antigens may occur in these disorders. Congenital adrenal hyperplasia is caused by mutations in the MHC Class III region genes encoding 21-hydroxylase.<sup>99</sup> Although linkage to specific HLA alleles is present in persons with congenital adrenal hyperplasia, direct genotyping is generally preferable for diagnostic purposes. Similarly, genetic hemochromatosis is caused by mutations in the HFE gene telomeric to the HLA-A locus.<sup>100</sup> Although the presence of A\*03 confers several-fold excess risk of hemochromatosis, the most direct route to genetic diagnosis is HFE genotyping.

### Pharmacogenetics

There appear to be HLA alleles that interact with other genetic and environmental factors to influence the outcome of this multifactorial disease. For instance, Steven Johnson Syndrome-like hypersensitivity reactions to the antiretroviral drug abacavir was linked to the presence of HLA-B\*57:01 in treated patients.<sup>101,102</sup> Near complete elimination of hypersensitivity morbidity was achieved by first screening patients to exclude 5–10% with B\*57:01 from exposure to

this agent. In this example, HLA allele identification serves as a pharmacogenomic test to stratify a patient population for targeted therapy. Other pharmacologic reactions associated with specific HLA alleles can be found in Table 54.4.<sup>86</sup>

### Summary

The MHC region, located on chromosome 6, represents one of the most polymorphic regions in all of the human genome. This exceptionally high degree of polymorphism appears to be maintained in part, to ensure appropriate responses to the infectious organisms encountered by the human population. However, as the function of HLA molecules is to present foreign peptides to the immune system, mutations within this system seem to be well tolerated. MHC polymorphisms have several implications in medicine and biology beyond driving a specific immune response: (1) MHC genetic variation can be exploited as a tool for anthropologic study of human migration and development; (2) specific HLA alleles are associated with a propensity to develop particular disease entities, especially autoimmune disorders and hypersensitivity reactions; and (3) they present a challenge in finding appropriately HLA-matched donors and recipients for both stem cell and organ transplantation.

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## CHAPTER 55

# Chimeric antigen receptor T cells and other cellular immunotherapies

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## Introduction

Immunotherapy may be in the form of cellular therapy, antibodies, cytokines, or other modalities that induce, enhance, suppress, or release suppression of the immune system response. *Adoptive* immunotherapy typically refers to treatment with a cellular product in order to re-establish or newly establish immune effector function. For these cell therapies, the donor may be autologous or allogeneic, and the product may be minimally manipulated (e.g., donor lymphocyte infusion) or the result of a complex isolation, expansion, and modification by genetic engineering (e.g., chimeric antigen receptor [CAR] T cells). Clinical use of CAR T cells has revolutionized the field of cellular immunotherapies in recent years and has led to US FDA approvals of the first gene-modified “living drugs.” This chapter reviews the history of cellular immunotherapy with a particular focus on CAR T cells to illustrate lessons learned through their development and commercialization. Many of these lessons are applicable to other cellular therapies and inform future directions.

## T-cell immunotherapy

### Introduction

T cells have a powerful ability to distinguish self from non-self via the T-cell receptor (TCR). TCR recognition of peptide antigens presented to naive T cells by major histocompatibility complex (MHC) molecules in conjunction with appropriate costimulatory signaling can elicit potent antitumor and antiviral effector function. Through this signaling, a naive endogenous T cell can be activated to proliferate, differentiate into effector- and memory-type T cells, and orchestrate a specific immune response including direct cytotoxicity, cytokine production, and appropriate immune regulation. This natural killing ability was recognized as potentially transferrable over 50 years ago in mouse foreign tumor studies.<sup>1</sup> Since that time, increased knowledge of T-cell biology and subset function and technical progress with expansion and manufacturing have improved the success and breadth of in vivo studies. The goal of adoptive T-cell immunotherapy is to introduce an antigen-specific arsenal to combat neoplastic or virally infected cells. Broadly speaking, immunotherapeutic T cells may be introduced

without redirection (nonspecific T cells) or following antigen exposure (antigen-specific), genetic engineering, or other modifications to direct T-cell specificity. In addition, T-cell immunotherapy ex vivo manipulation may be used to not only enhance specificity, but also potency. In this section, we provide an overview of endogenously derived (e.g., donor lymphocyte infusions) T-cell immunotherapies as well as those that are redirected through ex vivo manipulation (e.g., CAR T cells) to treat cancer and infection.

### T-cell therapy for cancer

In the last decade, engineered T-cell therapy and particularly CAR T-cell therapy has generated unprecedented clinical results and an explosion in T-cell immunotherapy research. However, T-cell treatments for cancer have been an active area of interest long before the first reports of CAR T-cell therapy in humans in 2011.<sup>2</sup> In fact, the biological and clinical developments of donor lymphocyte infusions (DLIs) and later tumor infiltrating lymphocytes (TILs) provided the framework for the first genetically modified T-cell therapies. DLIs relied upon the presence of antileukemic lymphocytes in the collected cellular product. Treatment with TILs takes this observation one step further, enhancing their activation state or preferentially expanding T cells with desired specificity ex vivo. Finally, genetically engineered T cells build upon these approaches to T-cell collection and expansion but rely upon introduction of DNA or RNA sequences to achieve expression of a novel receptor and therefore redirection. The next generation of engineered T cells will again expand upon the existing platform to include gene editing in addition to redirection through novel receptor expression.<sup>3,4</sup>

### Nonengineered cell therapy products

#### Donor lymphocyte infusions

Allogeneic hematopoietic stem cell therapy (HCT) as treatment for hematopoietic cancers is itself a form of highly effective cellular immunotherapy that has been recognized since the 1950s. Even before mechanisms were well understood, the indirect relationship between graft versus host disease (GVHD) and leukemia relapse became clear.<sup>5</sup> T cells were soon implicated for their roles in both processes, as T-cell depletion improved GVHD complications but led to higher relapse rates.<sup>6</sup> T cells are essential for controlling

tumor cells that survive any preparative regimen (i.e., the graft-versus-leukemia [GVL] effect), but the difficulty is balancing this benefit while preventing severe GVHD.<sup>7,8</sup> These discoveries led to a number of strategies to enhance the limited effect of DLI in non-CML transplantation protocols, where outcomes have been inferior. Higher doses of DLI, combined with lymphodepleting chemotherapy as well as unrelated donors, have been associated with increased GVHD risk, which limits efficacy.<sup>9</sup>

Specific CD4, CD8, and  $\gamma/\delta$  T-cell subsets continue to be explored as immunotherapeutic agents.<sup>7,10</sup> For example, the generation of CD4<sup>+</sup> T helper 2 (Th2) cells through ex vivo IL4 and IL2 stimulation and infusion into T-cell-replete allogeneic HCT patients led to accelerated lymphocyte reconstitution and increased inflammatory cytokine pathways without elevations in GVHD.<sup>11</sup> Another clinical trial infused rapamycin-resistant donor CD4<sup>+</sup> Th2/T helper 1 (Th1) cells after matched-sibling low-intensity-regimen HCT for refractory hematologic disease.<sup>12</sup> Recipients had preferential and rapid immune reconstitution with the CD4<sup>+</sup> Th2/Th1 cells, demonstrating the possibilities of achieving donor immunity (and thus GVL) with reduced-intensity preparative regimens. Acceptable GVHD rates were observed, making this platform an attractive candidate for future comparative efficacy studies.

Overall, DLIs have helped transplant patients overcome relapses of leukemia and low-grade lymphoma with rates of success ranging from 70% to 80% in chronic myeloid leukemia to 15–45% in acute myeloid leukemia (AML), 40–60% in multiple myeloma, 60% in low-grade lymphomas (including Hodgkin lymphoma), but only 5% in acute lymphoblastic leukemia.<sup>13</sup> The varying response rates and durations continue to be areas of active research, but likely relate to the regulation of T-cell recognition molecules, progression of disease, and tumor antigen phenotypes.

### Tumor-infiltrating lymphocytes

Many tumors are infiltrated by reactive T cells *in vivo*; although these T cells have specific antitumor activity, they can fail to control tumor growth.<sup>14</sup> Emerging evidence suggests that these TILs are suppressed by the upregulation of a variety of proteins.<sup>15</sup> Removing these TILs from the *in vivo* environment can facilitate activation and expansion. Additionally, host lymphodepletion and chemoradiotherapy prior to TIL transfer significantly enhance engraftment and efficacy of the transferred cells.<sup>16</sup> Subsequent reinfusion of this tumor-specific population has led to measurable responses in patients with metastatic melanoma, ranging from 49% to 72% depending on the preconditioning regimen.<sup>17</sup> Whereas the three- and five-year survival rates were only 36% and 29%, respectively, those patients who initially achieved complete tumor regression had rates of 100% and 93%. Other groups have reproduced these findings with initial response rates closer to 50%.<sup>18</sup> Efforts to extend this application to epithelial tumors have been generally unsuccessful. One theory suggests that the high number of mutations in melanomas makes this tumor well suited for T-cell-mediated therapy, whereas tumors predominated by epigenetic changes may be less immunogenic and thus less responsive to T-cell-mediated therapy.<sup>14,19,20</sup>

A recent exception to TILs' melanoma-limited efficacy is the durable response seen in a patient with cholangiocarcinoma.<sup>21</sup> Whole-exome sequencing of extracted TILs revealed a population of CD4<sup>+</sup> Th1 cells that recognized an HER2 mutation expressed by the patient's metastatic epithelial cancer. Infusion of ex vivo expanded TILs enriched for this mutation-specific population led to disease stabilization and regression in a dose-dependent manner.

This study underscores the concept that T cells, given the right microenvironment, stimulation, dose, and specificity, are effective tumor eradication tools. As our knowledge and ability to obtain detailed genetic information continue to increase, T cells are positioned as excellent targets for *ex vivo* engineering specificity, as addressed further in this chapter.

### Regulatory T cells

Immunotherapy with an additional CD4<sup>+</sup> subset, the regulatory T cell (Treg), has been explored to take advantage of its role in suppressing immune system activation and promoting tolerance.<sup>22,23</sup> Specifically, Tregs have been targeted for depletion and inactivation because they can effectively limit the expansion of tumor-specific T cells.<sup>24</sup> The best Treg receptor(s) to target for depletion is an area of active research; early clinical studies of CD25 blockade have shown mixed results for enhancement of antitumor activity in patients with metastatic melanoma and breast cancer.<sup>25,26</sup>

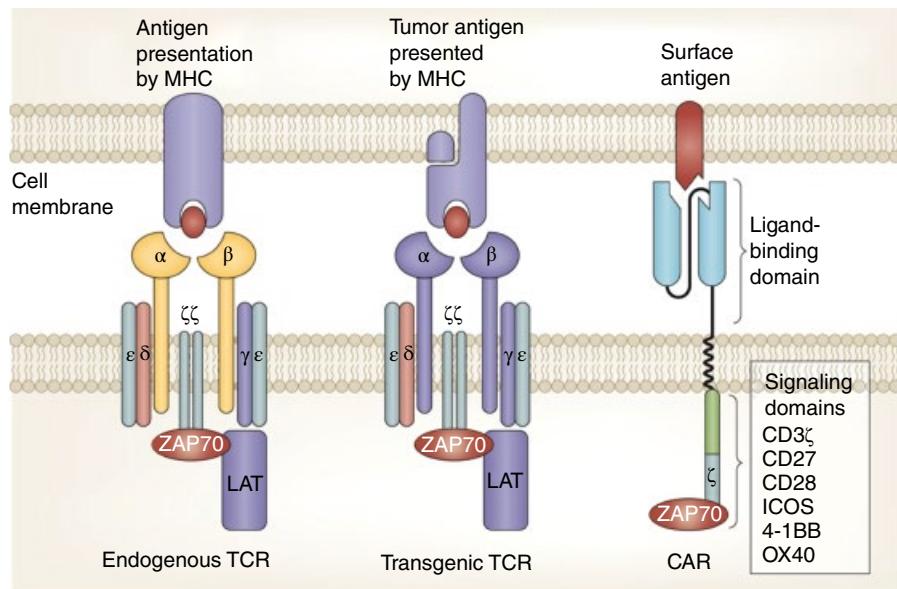
Interestingly, the majority of Treg cancer applications take a different approach than the direct antitumor effects sought with other lymphocyte subsets. Tregs can promote the immune tolerance that is essential to both solid organ transplant and HCT, with the latter showing promising clinical trial results. Infusion of CD25<sup>+</sup> Tregs harvested from partially HLA-matched human umbilical cord blood and expanded *ex vivo* by activation with anti-CD3/CD28 beads and recombinant human IL2 reduced the incidence of GVHD in 23 HCT patients, as compared to 108 controls.<sup>27</sup> Adult expanded Tregs from HCT HLA-haploididentical donors have also helped reduce chronic GVHD in high-risk acute leukemia patients.<sup>28</sup> Additional trials of adoptive Treg therapy are currently underway in the setting of solid organ and bone marrow transplant.

### Genetically engineered T cells

T cells can be genetically modified for cancer immunotherapy via three primary approaches: (1) modifying natural TCRs genetically to create receptors specific for a target antigen, (2) creating synthetic chimeric antigen receptors (CARs) that link a target-specific ligand (usually the variable region of an antibody) to a TCR intracellular domain that activates the T cell, and/or (3) using gene editing approaches such as zinc finger nucleases (ZFNs), transcription activator-like effector nuclease (TALENs), or clusters of regularly interspaced short palindromic repeats-Cas9 (CRISPR-Cas9).<sup>29</sup> Engineered expression of a novel receptor, whether it be a transgenic TCR or CAR, can redirect *in vivo* T-cell activity with high specificity and potency. This is a rapidly growing sector of biotechnology; a September 2013 meeting of the Office of Biotechnology Activities at the National Institutes of Health reported 111 registered protocols for the two approaches combined, with 90% of the TCR trials targeting solid tumors and 50% of the CAR trials focusing on hematologic cancers.<sup>29</sup> Since that time, clinical development of gene modified T-cell protocols has exploded. Recent examples of these clinical protocols include the first use of CRISPR-edited, TCR redirected T cells, and TALEN-edited allogeneic CAR expressing T cells.<sup>30,31</sup> While studies are ongoing, to date results have proven these therapies to be relatively safe and well tolerated.

### Engineered TCR-expressing T-cell therapy

Engineered expression of a full length transgenic TCR closely mirrors expression of a T cell's endogenous TCR with one crucial difference (Figure 55.1). The variable domains are specifically selected to confer TCR specificity and affinity. Once expressed in a T cell, this transgenic TCR allows for highly specific and potent



**Figure 55.1** Comparison of basic structures of native, transgenic, and engineered (CAR) T-cell receptors.<sup>21</sup> Fesnak *et al.* (2016).<sup>3</sup> With permission from Springer Nature.

in vivo activity against target-positive cells. One of the first studies of TCRs in human trials engineered T-cell expression of the melanoma antigen recognized by T cells 1 (MART-1). Transfer of these cells into 15 patients with metastatic melanoma resulted in high levels of engraftment (>10% of peripheral blood lymphocytes) for at least two months post infusion, and for one year in two patients who experienced regression of metastatic lesions.<sup>32</sup> Within several years, the applications extended beyond melanoma. Cancer-testis antigens (CTAs) are genes whose overexpression has been demonstrated in many carcinomas, hematologic malignancies, melanoma, and testicular cancers.<sup>33</sup> Transfer of T cells engineered with the CTA NY-ESO-1 provided tumor regression in 16 of 20 patients with NY-ESO-1-expressing tumors.<sup>34,35</sup> Next-generation NY-ESO-1 TCR expressing T cells with gene edited PD1, TCR alpha, and TCR beta loci have also been shown to be safe and well tolerated.<sup>31</sup> Others have targeted the Wilms tumor-1 antigen found at high levels on leukemic cells with TCR-transduced T cells.<sup>36</sup>

### Chimeric antigen receptor-expressing T-cell therapy

#### Biology

Whereas the use of engineered TCR-expressing T cells for recognizing cancer cells exploits the natural ability of T cells to kill their target, the design of TCRs for a particular application is not as straightforward as in, e.g., the creation of a monoclonal antibody to a target antigen through the use of animal immunization and B-cell cloning approaches or through the use of in vitro methods for antibody discovery like phage display. TCRs recognize their antigens as peptides expressed on cell surfaces in the context of the host's major histocompatibility complex (MHC). Thus, the design of a cancer-specific TCRs requires knowledge of the peptide sequences that are processed by the cell as well as the ability to design the amino acid sequences of TCR chains that are specific to those peptides when embedded in an MHC. Furthermore, at best, TCRs designed to recognize particular cancer antigen peptides may be MHC-restricted, i.e., only function in patients with that particular MHC. The recognition of a self-surface antigen on a cancer cell by an antibody is neither MHC-restricted nor limited to

reactivity with only certain peptides of the target. However, unlike T cells, the effector functions of antibodies with respect to the killing of cancer cells is generally limited to their ability to activate complement or induce antibody-dependent cellular cytotoxicity (ADCC). The concept behind CAR T cells is to exploit the antigen-recognition properties of antibodies with the effector (killing) ability of T cells. As shown in Figure 55.1, a CAR is a synthetic molecule that couples the antigen-binding portion of an antibody with the intracellular signaling machinery of a TCR complex to enable the CAR T cell to become activated through engagement of its antibody domain with a cancer cell target. The antigen-recognition portion is usually in the form of an scFv (single chain fragment variable) that comprises the heavy and light chain variable regions of an antibody expressed as a single polypeptide chain. As discussed below, the design of CARs has gone through several iterations referred to as “generations” in which additional signaling molecules have been used to affect cell effectiveness, persistence, and other properties.

First-generation CARs linked the scFv to the CD3ζ chain, a signal-transduction component of the T-cell antigen receptor.<sup>37</sup> The B-cell antigens CD19 and CD20 are the most common antibody targets for CAR hematologic protocols. These B-cell-specific CARs are designed to eradicate the neoplastic populations in B-cell lymphomas and leukemias and, by nature of CD19 and CD20 expression, also the nonneoplastic B cells. Although these early trials with anti-CD19 and anti-CD20 CARs were safe, efficacy was limited and detection of the transferred CTLs was short-lived (24 hours to 7 days).<sup>38</sup> This poor persistence was thought secondary to the absence of costimulation, and thus second-generation CARs incorporated dual signaling molecules such as CD28, 4-1BB, or OX-40.<sup>39</sup>

Indeed, direct comparisons of first- and second-generation CARs revealed improved expansion and persistence for those CTLs engineered with CD28 costimulation.<sup>40</sup> Clinical trials of these second-generation CARs have been directed toward CD19 in chronic lymphoid leukemia (CLL) and pediatric, adult, and relapsed B-cell acute lymphoblastic leukemia (B-ALL).<sup>41-44</sup> The clinical

efficacy and adverse event profile differ somewhat by indication, disease status, and prior treatment regimens. For example, patients with bulky CLL showed mixed responses in lymphadenopathy whereas in some studies all B-ALL subjects achieved complete molecular remission.<sup>41-46</sup> Although these results suggest that autologous CAR-expressing T cells have the potential to induce rapid remission and/or provide a bridge to curative transplant, the emergence of tumor cells with antigen evasion may mandate targeting multiple molecules. Further, persistence of autologous CAR T cells is crucial for long-term remissions.

Third-generation CARs add a second costimulatory domain. Early clinical trials with CD20-specific CARs coupled to CD28 and 4-1BB produced modest antitumor activity with relatively low circulating CTLs at one year.<sup>47</sup> Interestingly, this protocol used electroporated DNA plasmids as opposed to the retroviral vectors or transposons used in other work and proved not to be as effective for transformation.

Second- and third-generation CARs have shown extraordinary ability to affect antitumor response *in vivo*. Next-generation CAR T cells are likely to alter CARs or endogenous T-cell molecules to improve efficacy, persistence, and/or safety. CAR systems have been designed such that full activating signal is only transduced when two distinct antigens are present on the target cell.<sup>3</sup> Alternatively, the coexpression of a standard second-generation CAR with a suppressive CAR, combining an extracellular scFv with intracellular CTLA4 or PD1 signaling domains in lieu of the CD3 $\zeta$  signal domain, can prevent activation in the presence of the suppressive CAR target. CAR T cells can be engineered to coexpress chemokine receptors or cytokines that can recruit endogenous innate immune cells to improve trafficking to tumor and modifying immunosuppressive tumor microenvironment. Checkpoint receptors can be ablated from CAR T cells via gene editing to improve antitumor efficacy. Finally, T cells can be engineered to express a two-part CAR, wherein both primary and costimulatory signaling domains only colocalize and transduce signal when an adaptor molecule is provided. With this last approach, if the adaptor molecule is a bioavailable drug (e.g., rapamycin), the administration of this molecule may allow for titratable *in vivo* response.<sup>48</sup>

#### **Indications—leukemia**

CAR T cells have demonstrated unprecedented clinical efficacy treating refractory relapsed B-cell leukemias. The first reported clinical use of CAR T cells was published in 2011 and describes the use of CD19-directed CAR T cells to treat three patients with relapsed, refractory chronic lymphocytic leukemia.<sup>49</sup> All three subjects demonstrated robust and durable antitumor response in some cases lasting years. Subsequent trials in pediatric B-cell acute lymphoblastic leukemia demonstrated even more impressive responses, with 80–90% of patients experiencing complete remissions within three months of treatment. These initial response rates have been reproducible across institutions and with the use of different anti-CD19 CAR T-cell drug products.

Other tumor targets, most notably CD22, have been targeted by CAR T cells in patients with B-cell leukemia. Patients who previously failed CD19-directed CAR T-cell therapy have been treated with CD22-CAR T cells with 70% complete remission rate.<sup>50</sup> Promising results support the use of CD22-targeted CAR T cells either alone or in conjunction with CD19-directed CAR T cells to treat a variety of B-cell malignancies.<sup>51,52</sup>

While many B-cell leukemia targets are under active investigation, significant challenges limit durability of overall response.

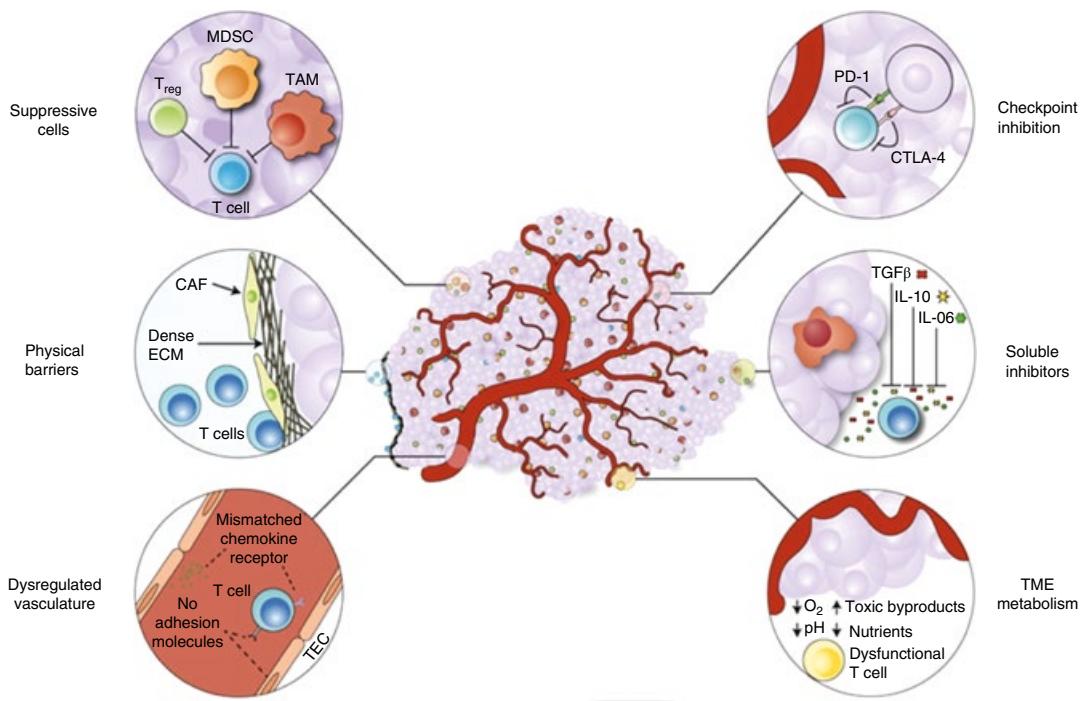
Specifically, failed CAR T-cell persistence after transfer can lead to relapse in approximately half of those treated with CD19-CAR T cells. Persistence has been enhanced with better preinfusion lymphodepletion; however, these regiments come with their own risks. It is worth noting that the manufacture of autologous CAR T therapies for patients with refractory relapsed disease begins with collection of T cells from patients who are often treated with years of cytotoxic chemotherapy. A growing body of evidence suggests that cumulative treatment burden impairs T-cell function in ways that could decrease *in vivo* persistence.

If autologous T cells provide suboptimal material for manufacturing long-lasting CAR T cells, then allogeneic CAR T-cell therapy is one solution to this problem. Allogeneic CAR T cells allow for preselection of optimal donors and collection of T cells that have not been exposed to high levels of cytotoxic therapy. However, allogeneic CAR T cells, unless modified, continue to express both the endogenous TCR and the endogenous MHC molecules. If reinfused into an allogeneic recipient, the allogeneic CAR T cell could recognize host via the endogenous TCR and cause graft-versus-host disease. Alternatively, donor MHC molecules may mark the allogeneic CAR T cell as foreign and lead to rejection by the recipient. Gene editing shows promise in efficiently ablating these molecules from the surface of CAR T cells; however, it is still unclear what the tolerable thresholds are.

#### **Indications—other malignancies**

Compared to leukemia, CAR T-cell therapy response rates lag for B-cell lymphoma, multiple myeloma, and other hematologic malignancies. B-cell maturation antigen (BCMA)-directed CAR T cells demonstrated a 73% overall response rate in patients with refractory/relapsed multiple myeloma.<sup>53</sup> CD19-directed CAR T cells led to overall response rates of around 50% of patients with B-cell lymphomas.<sup>54</sup> To date, CAR T-cell therapies against solid tumors have demonstrated lower response rates. These results are encouraging nonetheless, particularly given that these clinical responses are observed in refractory/relapsed populations for whom little or no alternative therapies are available and prognosis is already poor. Nonetheless, overall and complete response rates for CAR T-cell therapies seen when treating non-B-cell leukemia hematologic malignancies are consistently below those seen when treating leukemias.

There are many possible explanations for this discrepancy as summarized in Figure 55.2 including a tumor microenvironment comprising immunosuppressive cells, soluble immune inhibitors, and unfavorable metabolic conditions.<sup>55</sup> Many cancers, including lymphoma and solid tumors, are capable of suppressing the endogenous antitumor immune response.<sup>56</sup> Like their endogenous counterparts, the current generation of CAR T cells, such as those that are currently commercially approved, are also subject to tumor-mediated immunosuppression. For an immune cell to generate an antitumor response, it must have access to the tumor, activate at the appropriate site, and generate a robust and sustained effector function. Unlike leukemia, solid tumors may present physical barriers by generating fibrosis and necrosis, making tumor sites inaccessible to immune cells. Further, the overexpression of immune checkpoint signaling molecules (e.g., programmed cell death protein 1 [PD-1], its receptor PD-L1, cytotoxic T lymphocyte-associated protein 4 [CTLA-4], T-cell immunoglobulin and mucin-domain containing-3 [TIM3], and lymphocyte-activation gene 3 [LAG3]) leads to blockade of activation and downstream effector function of infiltrating endogenous and CAR T cells.<sup>57</sup>



**Figure 55.2** Mechanisms of immune suppression in the tumor microenvironment. Labanieh *et al.* (2018).<sup>55</sup> With permission from Springer Nature.

The challenges posed by a tumor microenvironment that is inhospitable to immune cell infiltration and activation is made worse by a limited number of optimal tumor targets. A large part of CD19-directed CAR T-cell success can be attributed to the features of CD19 which make it a near-ideal tumor target. CD19 is expressed solely on B cells though not just on malignant B cells which is why technically CD19 is not “ideal.” The majority of B-cell leukemias express CD19, and a given case of CD19-positive B-cell leukemia is expected to express CD19 on all of its malignant cells. Finally, the on-target, off-tumor effect that results in B-cell aplasia is tolerable in that patients can live without normal B cells provided their immunoglobulin levels are maintained with regular infusions of intravenous gamma globulin. Together, these features make CD19 an excellent tumor target for CAR T-cell therapy. For most solid tumors, such an ideal tumor target rarely exists. Targets such as EGFRvIII are highly specific for glioblastoma but are only expressed in a subset of cases and within a given tumor show high expression heterogeneity from cell to cell.<sup>58</sup> Other solid tumor targets such as mesothelin are overexpressed in a number of different cancers and can be uniformly expressed on a given tumor; however, mesothelin is widely expressed in many benign tissues. Lack of optimal tumor targets force solid tumor CAR T-cell therapies to balance efficacy against toxicity, limiting the therapeutic window.

Next-generation CAR T cells aim to overcome the limitations highlighted here. Concurrent chemotherapy, antiangiogenic therapy, or radiation in combination with CAR T cells can enhance tumor infiltration.<sup>59–62</sup> Alternatively, CAR T cells can be engineered to coexpress chemokine receptors to improve homing and infiltration.<sup>63,64</sup> CAR T-cell therapy can also be combined with checkpoint blockade therapy to improve CAR T-cell function once at the tumor site. Checkpoint blockade therapy in the form of monoclonal antibodies against PD-1, PD-L1, or CTLA-4 can be used to disrupt suppression of CAR T cells. While this combination therapy has been shown to enhance CAR T-cell efficacy, systemic

checkpoint blockade therapy is associated with its own risks. A more targeted approach is to engineer CAR T cells to ablate checkpoint receptor expression.<sup>65,66</sup> In this way, only those CAR T cells redirected toward a target antigen are unable to be suppressed by checkpoint signaling, therefore limiting potential checkpoint blockade-associated autoinflammation.

Improving upon the current solid tumor target selection process will require re-engineering of the current CAR T-cell platform. Next-generation CAR T cells redesign the receptor itself to only provide robust activation signals when two targets are present (e.g., “dual CAR T cell”) or when one target is present but another is absent (e.g., “suppressive CAR T cell”). These redesigned CAR T cells allow for Boolean-gated activation and expand the pool of potential targeting strategies. This is most important for solid tumor targets for the reasons mentioned above. For example, a dual CAR against EGFRvIII and a second glioblastoma marker could improve efficacy, whereas a suppressive CAR that activates in the presence of mesothelin but inactivated in the presence of a benign tissue marker could limit off-tumor effects.

### T-cell therapy for viral infections

T-cell therapies for viral infection rely on the ability of T cells to detect viral peptides presented on the surface of infected cells. Alternatively, if T cells are a reservoir for infection, as is the case with HIV, T therapies may generate infection-resistant T-cell populations to reestablish functional T-cell immunity.<sup>67</sup> Recipients of hematopoietic cell transplants (HCTs) often lose the bulk of their cell-mediated immunity and become susceptible to viral infections that carry high morbidity and mortality. These patients are at particular risk for reactivation of cytomegalovirus (CMV) leading to enteritis and pneumonia, Epstein–Barr virus (EBV) causing post-transplant lymphoproliferative disease (PTLD), and adenovirus-associated enteritis, hepatitis, hemorrhagic cystitis, and pneumonia.<sup>68</sup> Effective antiviral agents and anti-B-cell CD20-mediated therapy have

improved many of these complications, but negative side effects and treatment failures are still too common.

The first reports of viral-specific therapy employed autologous fibroblasts as APCs for stimulation and expansion of sibling donor CD8<sup>+</sup> T cells pulsed with CMV.<sup>69,70</sup> The CMV-specific CD8<sup>+</sup> population was infused into recipients of matched sibling donor grafts, who showed no adverse effects and successful reconstitution of CD8-mediated immunity for at least eight weeks. Manufacturing approaches include retroviral transfer of CMV-specific T-cell receptors (TCRs) for HLA-matched immunotherapy in HCT recipients from CMV-negative donors.<sup>71</sup> Multimer-based selection of antigen-specific T cells has also been applied to CMV reactivation disease.<sup>72,73</sup> In these assays, reactive T cells are enriched from a donor population using peptide multimers bound to magnetic particles. Although initial clinical study results are promising, the strategy requires a high donor blood volume as well as HLA matching and a high baseline frequency of viral-specific T cells.

Manufacturing protocols must be adapted to create T cells capable of targeting Epstein-Barr, adenovirus, and others. Latency type III EBV-specific T cells can be generated by repeated stimulation from irradiated lymphoblastoid cell lines (LCLs) produced from donor peripheral blood mononuclear cells infected with a laboratory EBV strain.<sup>74,75</sup> Multiple clinical studies have demonstrated the efficacy of this protocol for both prophylaxis and sustained remission in more than 70% of post-HCT EBV-associated PTLDs.<sup>76–78</sup> In contrast to EBV and CMV, only a minority of healthy donors have adenovirus-specific T cells, presenting a challenge for source availability. In a manner similar to that discussed for CMV-negative donors, transfecting naive T cells with adenovirus-specific TCRs may prove to be a productive strategy. Positive *in vitro* results have recently been published applying this technology to TCR-transfected  $\gamma/\delta$  subset T cells, which are not alloreactive and thus can be used in mismatched donor-recipient pairs.<sup>79</sup> IFN $\gamma$  capture is a promising emerging selection strategy. Donor T cells are challenged with viral-specific antigens, and the activated IFN $\gamma$ -secreting subset is isolated. Advantages of this approach include shortened manufacturing times and no HLA restriction. CMV-, EBV-, and adenovirus-specific CTLs have been tested in patients with active disease, with response rates of at least 83%, 50%, and 44%, respectively.<sup>10,80–83</sup>

Expanded viral coverage has been achieved from CMV-positive donors using monocytes and EBV-LCLs transfected with adenoviral vectors expressing CMV antigens, producing a single culture of CMV-, EBV-, and adenovirus-specific CTLs. Infusion of these trivirus-specific cells provided immune reconstitutions for CMV and EBV, but response to adenovirus only in the context of reactivation or active infection.<sup>84</sup> Comparable results were observed in patients receiving CMV-negative donor bivirus-specific CTLs.<sup>85</sup> Third-party T-cell lines with common polymorphisms have been used in the treatment of EBV, CMV, and adenovirus.<sup>76,86,87</sup> Generally, the results of these studies have shown significant rates of remission for patients with active infections, although of lower magnitude than traditional specific donor-derived CTLs. Of note, the risk of alloreactivity does not appear to have increased with one case-reported exception of bystander-induced liver GVHD.<sup>88</sup>

Treatment for long-term control of HIV infection has greatly advanced over the previous decades. Curative therapies have been more elusive. In 2009, an HIV1+ patient with acute myeloid leukemia received a stem cell transplant from a CCR5 delta32/delta32 donor and demonstrated undetectable viral load. This suggested the ability to use infection-resistant T-cell therapy to cure or control HIV. Adoptive transfer of ex vivo, zinc-finger nuclease

CCR5 ablated CD4 T cells demonstrated postviral rebound control of viremia in HIV positive patients.<sup>67</sup> Alternatively, CD4 T cells redirected to HIV-infected cells by a CAR and made infection resistant by expression of a C34-CXCR4 fusion are capable of generating specific antiviral response.<sup>89</sup> Given the decades long persistence of gene-modified cells in HIV patients, these therapies hold great promise in long-term control.<sup>90</sup>

### Dendritic cell immunotherapy

DCs, named for their tree-like cytoplasmic projections, are innate hematopoietic cells that reside in all body tissues, including the lymph tissue.<sup>91</sup> Through environmental surveillance, DCs capture protein antigens and present them as peptides in both MHC class I and II molecules (as well as lipid antigens in nonclassical MHCs).<sup>92</sup> Through these DC-initiated interactions, naive T cells are differentiated into antigen-specific and effector T cells, Th cells can be expanded, and B cells, NK cells, and mast cells may be stimulated. Some DC subsets, such as immature DCs in peripheral tissues, can present self-antigens and induce immune tolerance through T-cell depletion or activation of regulatory and suppressor T cells. DCs are known to infiltrate the tumor microenvironment, picking up antigens from tumor cells.<sup>93</sup> Both protumor and antitumor responses are generated by DC interactions.<sup>94–96</sup> As such, DCs engineered to enhance antitumor or prevent protumor activity are a potentially effective path to antitumor immunity.

*Ex vivo* generated DC vaccines have been used with varied levels of success for the treatment of multiple myeloma, colon cancer, renal cancer, prostate cancer, and advanced-stage melanoma.<sup>92</sup> DCs are generally derived from hematopoietic progenitor cells or monocytes with cytokine cocktails, the exact nature of which can yield improved antitumor activity.<sup>93</sup> DC culturing *ex vivo* helps avoid some of the functional deficiencies and tumor tolerance observed with DCs developed in the tumor microenvironment.<sup>97</sup> The selection of the antigen for DC processing is also crucial to immunogenicity. DC loading with nonmutated self-antigens can lead to negative selection due to high-avidity self-antigen, and using unique mutated tumor antigens will require highly patient-specific vaccine formulations.<sup>98</sup> Importantly, dendritic cell vaccines expand the *in vivo* T-cell response to neoantigens, potentially overcoming poor naturally occurring antitumor T-cell effector function.<sup>99,100</sup>

An alternative to single antigen loading is fusing autologous DCs with patient-derived whole tumor cells as an antigen source via electrofusion or polyethylene glycol.<sup>101–103</sup> Processed antigens are presented in both MH class I and II, generating both CD4- and CD8-specific responses. These preparations show increased *in vitro* tumor killing compared to T cells stimulated by DCs pulsed with tumor lysate or apoptotic bodies.<sup>104</sup> Early clinical studies in patients with renal cell and breast cancer demonstrated the excellent safety profile of the DC-tumor cell fusion vaccines, as well as their ability to expand tumor-specific T cells.<sup>105</sup> More recent studies have shown significant clinical success with DC-myeloma cell hybridomas in the period following autologous stem cell transplant leading to the expansion of CD4<sup>+</sup> and CD8<sup>+</sup> myeloma-specific T cells. Seventy-eight percent of the phase II study patients achieved a best response of complete response (CR) or very good partial response, and 47% achieved a CR or near CR.<sup>101</sup>

### NK-cell immunotherapy

NK cells are lymphocytes of the innate immune system responsible for surveillance of malignant transformation or infection. They become educated and acquire function by interaction with MHC class I molecules. Malignant and viral transformed cells may lose

expression of MHC class I in a process of “loss of self” and as a result become “susceptible” to NK-cell killing.<sup>106–108</sup> In the clinic, haploidentical NK cells can be adoptively transferred to treat cancer. Persistence and *in vivo* expansion of NK cells depend on lymphodepleting chemotherapy to make space, eliminating suppressor cells, and releasing endogenous IL15. *In vivo* expansion is also enhanced by cytokine administration of IL2 or IL15. It is believed that IL15 may be superior because IL2 has the downside of stimulating CD25<sup>hi</sup> Tregs. Recent developments of specific NK-cell engagers may help address the limitations of NK-cell therapy, including the complexity of exporting cell therapies and lack of specificity.

Until the discovery of NK-cell receptors that recognize class I MHC (killer immunoglobulin-like receptors [KIRs] and the lectin NKG2 receptors representing the main families), it was assumed that NK cells kill targets in an MHC-unrestricted fashion.<sup>109</sup> Karre and Ljunggren overturned this belief in 1985 with their discovery that NK-cell killing is directed to targets with “missing self.”<sup>110</sup> Inhibitory KIR and NKG2A recognize classical class I MHC or nonclassical HLA-E, respectively, to prevent lysis and thereby induce NK-cell tolerance. Both of these receptor families contain activating receptors and thus engender even greater complexity.<sup>111</sup> Although activating KIR2DS1 can recognize HLA-C2<sup>122</sup> and KIR2DS2 recognizes HLA-A11<sup>112</sup>, many other activating KIR ligands are unknown. Like NKG2A, NKG2C can bind to HLA-E.<sup>113</sup> Notably, NKG2C is an activating receptor of particular importance that is induced by CMV infection.<sup>114,115</sup>

Under normal homeostatic conditions, a balance of activating and inhibitory signals tightly controls NK-cell function. Activating NK-cell receptors include natural cytotoxicity receptors NKp30, NKp44, and NK46, and others such as NKG2D and DNAM-1 that are constitutively expressed on all NK cells.<sup>116,117</sup> Activating receptors recognize stress-induced molecules, HLA class I-related MICA and MICB, class I-like CMV-homologous ULBP proteins, and ligands CD155 (PVR) and CD112 (Nectin-2).<sup>118</sup> Tumors vary in these activating ligands they express. *In vitro*, NK cells can mediate the direct killing of freshly isolated human tumor cells from AML, acute lymphoblastic leukemia, multiple myeloma, neuroblastoma, and ovarian, colon, renal cell, and gastric carcinomas.<sup>119,120</sup> NK cells can also be activated or primed directly by cytokines. After incubating NK cells with cytokines, in particular IL2, IL12, IL18, or IL15, NK cells acquire the capacity to lyse a broad array of fresh and cultured tumor targets not normally sensitive to NK lysis.<sup>121</sup> Furthermore, cytokine-activated NK cells are synergistic with monoclonal antibodies against resistant cell lines *in vitro* and in mouse xenograft models.

Early clinical trials of *ex vivo* IL2-activated autologous NK cells followed by daily subcutaneous IL2 in patients show adoptive transfer of NK cells to be safe, but with limited efficacy in several indications.<sup>122</sup> Because self-MHC can inhibit NK cells, allogeneic NK-cell therapy was pursued. In clinical trials using allogeneic T-cell-depleted HCT from haploidentical donors in patients with AML, Rugierri *et al.* showed that NK-cell cytotoxicity is enhanced if a KIR-HLA class I mismatch occurs. Allogeneic donor-derived NK cells were not associated with GVHD<sup>123,124</sup> suggesting a role for NK-cell therapy in the absence of HCT for patients with AML.

### Mesenchymal stromal cell immunotherapy

Mesenchymal stromal cells (MSCs) were first described in 1968 by Friedenstein *et al.* as bone-marrow-derived, adherent, fibroblast-like cells capable of differentiation to bone.<sup>125</sup> Since then, MSCs have been isolated from a variety of tissue sources, including

umbilical cord blood, Wharton’s jelly, and adipose tissue,<sup>126</sup> and an abundance of studies exploring a wide array of potential clinical utilities have been undertaken. In an initial effort to better characterize MSCs, Dominici *et al.* determined minimal criteria for defining these cells.<sup>127</sup> Subsequent work has moved characterization forward; however, there is still a need for further efforts.<sup>128</sup>

Basic research has suggested several possible mechanisms of action, including the ability of MSCs to home to injured tissue and to secrete bioactive molecules to stimulate recovery/repair and inhibit additional inflammation (Figure 55.3).<sup>129</sup> Additionally, many studies have shown that allogeneic MSCs lack immunogenicity and are essentially immunologically privileged.<sup>130</sup>

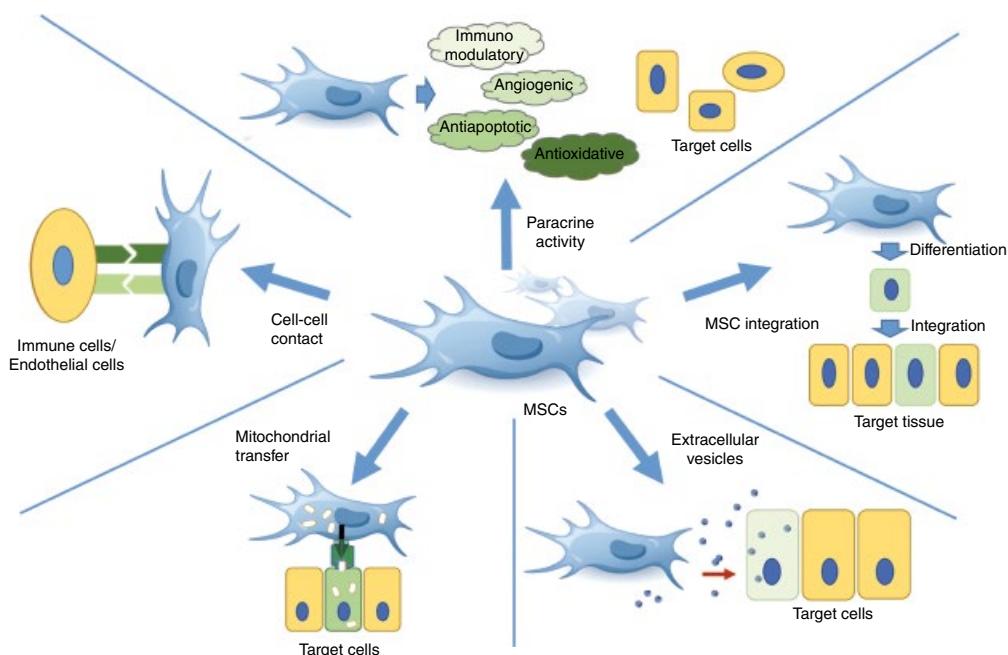
Clinical applications of MSCs can be categorized with some overlap into regenerative medicine and immunomodulation or immunotherapy.<sup>130</sup> As of 2020, more than 300 MSC clinical trials have been completed.<sup>131</sup> Several MSC-based therapies have been approved for use internationally.<sup>132</sup> Nonetheless, clinical trial results have varied significantly. While this could be due to a number of factors, MSC source material likely contributes to some of these discrepancies. It remains to be determined which source is best, and it may be that a certain source is better for a given application. In the case of marrow, donor age has been shown to affect MSC quality and quantity.<sup>133</sup> The importance of age is further exemplified by studies indicating a 2-log greater number of MSCs in umbilical cord blood as compared to adult marrow.<sup>134</sup> In addition to different sources of starting material, studies have involved both autologous and allogeneic (related and unrelated) donations. In trials involving allogeneic donors, the concept of the “qualified” donor has surfaced.<sup>135</sup> As our understanding of MSCs advances, the need for a more robustly characterized, or “qualified,” donor may be appreciated.

Although MSC manufacturing methods have advanced significantly over the past decade, it is still too early to propose standardization of manufacturing. This is particularly true given the variability in starting material, product characterization, as well as dose and administration. Generally, MSCs have been shown to be safe and well tolerated, and several studies point toward efficacy in the immunomodulation/immunotherapy application, making the future appear promising for this cell type. Better understanding of basic immunomodulatory mechanisms will allow for more effective MSC therapies.

### Conclusions

Several adoptive immunotherapies have shown considerable promise in the clinical arena, and their applications have expanded substantially in recent years. Early studies involved nonspecific T cells and lymphokine-activated killer cells, but subsequent studies have applied the knowledge gained in cell biology and immunology to exploit or harness the function of various subsets of T cells, DCs, NK cells, and MSCs. Advances in technology have further enhanced our ability to bring the next generation of cellular immunotherapies into the clinic.

Development of redirected T-cell therapy beginning with observations in allogeneic stem cell transplant and culminating in successes with CD19 CAR T-cell therapy taught us many lessons that are generally applicable to all of cell therapy. Variable starting material and dynamic cell populations behavior in culture limit the ability to generate reproducible final drug products when applying standardized processes. Further, working in closed systems and regulated environments present obstacles to efficient scale-up and scale-out. These major challenges apply to all cell therapies to varying degrees and pose a threat to cell therapies’ sustainable role



**Figure 55.3** Mechanisms underlying MSC-based therapy. Fan *et al.* (2020).<sup>129</sup> Springer Nature / Public Domain.

in the immunotherapy armamentarium. Nonetheless, unprecedented clinical results, particularly in patients with otherwise terminal disease, motivate investigators to contend with and overcome these challenges.

Next-generation cellular immunotherapies promise to expand indications and improve safety. CAR expression in non-T cells, such as macrophages, is a novel approach to the treatment of solid tumors.<sup>136</sup> Compared to T cells, macrophages may have superior ability to penetrate solid tumors and overcome tumor mediated immunosuppression. Anti-HER2 CAR macrophages show potent antitumor activity in humanized mouse models and are under active clinical investigation.<sup>137</sup> CAR expression in NK cells holds similar promise. Incredibly, early-phase CAR-NK-cell trials demonstrated significant complete response rates in patients with CD19 cancers with no associated cytokine release syndrome, avoiding the major adverse effect of most CAR T-cell therapies.<sup>138</sup>

Beyond malignancy, redirected T cells have been designed to treat autoimmune disease. Pemphigus vulgaris is an autoimmune disease in which patients generate autoantibodies against desmoglein 3 which disrupt tissue architecture leading to mucosal blisters. Lee *et al.* generated a chimeric receptor in which a portion of the desmoglein 3 protein (the autoantigen), rather than an antibody fragment, is fused to the transmembrane, costimulatory, and CD3ζ signaling domains. When transduced in T cells, this chimeric auto antigen receptor (CAAR) leads to the specific elimination of autoreactive B-cell clones in preclinical models by engaging cell surface (auto)immunoglobulin.<sup>139</sup> Others have developed similar approaches such as using T regulatory cells to suppress anti-FVIII antibody production in a targeted fashion.<sup>140–142</sup> Though not targeted to specific auto- or alloantibody-producing cells as in the above approaches, the infusion of “conventional” autologous CD19-directed CAR T cells was shown to be effective at inducing rapid remission in a case of refractory systemic lupus erythematosus (SLE).<sup>143</sup> Infusion was subsequently associated with the disappearance of double-stranded DNA serum autoantibodies,

normalizations of C3 and C4 complement levels, decreased proteinuria, and reduction of an SLE disease activity index score from 16 to 0. These striking results suggest that CD19-expressing plasma blasts may be the major source of pathogenic autoantibodies in SLE and suggest the exciting possibility that other autoantibody-mediated autoimmune diseases may be amenable to nonspecific B-cell-targeted CAR T therapy.

In the age of clinical scale gene editing, these and many other exciting scientific developments have translational prospects. Efficient and safe product development still poses a number of challenges. Despite prior clinical successes, new cellular immunotherapy drug development can be a financially risky endeavor. Commercial success is by no means a guarantee; less than a decade ago, the world's first cancer vaccine bankrupted its manufacturer.<sup>144</sup> In this risky environment, cellular immunotherapy development requires significant upfront investment to scale up, scale out, navigate the regulatory system, and build necessary infrastructure. Uncertainty about future reimbursement adds to the risk of early-phase investing. Autologous therapies in particular are subject to extraordinary manufacturing costs as there can be no batch processing to take advantage of economies of scale. These operational challenges must be overcome to successfully commercialize cellular immunotherapies. Streamlined regulatory processes, technologic advances in automated processing, and expansion of allogeneic platforms all may potentially shift the balance to effective and efficient translation of next-generation therapies.

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# Gene therapy applications to transfusion medicine

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## Introduction

The delivery of genetic material into a patient's cells as a medical therapy is referred to as *gene therapy*. Since the first human studies of gene therapy on Severe Combined Immunodeficiency (SCID) in the early 1990s, gene therapy has evolved to have diverse and creative applications across many conditions.<sup>1,2</sup> Here, we review the multiple variables to be considered when gene therapy is performed based on the type of genetic material to insert, the target cells, and the route of administration with a focus on applications of gene therapy to transfusion medicine.

Traditionally, transfusion medicine has provided supportive therapy for patients with inherited hematologic diseases rather than a curative treatment. For example, chronic transfusion can be used to treat anemia associated with sickle cell disease (SCD) or thalassemia, and the administration of clotting factor concentrates can be used to treat or prevent hemorrhage in patients with hemophilia. However, in the future, novel therapies directed at the genetic basis of these diseases may reduce, or even prevent, the need for such patients to require chronic supportive therapy. In this transformation, transfusion medicine will be involved in providing gene-modified cells for curative treatments.

## Gene therapy and transfusion medicine

Gene therapy falls within the purview of transfusion medicine when it requires the infusion of either gene-modified cells or direct administration of vectors that contain the therapeutic genetic material. In addition, transfusion medicine will surely be impacted by gene therapies used to treat diseases that are currently managed with transfusion or factor infusion. For example, hemophilia B, a disease in which patients lack expression of functional clotting factor IX, has been successfully treated with gene therapy in small, early-phase clinical trials.<sup>3</sup> Gene therapy also shows promise as a possible treatment for sickle cell anemia and hemophilia A.<sup>4,5</sup>

Three major considerations for the design of a gene therapy strategy are which gene to insert, which vector to use, and how to administer the vector. Because hematopoietic stem cells can be mobilized, modified, and reinfused to patients, many hematologic disorders are uniquely suited to gene therapy. At the same time, hematological disorders also pose several unique challenges. This is due to the difficulty of delivering the often large genes required to

treat hematologic disease and the risk of malignancy from gene insertion. Yet another challenge is to isolate hematopoietic cells in suitable quantities for transduction-transfection and to support them ex vivo in culture, which is required for most gene modification approaches specific to the hematopoietic system.

## Gene selection and targeted insertion

Based on the disease to be treated, the gene that will be inserted can have a variety of attributes. The gene can provide a functional form of a missing or defective gene, it can augment, inhibit, regulate the expression of a dysfunctional gene, or it can regulate cell survival. Ideal vectors should be designed to target specific cells. The importance of controlling where a gene therapy vector integrates into the genome became clear during the initial gene therapy trials for X-linked immunodeficiency (SCID-X1). Patients with SCID-X1 lack expression of the common gamma chain called CD132 or IL2RG, which serves as a coreceptor for at least six different cytokines. In the initial clinical studies, the retroviral vectors used to deliver the correct copy of the gene integrated into genomic sites that activated the nearby proto-oncogene LMO2. Together with induction of LMO2, overexpression of the common gamma chain caused leukemia in treated patients.<sup>6</sup> The importance of targeting the gene therapy vectors to specific cells was illustrated in a gene therapy trial for hemophilia B, in which the vector itself was administered to the patients. This trial was stopped after the viral vector was detected in a subject's semen, raising concern that a therapeutic gene could be inherited by a subject's future offspring.<sup>7</sup> The health concerns that arose during these initial clinical studies led investigators to return to the bench to develop improved safer approaches for gene therapy. Recent advances in gene therapy strategies and the improvement of existing technologies have reopened the prospect of using gene therapy in the clinic.

## Gene therapy administration

Gene therapy vectors can be delivered to cells ex vivo and then returned to the patient. Alternatively, nonviral and replication-incompetent viral vectors can be directly administered to the patient for transduction of target cells in vivo. There are several benefits to the ex vivo approach (see Table 56.1).<sup>8</sup> By not administering

**Table 56.1** Advantages and Disadvantages of Different Modes of Vector Administration

Manner of Vector Administration	Ex vivo	In vivo
Possible advantages	<ul style="list-style-type: none"> <li>Ability to select transduced cells prior to reinfusion</li> <li>Minimizes infection of nontarget cells</li> <li>Exposes patient to smaller dose of viral vector</li> <li>Ability to insert a suicide gene to inactivate a therapeutic gene</li> </ul>	<ul style="list-style-type: none"> <li>No need to maintain ex vivo fully functional cells that can engraft long term after administration to the patient</li> </ul>
Possible disadvantages	<ul style="list-style-type: none"> <li>Can be technically difficult to maintain and transduce fully functional cells ex vivo</li> </ul>	<ul style="list-style-type: none"> <li>Exposes patient to larger dose of viral vector</li> <li>Vector may have unforeseen effects on unintended target cells</li> <li>Immune system may target the vector for destruction</li> </ul>

the viral vector systemically, the ex vivo approach minimizes infection of nontarget cells, significantly decreases the potential risks of exposing the patient to large amounts of viral vector, and allows for the confirmation of cell transduction prior to reinfusion. On the other hand, direct administration of vectors to the patient overcomes the challenge of maintaining fully functional cells during ex vivo culture. Because there are risks associated with transgene expression in unintended host cells, it is critical that a vector administered in vivo has target specificity or minimal toxicity if expressed by cell types other than the intended target.

### Vector selection

The ideal vector should have no toxicity, lead to minimal inflammation, have a large gene carrying capacity, and should be able to efficiently deliver the gene of interest to the host cells. In addition, vectors should be able to target specific cell types and genetic integration sites within the host. Other factors to consider are whether gene expression needs to be inducible and if lifelong expression is necessary. Unfortunately, a single vector that achieves all of these goals is not yet available. However, based on the above needs, a suitable vector can often be selected. Many research laboratories are developing strategies to optimize current vectors to meet the criteria for an ideal vector. The most well-established viral vector systems for clinical trials are retroviruses, lentiviruses, adenoviruses, and adeno-associated viruses. (Note that the use of retroviral vectors in T cell therapies is covered in Chapter 55, “Chimeric antigen receptor T cells and other cellular immunotherapies,” but such vectors are not limited to use in immunotherapy.) Table 56.2 summarizes the salient features of these viral vectors. Nonviral techniques that are under development for gene therapy are discussed below.<sup>9</sup>

### Ensuring viral vectors are replication incompetent

For safety reasons, all viral vectors for gene therapy must be incapable of replication in the human host. For example, replication-incompetent retroviruses (RIRs) are produced by the removal of the *gag*, *pol*, and *env* genes, which are the genes essential for viral replication and native virus tropism. Before viral vectors can be used in clinical trials, each batch must be tested extensively to ensure that no replication-competent particles are present.

### Risks

The major risks associated with viral vector-based gene therapy are the generation of replication-competent viruses and gene integration that could lead to the activation of oncogenes or loss of function

**Table 56.2** Common Viruses that Have Been Modified for Gene Therapy Research

Family/Subfamily and Characteristics	Specific Virus
<b>Retroviruses</b> (single-stranded RNA, maximal insert size ~8 kb)	Murine leukemia virus Spleen necrosis virus Rous sarcoma virus Avian leukocytosis virus Human immunodeficiency virus, type 1 Human immunodeficiency virus, type 2 Foamy virus Adenovirus, type 5
Oncoretroviruses	
Lentiviruses	
Spumaviruses	
<b>Adenoviruses</b> (double-stranded DNA, maximal insert size ~ 8 kb for first generation; up to 37 kb for new generation)	
<b>Adeno-associated virus</b> (single-stranded DNA, maximal insert size ~ 5 kb)	Adeno-associated virus, type 2 (AAV2), AAV5, AAV6, AAV2/6, AAV8

of tumor suppressor genes. Evidence of the importance of the continual evaluation of risks related to gene therapy is highlighted by the recent revelation that two patients who participated in an NHLBI-funded trial of a lentiviral vector for the treatment of sickle cell disease went on to develop myeloid neoplasms, leading to the pausing of the trial in February 2021 in order to assess whether the leukemia was due to the chemotherapy administered prior to transplantation with modified cells or to the modified cells themselves.<sup>10</sup>

### Recombination to form replication-competent viruses

Replication-competent viruses require the presence of the *gag*, *pol*, and *env* genes encoding the retroviral core structure, reverse transcriptase, and the viral envelope proteins, respectively. Such replication-competent viruses can develop either by the recombination of the constituent parts of the vector system with separately encoded viral sequences in the vector packaging cell lines at the time of viral production or by the activation of endogenous proviral sequences in cells after infection with replication incompetent vectors. The significant risks of retroviral reactivation for gene therapy were elucidated when primate studies were performed in the early 1990s. In these studies, CD34-selected primate marrow cells were infected with what was thought to be pure replication-incompetent virus produced by packaging cell lines. There were, however, some replication-competent viruses that had not been detected by existing assays. Upon infusion, three of eight primates subsequently developed lymphomas containing the active, rearranged retrovirus.<sup>11</sup> Since that time, multiple approaches have been incorporated

to optimize safety by improved assays for any replication-competent vectors, and minimizing the regions of homology between the vectors, packaging cell lines, and endogenous retroviruses. Currently, clinical trials require stringent testing to guarantee that the retroviral vector to be used in humans is entirely replication incompetent.

### Insertional mutagenesis

Genotoxicity occurs when genetic material is inserted in proximity to a proto-oncogene or disrupts expression of a tumor suppressor gene. The presence of the strong viral promoter near a proto-oncogene can lead to transcriptional activation of the gene. This has led to the development of malignancy in patients enrolled in a clinical trial for X-linked immunodeficiency.<sup>6,12–14</sup> In addition, insertion of a transgene into a portion of the genomic DNA that is essential for cell survival can lead to disruption of critical cell processes, resulting in cell death.

Advancements have allowed gene therapy to become more precise. For example, it is now possible to use AAV to insert a therapeutic gene at a specific and desired location.<sup>15</sup> The groundbreaking work on Clustered Regularly-Interspaced Short Palindromic Repeats (CRISPR), which led to the winning of the 2020 Nobel Prize in Chemistry to Dr. Jennifer Doudna and Dr. Emmanuelle Charpentier, has paved the way for targeted gene therapy, opening the door for many potential breakthroughs.<sup>16,17</sup>

### Nonviral gene therapy vectors

Nonviral vectors for gene therapy are also undergoing intensive investigation.<sup>18</sup> Unlike viral vectors, nonviral methods do not need to overcome the extensive immune mechanisms that inhibit transduction *in vivo*. There is also no risk of replication-competent forms of the vector. These methods include DNA microinjection, chemical transfection of exogenous DNA, or nanoparticles filled with DNA or RNA. This is the approach used for the SARS-CoV-2 vaccines produced by Moderna and Pfizer, as discussed below.<sup>19</sup>

Nanoparticles have numerous utilities. When it comes to vaccines, they can be crosslinked to a protein antigen to promote antigen immunogenicity while decreasing antigen degradation.<sup>20,21</sup> When protein and RNA-based vaccines are administered with nanoparticles, there is little to no risk of incorporation into the host genome, and the RNA vectors and proteins they encode are short-lived. With these advantages in mind, beyond their practical applications to infectious diseases, there has also been interest in their utility to other scopes of medical practice such as the development of cancer vaccines, in order to boost the host immune system to achieve antitumor activity.<sup>21</sup>

### Gene editing

Various gene-editing approaches, such as transcription activator-like effector nucleases (TALENs), zinc finger nucleases (ZFNs), and CRISPR with CRISPR-associated 9 (Cas9), have been described.<sup>22</sup> All three of these technologies use exogenous programmable nucleases (e.g., FokI, Cas9, and Cpf1) that are designed to generate sequence-specific double-strand breaks (DSBs) of the DNA. DSBs activate endogenous cellular repair mechanisms that will induce homology-directed repair (HDR) of the mutated sequence in the patient's DNA in the presence of donor DNA containing the corrected sequence as template.<sup>23</sup> Gene editing can be used to disrupt, add, or correct genes in animal cells.<sup>22</sup>

Studies that have assessed the possible application of gene-editing techniques to hemoglobinopathies are discussed in the

"Transfusion-medicine-related gene therapy trials" section. Notably, gene-editing approaches have also shown promise for other conditions. For example, a trial of HIV patients treated with autologous T cells that were engineered to have dysfunctional CCR5 genes using a targeted ZFN was reported in 2014.<sup>24</sup> Although 1 of the 12 patients in the trial had a transfusion reaction to the infusion of T cells, the study found that the infusion of these engineered cells appeared to be safe.<sup>24</sup> Long-term monitoring of study participants is ongoing to confirm the safety of this approach. There are currently at least 19 interventional clinical trials with CRISPR/Cas9 gene-editing technology, spanning from blood diseases to cancer and HIV.<sup>23</sup>

### Transfusion-medicine-related gene therapy trials

Herein, we review select conditions for which gene therapy is rapidly progressing as a novel treatment strategy.

#### Hemophilia B

Hemophilia B is an X-linked genetic disease that results in bleeding due to reduced or absent activity of factor IX, a serine protease synthesized in the liver. At present, bleeding prevention in hemophilia B is typically accomplished via administration of recombinant factor IX concentrates.

Treatment alternatives that do not require factor administration are needed. Severe hemophilia B is an attractive target for gene therapy because even a small increase in endogenous factor IX activity could reduce dependence on factor IX infusions and prevent spontaneous bleeding episodes. In the late 1990s, researchers using various animal models of hemophilia B found that gene therapy with an adeno-associated virus (AAV) vector could be used to induce sustained circulation of factor IX.<sup>25,26</sup> Shortly thereafter, a human trial established that the administration of an AAV vector to human subjects was safe.<sup>27</sup> A subsequent study of seven patients with severe hemophilia B showed that treatment with a recombinant AAV vector (rAAV-hAAT-F.IX) led to only transient elevations in circulating factor IX.<sup>28</sup> Because the reduction in factor IX activity was associated with an increase in hepatic transaminases, the study authors concluded that immune-mediated destruction of transduced hepatocytes expressing the AAV capsid may have occurred. In contrast to animal models, humans are frequently infected by AAV during childhood. This may explain why animals were capable of a sustained response to AAV-mediated gene therapy whereas humans generated a secondary immune response that led to the destruction of transduced hepatocytes.

Early-phase trials have established the potential for AAV-mediated gene therapy approaches to improve bleeding outcomes in patients with hemophilia B.<sup>29,30</sup> However, controlling immune responsiveness to engineered cells remains a barrier.<sup>29</sup> It appears that the immune response to engineered cells is primarily driven T cells and can result in loss of transduced gene product in a dose-dependent manner.<sup>28</sup> Consideration of the vector quantity that is required to attain sufficient factor IX expression level to provide clinical benefit, without triggering an unfavorable immune response, is an important topic for continued evaluation.<sup>29</sup>

#### Hemophilia A

Hemophilia A is caused by a deficiency in factor VIII, which is the only clotting factor synthesized by endothelium (rather than hepatocytes). Similar to factor IX deficiencies, even a small increase in factor VIII activity would be expected to protect patients from

many bleeding episodes. Therefore, hemophilia A is another appealing target for gene therapy.

However, unlike factor IX, factor VIII is a large protein that is difficult to efficiently express in vitro. Attempts to induce cultured cells to produce factor VIII can result in the production of misfolded factor VIII protein, which can, in turn, activate the unfolded protein response and trigger apoptosis of the cells. Also, because the gene encoding factor VIII is so large, in the absence of complex laboratory manipulations, it is too large to fit into the AAV vector.<sup>31</sup> Finally, patients with hemophilia A are more likely to develop circulating inhibitors to factor VIII. Such inhibitors result in factor neutralization and require bypass factor therapy, such as factor VII. For these reasons, gene therapy for hemophilia A lags behind gene therapy for hemophilia B.

The complexity of the immune response in this application is demonstrated by the results of a recent phase I/II study on valoctocogene roxaparvovec (BMN 270), which uses an AAV-based vector encoding factor VIII. Reassuringly, this study did not identify the development of factor VIII inhibitors.<sup>32</sup> However, antibodies to the AAV5 capsid were detected.<sup>32</sup> While immunity to the AAV5 capsid was not harmful to study participants, this reactivity will need to be further addressed.<sup>32</sup>

### Hemoglobinopathies and thalassemias

Much like hemophilia, the hemoglobinopathies are attractive targets for gene therapy. Sickle cell disease (SCD) and thalassemias are some of the most common single-gene defects worldwide. SCD affects approximately 275,000, and β-thalassemia affects approximately 56,000 newborns every year.<sup>33</sup> Current treatment for these diseases is primarily supportive therapy, which includes chronic transfusion for the management of sickle crises and/or anemia. However, this approach comes with several consequences, including alloimmunization to minor red blood cell antigens and iron overload, and even under ideal conditions transfusion does not prevent all morbidities associated with these chronic diseases.<sup>34</sup> Thus, a curative therapy is highly desirable. One option is hematopoietic stem cell transplantation, although this approach usually requires a matched sibling donor who is not affected.<sup>35</sup> Alternative therapies that lead to decreased reliance on transfusion and other supportive therapies would be beneficial. Compared to bone marrow transplantation, gene therapy would bypass the need to find a donor and the risks of long-term immunosuppression.

These chronic anemias have traditionally been difficult to treat with gene therapy as they require large genetic sequences with stable, long-term expression to be effective.<sup>4</sup> In addition, they require a high level of gene expression for correction, which is difficult to achieve with traditional vectors.<sup>4</sup> Mouse models of SCD have demonstrated that lentiviral vectors carrying an antisickling globin are able to persistently express the globin gene at levels high enough for symptomatic correction.<sup>36</sup> Understanding of hereditary persistence of fetal hemoglobin and its well-characterized protective effects in SCD has sparked special interest in exploring gene therapy as a means to turn on the beta chain of fetal hemoglobin (the γ-globin gene) to produce this protective phenotype.<sup>37</sup> As discussed below, inactivation of the transcriptional repressor BCL11A can lead to reactivation of endogenous γ-globin gene expression, making BCL11A and its genomic binding sites excellent targets for gene-editing approaches.

Clinical trials for the treatment of β-thalassemia with gene therapy have progressed more quickly than for SCD. Approaches that use viral-mediated addition of a γ-globin gene expressed from a

**Table 56.3** Some Key Ongoing Clinical Trials in the Realm of Gene Therapy

Condition	Clinical Trials (with ClinicalTrials.gov Identifier)
Sickle cell disease (SCD)	Gene transfer for sickle cell disease: Single infusion of autologous bone-marrow-derived CD34+ HSC cells transduced with the lentiviral vector containing a short-hairpin RNA targeting BCL11a (NCT03282656) A study evaluating gene therapy with BB305 lentiviral vector in sickle cell disease: genetic LentiGlobin BB305 drug product for SCD (NCT04293185) Stem cell gene therapy for sickle cell disease: biological βAS3-FB vector-transduced peripheral blood CD34+ cells (NCT02247843) CSL200 gene therapy in adults with severe sickle cell disease: biological: autologous enriched CD34+ cell fraction that contains CD34+ cells transduced with lentiviral vector encoding human γ-globin G16D and short-hairpin RNA734 (NCT04091737)
Hemophilia A	Study to evaluate the efficacy and safety of PF-07055480 in moderately severe to severe hemophilia A adults: biological recombinant AAV2/6 human factor VIII gene therapy (NCT04370054) Lentiviral FVIII gene therapy: biological YUVA-GT-F801 (NCT03217032) A gene transfer study for hemophilia A: genetic SPK-8011 (NCT03003533) ASC-618 gene therapy in hemophilia A patients: biological ASC-618 (NCT04676048)
Hemophilia B	A factor IX gene therapy study (FIX-GT): biological FLT180a (NCT03369444) Lentiviral FIX gene therapy: biological YUVA-GT-F901 (NCT03961243) A study to evaluate the efficacy and safety of factor IX gene therapy with PF-06838435 in adult males with moderately severe to severe hemophilia B: biological PF-06838435/fidanacogene elaparvovec (NCT03861273) A study to evaluate the efficacy and safety of valoctocogene roxaparvovec, with prophylactic steroids in hemophilia A: biological valoctocogene roxaparvovec (NCT04323098)
Beta thalassemia	Gene therapy for beta-thalassemia major using autologous hematopoietic: genetic autologous CD34+ cells genetically modified (NCT03276455) Safety and efficacy evaluation of gamma-globin reactivated autologous hematopoietic stem cells: biological gamma-globin reactivated autologous hematopoietic stem cells (NCT04211480) A safety and efficacy study evaluating CTX001 in subjects with transfusion-dependent beta-thalassemia: biological CTX001 (NCT036555678)

β-globin promoter to ensure expression in adults have been promising in mouse models.<sup>38</sup> A single patient with β<sup>E</sup>/β<sup>0</sup>-thalassemia, who lacked an HLA-matched hematopoietic stem cell donor, was reported to have achieved transfusion independence three years after undergoing transplantation with autologous cells that had undergone lentiviral β-globin gene transfer ex vivo.<sup>39</sup>

Other novel approaches to hemoglobinopathies use a gene-editing technique. Early studies using induced pluripotent stem cells showed that TALENs and CRISPR/Cas9 could be used to correct mutations causing β-thalassemia,<sup>40,41</sup> and CRISPR/Cas9 and ZFNs could be used to correct the SCD mutation.<sup>42,43</sup> TALENs have been reported to correct mutations causing α-thalassemia major in induced pluripotent stem cells.<sup>44</sup> It is hoped that gene therapies for these conditions will continue to progress quickly through clinical trials. Some key trials are discussed in detail below. For a more comprehensive list of current clinical trials, see Table 56.3.

### BCL11A targeted therapy (lentiviral approach)

One recent trial leveraged a lentiviral vector targeting BCL11A in autologous CD34+ cells with the goal of increasing fetal

( $\gamma$ -globin-containing) hemoglobin among patients with sickle cell disease.<sup>45</sup> The approach used a short hairpin RNA (shRNA) targeting the BCL11A gene.<sup>45</sup> The infusions were well tolerated, with all toxicity believed to be linked to the preparative chemotherapy.<sup>45</sup> Remarkably, the investigational therapy resulted in the production of fetal hemoglobin as well as improved clinical parameters.<sup>45</sup>

### BCL11A-targeted therapy (CRISPR approach)

Investigators reported the use of CRISPR-Cas9 to target BCL11A in autologous patient cells obtained from one patient with sickle cell disease and another with transfusion-dependent thalassemia.<sup>46</sup> Twelve months after myeloablation, and infusion of these autologous CRISPR-modified cells, both patients demonstrated increases in fetal hemoglobin, signifying successful engraftment of the modified stem cells.<sup>46</sup> Incredibly, neither patient required transfusions and sickle cell pain crises were eliminated, thus correlating engraftment with improved clinical markers.<sup>46</sup>

### Adverse effects

In early 2021, a clinical trial for severe sickle cell disease was stopped after two participants who had received a lentiviral vector-based investigational treatment unexpectedly developed myeloid neoplasms (NCT02140554).<sup>10</sup> This raised concerns for potential side effects of gene therapy stemming from lentiviral vectors more generally. As a measure of caution, the National Heart, Lung, and Blood Institute (NHLBI) halted a separate gene transfer study for sickle cell disease (NCT03282656) targeting BCL11A pending further study of the myeloid neoplasms that occurred in NCT02140554.<sup>10</sup> The evaluation of this safety concern will be key to the future development of lentiviral gene therapy for sickle cell disease as well as other chronic anemias.

### Wiscott-Aldrich syndrome (WAS)

WAS is a rare, X-linked immune deficiency caused by mutations in the WAS gene. Patients classically present with thrombocytopenia with small platelets, susceptibility to infections, and eczema.<sup>47</sup> Patients also have an increased risk for the development of autoimmune diseases and cancer.<sup>47</sup> Patients may be treated with a stem cell transplant if an HLA-matched donor is available. Patients with WAS may come to the attention of the transfusion service as a result of bleeding in the context of thrombocytopenia.

A phase I/II clinical trial of three pediatric patients with WAS, who lacked an HLA-matched donor or were otherwise ineligible for stem cell transplant, was reported in 2013.<sup>48</sup> In this trial, autologous CD34+ cells were transduced with a normal WAS gene ex vivo using a lentiviral vector. When the engineered hematopoietic stem cells were infused, they engrafted successfully. The patients went on to demonstrate improvements in hemostasis and immunity.<sup>48</sup> In recent years, data from additional clinical trials have also demonstrated the short-term and medium-term efficacy of gene therapy using a lentiviral vector for WAS.<sup>49</sup> There has been some restoration of immune function post therapy with protection from infectious agents and improved control against autoimmune disease.<sup>49</sup>

Promising beginnings for CRISPR/Cas9-based editing as a future treatment for WAS have also been reported. In a mouse model of WAS, genetically modified cells persisted for at least 26 weeks after treatment, and the approach was not associated with overt toxicity to the animals. In human cells, CRISPR/Cas9 has been shown to correct the majority of WAS gene mutations in vitro, without interfering with basic cellular machinery.<sup>50</sup>

### Severe combined immunodeficiency

Finally, in the realm of genetic immunodeficiencies, severe combined immunodeficiency (SCID) deserves a brief mention. Adenosine deaminase (ADA) is responsible for approximately one-half of the cases of autosomal recessive SCID.<sup>51</sup> Patients with ADA-SCID have a high risk of infections by opportunistic organisms at an early age, and up to this point, definitive treatment has relied predominantly on bone-marrow transplantation from a histocompatible-related donor.<sup>51</sup> Despite its rarity, SCID is considered a prime candidate for gene therapy, and since the late 1990s, scientists have been exploring gene therapy via gamma-retroviral vectors using multiple infusions of genetically corrected autologous peripheral blood lymphocytes.<sup>51,52</sup> After a decade of efforts, the introduction of low-intensity conditioning represented the crucial step in achieving stable gene-corrected hematopoietic stem cell engraftment and therapeutic levels of ADA-expressing cells.<sup>52</sup> Finally, in 2016, retroviral therapy received marketing approval in Europe for patients with ADA-SCID without a suitable human leukocyte antigen-matched related donor, becoming the only viral-based gene therapy protocol approved for ex vivo CD34+ gene therapy.<sup>52</sup> Overall, the safety and efficacy of viral vector-based treatments for SCID has fundamentally revolutionized the treatment of this rare disease.<sup>52</sup>

### Gene therapy and vaccines: a unique frontier of exploration

In early 2020, COVID-19, a respiratory and vascular multisystem disease caused by SARS-CoV-2, rapidly transformed into a pandemic.<sup>53</sup> The pandemic resulted in expedited funding and research efforts aimed at containing the crisis, and resulted in the first widespread use of mRNA-based vaccines.<sup>54,55</sup> Two such vaccines, one made by Pfizer in collaboration with BioNTech and the other by Moderna in collaboration with United States National Institute of Allergy and Infectious Diseases, utilize lipid–nanoparticle-encased RNA molecules.<sup>55</sup> Mechanistically, after the mRNA is taken up by human cells, it encodes a modified (stabilized) version of the SARS-CoV-2 spike protein.<sup>54</sup> Consequently, an immune system response is generated.<sup>55</sup> Data from clinical trials has shown that both vaccine candidates are over 94% effective in preventing symptomatic SARS-CoV-2 infection.<sup>54,55</sup> While side effects from the vaccines appear to be mild overall,<sup>55</sup> the ongoing global vaccinations are likely to shed light on population-wide efficacy and unknown potential long-term side effects. Furthermore, if successful and safe in the long term, the development of other genetically based vaccines seems likely.

### Summary

Gene therapy continues to offer much promise for the treatment of genetic and acquired diseases. Transfusion medicine laboratories are currently involved in clinical gene therapy trials and, in the future, as these therapeutic modalities become more mainstream, will likely play a role in what may be “routine” administration of gene therapy vectors and vector-infected cells. Finally, we have listed, in tabular format (Table 56.3), some of the ongoing gene therapy clinical trials for selected pathologic conditions.

### Disclaimer

The authors have disclosed no conflicts of interest.

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## CHAPTER 57

# Tissue engineering and regenerative medicine

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As the number of individuals awaiting organ transplant continues to increase, tissue engineering and regenerative medicine technologies present promising new options for addressing the critical tissue and organ shortages that currently exist.<sup>1</sup> This interdisciplinary field integrates engineering and life science principles in an effort to restore function to impaired tissues and organs.<sup>2</sup> Sophisticated grafts, often in combination with cellular therapies, are designed to mimic native tissues in an effort to promote or enhance the body's intrinsic regenerative capacity to restore tissue integrity. The fields of tissue engineering and regenerative medicine have become expansive and diverse in the last few decades, creating the potential for patient-specific, engineered treatments. Great progress has been made in identifying biomaterials that support the healing process. However, only a limited number of approaches that incorporate cellular strategies have been successfully translated and approved by the FDA for commercialization,<sup>3–5</sup> even though the use of cellular components in tissue engineering has been shown to improve the extent of regeneration and increase scaffold integration to the native tissue.<sup>6</sup> The inclusion of biochemical factors and functional groups in natural and synthetic scaffold materials provide a microenvironment that promotes cell attachment, differentiation, and migration to facilitate tissue repair,<sup>7</sup> while the scaffold architecture and porosity support vascularization and nutrient diffusion to nascent tissue.<sup>8,9</sup> Decellularized tissues from allogenic and xenogeneic sources have demonstrated potential in a number of applications,<sup>10,11</sup> and 3D printing provides a path forward for fabricating networks that mimic the native tissue matrix with high fidelity.<sup>12</sup> New strategies for large scale in vitro cell expansion and tissue maturation will advance the prospect of "off-the-shelf" regenerative therapies that meet FDA guidelines and are readily available for patient treatment.<sup>5</sup>

In the previous edition of this book, Niklason et al. provide a comprehensive review of tissue engineering and regenerative medicine technologies related to skin, blood vessel, bone, cartilage, cardiac, urology, and corneal tissue. In this edition, we have added several additional areas including lung, reproductive system, peripheral nerve, skeletal muscle, gastrointestinal system, and renal tissue engineering, as well as provided review of some of the latest advances.

## Lung Tissue Engineering

Pulmonary disease is a worldwide public health problem. Recent developments in stem cell biology and lung tissue engineering have shown promise in addressing various pulmonary diseases, including chronic obstructive pulmonary disease,<sup>13</sup> congenital lung hypoplasia,<sup>14</sup> sarcoidosis,<sup>15</sup> cystic fibrosis,<sup>16</sup> interstitial fibrosis,<sup>17</sup> and pulmonary hypertension.<sup>18</sup> While lung transplantation appears to be the ultimate remedy for advanced lung diseases, significant shortages of compatible donor organs is an issue and transplant recipients must endure lifelong immunosuppression. Engineering an entire lung using autologous cells or allogenic stem cells seems optimal for increasing organ availability and minimizing the need for post-transplantation immunosuppression.

## Scaffolds

Current lung tissue engineering research has focused on generating whole lung or constituent lung parts by seeding cells onto scaffolds with the ultimate aim of grafting these constructs into the human body.<sup>19</sup> Generally, these scaffolds are classified into two groups, based on the source or origin: natural and synthetic. Natural, extracellular matrix (ECM)-derived scaffolds obtained from decellularized donor lungs retain the unique structural and functional architecture of this complex tissue.<sup>20</sup> Developing whole lung decellularization procedures that yield native matrices is a rapidly growing field. Studies have addressed the utilization of different reagents and techniques to preserve structural proteins such as collagen, laminin, elastin, fibronectin, and other growth promoting biomolecules that are essential for regulating cellular behavior. Decellularization processes have included detergents, such as sodium dodecyl sulfate (SDS), 3-[3-Cholamidopropyl] dimethylammonio]-1-propanesulfonate hydrate (CHAPS), and Triton X-100, which have been utilized to remove cells from whole lung tissue. The zwitterionic detergent CHAPS has been shown to preserve airway and vascular structures with retention of collagen and elastin but does not conserve glycosaminoglycans (GAGs).<sup>21</sup> Decellularization with SDS also maintains alveolar and vascular structures,<sup>22</sup> while decellularization with Triton X-100 and SDS with DNase allows for retention of collagen, elastin, laminin, and GAGs.<sup>23</sup> Understanding the relative roles of lung tissue ECM

elements in cell attachment, differentiation, migration and immunogenicity will help to identify the advantages and disadvantages of each technique. These decellularized matrices have been shown to be successfully reseeded with pulmonary cells such as epithelial,<sup>21</sup> endothelial cells,<sup>22</sup> and fetal alveolar type II cells.<sup>23</sup> Notably, in experimental models, instances of gas exchange have been observed for short-term intervals.<sup>21</sup>

Other examples of natural scaffolds include biological materials such as collagens, proteoglycans, and Matrigel®. In a study using fetal rat lung cells, Gelfoam®, a gelatin-based scaffold, was grafted in combination with the cells into lung parenchyma. The rat pulmonary cells formed into “alveolar-like structures” at the border between the Gelfoam sponge and the surrounding lung tissue and positive immunohistochemical staining for epithelial and endothelial cells.<sup>24</sup> Another study performed with fetal rat lung cells utilized a scaffold consisting of a Type I collagen-chondroitin 6-sulfate matrix and demonstrated cell forming and maintaining alveolar-like structures.<sup>25</sup> Natural ECM hydrogels such as Matrigel and Type I collagen gels are rapidly growing in use due to their biochemical and mechanical features, which provide an appropriate 3D matrix for pulmonary cell morphogenesis and differentiation.<sup>26,27</sup>

Synthetic materials such as polyglycolic acid (PGA), poly lactic-co-glycolic acid (PLGA), and poly-D,L-lactide (PDLLA) are also being extensively studied for lung tissue engineering due to their porous properties, elasticity, and biodegradability.<sup>28–31</sup> Synthetic polymers, however, often fail to drive the differentiation of cultured cells<sup>32</sup> and frequently initiate an inflammatory response. Surface modification of these synthetic matrices with natural ECM proteins such as laminin, collagen, or Matrigel can facilitate cell attachment and subsequently support the development of a pulmonary cell phenotype.

### Cell source

The lung is a highly complex organ comprised of diverse cell types including parenchymal, ductal, vascular, and structural cells (e.g., fibroblasts), most of which would be necessary in order to engineer a replicate organ.<sup>33,34</sup> The use of autologous cells reduces the potential for rejection and therefore the need for immunosuppressive agents. Despite extensive research into creating lung tissue constructs using primary pulmonary cells, these cells can rapidly lose phenotypic marker expression in vitro and the expansion to large numbers of cells can be difficult and costly.<sup>35–40</sup> A key challenge for the reconstruction of human lung tissue is the lack of an abundant source of pulmonary epithelial cells.<sup>41–43</sup> Utilization of stem cells presents an alternative avenue that may overcome the limitations experienced with primary cells.

Stem and progenitor cells, including cells such as embryonic stem cells (ESCs), induced pluripotent stem cells (iPSC), lung stem cells, umbilical blood cord (UBC) stem cells, and mesenchymal stem cells derived from bone marrow or adipose tissue, have been extensively studied in the lung tissue industry. Embryonic stem cells are true pluripotent stem cells with the capacity to differentiate into all three germ layers and evolve into any cell lineage, and have therefore been used to generate upper airway epithelial cells, alveolar epithelial type II (AECII)-like cells, and pneumocytes.<sup>24,31,44</sup>

In addition to rapid in vitro expansion, a beneficial effect of using mesenchymal stem cells (MSCs) in tissue engineering is that they secrete paracrine factors that modulate the immune response. In a recent study using MSC microvesicles in an ex vivo *E. coli*-injured human lung model, alveolar fluid clearance was restored, and a reduction in inflammation and antimicrobial activity was observed.<sup>45</sup>

Mesenchymal stem cells have also demonstrated the capacity to differentiate into Type I collagen-producing fibroblast-like cells<sup>46</sup> and lung epithelial phenotypes<sup>47</sup> when grown on a decellularized lung scaffold, supporting a potential role for these cells in addressing pulmonary cell therapies and tissue engineering.

Induced pluripotent stem cells are an attractive cell source for the generation of lung epithelial cells as they have the potential to avoid immune rejection and can be expanded to cell numbers necessary for repopulating decellularized human-sized lung scaffolds. Additionally, these stem cells can be induced into a population of type II epithelial-like progenitor cells using a stepwise differentiation protocol<sup>48</sup> and have been shown to repopulate the alveolar and airway compartments of decellularized lungs.<sup>49</sup> Introducing differentiated iPSCs into decellularized lung scaffolds has been shown in multiple studies to result in perfusable and functional vascular lumens.<sup>50,51</sup>

### Bioreactors

Bioreactors provide precisely controlled physicochemical factors such as pH, O<sub>2</sub>, CO<sub>2</sub>, and metabolites for supporting the growth of engineered tissues.<sup>32,52</sup> In addition to adequate nutrient transfer, oxygen transport, and waste removal, lung tissue bioreactors require mechanical stimuli, including an air-liquid interface and ventilation generated by negative-pressure to support pulmonary tissue development.<sup>27</sup> A decellularized scaffold is often perfused via cannula at physiological pressure, and a syringe pump is used to simulate negative-pressure ventilation by inhalation and exhalation of liquid medium.<sup>53</sup> Microfluidic-based bioreactors can be cost-effective and high throughput, which is ideal for pharmaceutical and toxicity studies. Macroscale automatable closed systems that benefit from pressure-controlled perfusion and ventilation would be necessary, however, for generating transplantable lungs that would progress into clinical therapies. Notably, a fully automated high-throughput bioreactor system capable of ex vivo lung recellularization and biomimetic culture under varying perfusion and ventilation parameters has been reported and can easily be scaled to support the maturation of 20 or more cultured lungs.<sup>54</sup>

### Bioprinting

Three-dimensional (3D) bioprinting has recently emerged as a potential method for generating bioengineered tissues or supporting structures. The lung is a large complex tissue containing multiple cell types, interconnected bifurcating airways, and varying degrees of vasculature all of which pose significant fabrication challenges. A major advantage of bioprinting is the ability to print complex patterns within a large geometry. There are currently no published reports on attempts to 3D print whole lung tissues that are capable of gas exchange; however, 3D-printing technologies have advanced enough for printing supporting structures such as trachea and bronchus.<sup>55,56</sup> In general, most studies addressing tracheal construction combine biological hydrogels seeded with cells that surround a sturdy polymer structure. For example, a tissue-engineered trachea was created by assembling chondrocyte-seeded, functionalized gelatin sponges into the grooves of a 3D-printed polycaprolactone (PCL) bellows scaffold; the construct demonstrated similar mechanical behavior to that of a native trachea and substantial regeneration of tracheal cartilage in vivo.<sup>57</sup> Similarly, a 3D-printed biodegradable PCL tracheal scaffold impregnated with chondrocyte laden hydrogel was grafted in a rabbit animal model and demonstrated cartilage formation.<sup>58</sup> 3D printing has been applied clinically for the treatment of tracheobronchomalacia<sup>59</sup> and

tracheal collapse with 3D-printed patient-specific tracheal splints for supporting structures.<sup>60,61</sup> The future of lung bioprinting will depend upon overcoming multiple obstacles including cell sourcing, maintaining lumen patency, maintaining a sealed air/fluid interface, and developing a dynamic bioreactor to provide efficient gas and nutrient transport while maintaining mechanical stimulation.<sup>62</sup>

### **Cell therapy for pulmonary complications of COVID-19**

The coronavirus, SARS-CoV-2, has caused a novel coronavirus disease (COVID-19) that has spread worldwide since its outbreak in 2019. COVID-19 has the potential to cause severe pulmonary complications such as pneumonia and acute respiratory distress syndrome (ARDS). Severe ARDS is often accompanied by a massive attack of proinflammatory cytokines and chemokines, which can cause vascular leakage and tissue inflammation in the affected organs. Immunomodulatory intervention is therefore desired in order to mitigate disease progression and severity.<sup>63</sup> Stem cells, natural killer (NK) cells, T cells, dendritic cells (DN), and exosomes have been identified as leading therapeutic candidates for regenerating damaged lung tissue.

Among cell-based therapies currently registered in clinical trial for COVID-19, the most prevalent approach has been mesenchymal stem cell (MSC) therapy. Mesenchymal stem cells can easily be isolated from a variety of tissues and are well known for possessing immune-modulatory capabilities that manifest through multiple mechanisms.<sup>64</sup> In clinical studies for COVID-19 treatments, the use of human umbilical cord mesenchymal stem cells (hUCMSCs) was shown to produce inflammatory remission in a patient with severe level symptoms.<sup>65</sup> Similarly, case reports have indicated that clinical improvements and changes in the inflammatory and immune cell populations occurred following MSC transplantation in critically ill patients with COVID-19.<sup>66,67</sup> Wharton's jelly-derived MSCs (hWJCs) were additionally reported as treatment for a patient with severe symptoms due to COVID-19 pneumonia. Significant improvement was observed two days following hWJC transplantation.<sup>68</sup> Exosomes derived from allogeneic bone marrow MSCs (ExoFlo™) and administered as treatment in 24 patients exhibiting severe symptoms due to COVID-19 also produced significantly improved clinical status and oxygenation.<sup>69</sup> These reports demonstrated tolerance for allogenic MSCs as well as promising clinical outcomes in COVID-19 treatment.

Adoptive NK and chimeric antigen receptor (CAR)-NK cells provide another promising approach to treating severe COVID-19 symptoms.<sup>70,71</sup> The NK cells are innate immune responders critical for viral clearance and immunomodulation. Several clinical trials have been initiated to evaluate the safety and immunogenicity of adoptive and CAR-NK cells.<sup>71</sup> SARS-CoV-2-specific T cells from recovered COVID-19 patients maintained an immune response six months following infection.<sup>72</sup> These allogeneic virus-specific T-cells have therefore been introduced as an emergency treatment for critically ill patients (ClinicalTrials.gov Identifier: NCT04401410 and NCT04351659).

### **Reproductive System Tissue Engineering**

Reproductive system disorders are generic terms relating to diseases that damage reproductive tissues. These include congenital and acquired abnormalities, malignant tumors, trauma, infectious etiologies, inflammation, and iatrogenic injuries.<sup>73</sup> At present, no

effective treatments that target congenital and acquired abnormalities, malignant tumors, and traumatic injuries exist. Tissue for autologous grafting is specialized and therefore limited, and may lead to secondary donor site damage. Allogeneic transplantation faces issues regarding donor shortages, as well as problems with tissue rejection and compatibility. The application of regenerative medicine technologies as a biological alternative is currently under consideration for treating several types of reproductive system disorders.

### **Scaffolds**

A wide variety of biomaterials have been utilized for scaffolding in reproductive tissue engineering including acellular matrices, collagen, fibrin, silk, and synthetic materials such as PLA, PLGA, and PCL. Autologous transplantation of murine ovarian cells in a fibrin scaffold resulted in survival and growth of the ovarian follicles, suggesting that fibrin may be a promising candidate for the construction of an artificial ovary.<sup>74</sup> It was reported that both MSC-seeded and acellular silk scaffolds restored urethral function; notably, the cell-seeded scaffolds improved neo-tissue formation and mechanical strength.<sup>75</sup> Various forms of decellularized tissues have been applied in developing engineered ovarian,<sup>76–78</sup> uterine,<sup>79–81</sup> testis,<sup>82–84</sup> and placenta<sup>85,86</sup> tissues. A bioengineered uterine patch made from decellularized uterine scaffold seeded with primary uterine cells and MSCs was used to repair a partial defect in a rat uterus. Host cell infiltration and uterus-like tissue structure were observed in the implanted patch.<sup>80</sup> An autologous-cell, tissue-engineered vaginal organ constructed by seeding vaginal smooth muscle and epithelial cells on small intestinal submucosa (SIS) segments has been investigated in a pilot cohort study. No long-term postoperative surgical complications were noted, and the reconstructed tissues displayed trilayered structure similar to native vaginal tissues with phenotypically normal smooth muscle and epithelial cells.<sup>87</sup>

### **Cell source**

Cells for reproductive tissue engineering include both autologous and allogeneic sources; however, autologous cells are preferred. Ideally, the cells are isolated from patient biopsy tissue and expanded *in vitro* for subsequent reimplantation. Some patients, however, do not have sufficient functional tissue from which to procure a biopsy. In these cases, alternative approaches such as stem cells (either autologous or allogeneic) may also be considered for use in engineering a regenerative therapeutic.<sup>88,89</sup>

Autologous vaginal epithelial and smooth muscle cells have been studied extensively in combination with scaffold materials such as polyglycolic acid (PGA),<sup>90</sup> hyaluronic acid embedded gauze,<sup>91</sup> and SIS<sup>87</sup> for reconstructing vaginal tissue. Clinical studies employing these methodologies suggest that the restoration of normal epithelium can be achieved, and that, for long term, a normal range of function could be established, as determined using the Female Sexual Function Index (FSFI).<sup>87,91</sup>

A series of studies have also demonstrated that the penile corporal body can be replaced using naturally derived collagen matrices seeded with human corporal SMC and endothelial cells (EC).<sup>92–94</sup> In a rabbit model of penile defect, the implantation of an engineered penile tissue segment resulted in the restoration of structural integrity as reported by cavernosography.<sup>93,94</sup> Similarly, the restoration of functional integrity was demonstrated in rabbits following the implantation of a full-length engineered penile corpora. Sperm presentation on vaginal swabs and pregnancy rates for mated female rabbits were 83% and 30%, respectively. Most untreated

control rabbits, however, did not attempt copulation after introduction to their female partners; all vaginal swabs were negative for sperm, and none of the females were impregnated.<sup>95</sup>

Studies have also shown that the restoration of fertility in prepubescent boys who have been subjected to cancer treatments may be achievable through preemptive collection and cryopreservation of their spermatogonial stem cells (SSCs).<sup>96</sup> Autotransplantation of the SSC would then occur at a later point in time, following cessation of the treatments, and would enable these individuals to mature into functionally reproductive adults.

A regenerative-based approach to creating testicular prostheses has also been pursued. Cartilage-based testes engineered from chondrocytes that have been cultured in testis-shaped PLA scaffolds formed the basis for this methodology. These prostheses can be created in bioreactors and designed to release testosterone over a prolonged period of time,<sup>97</sup> providing a regenerative mechanism for individuals who require testicular prostheses and chronic hormone supplementation.

### **Bioprinting**

Recent advancements in 3D printing technologies have elevated the geometric precision of this technique, allowing for sophisticated biomimicry and customization. Prosthetic ovaries that maintain the tortuous distribution patterns of ovarian follicles, for example, have been produced with bioprinted gelatin and electrospun polycaprolactone (PCL) materials,<sup>98,99</sup> and 3D-printed alginate scaffolds have been developed for generating testes organoids.<sup>100</sup> Additionally, extrusion-based 3D bioprinting with human trophoblast-cell-laden gelatin/methacrylate hydrogel bioinks has been applied in studies addressing the impact of trophoblast migration on preeclampsia.<sup>101</sup> Bioprinting human mesenchymal stem cells onto conventional PCL meshes has been evaluated as a potential path forward in treating pelvic organ prolapse; improved tissue integration and reduced inflammation were demonstrated compared to cell-free meshes.<sup>102</sup> Continued development of 3D-printing and bioprinting techniques will aid in the advancement of customizable scaffolds that can be engineered to support cell-specific niches and provide clinical solutions for patients.

### **Conclusions and future development**

Numerous efforts have been made toward developing and enhancing tissue-engineered methodologies for reproductive tissues. Translation of these approaches into human clinical trials has been limited; however, preclinical studies have demonstrated tremendous potential for advancing these important regenerative therapies into the clinic to provide restorative and reconstructive alternatives in the near future.

### **Peripheral Nerve System Tissue Engineering**

The peripheral nervous system (PNS) is a vast neural network that transmits motor, sensory, and autonomic information from the central nervous system (brain and spinal cord) to all regions of the body.<sup>103</sup> Peripheral nerve injury (PNI) often occurs during incidents of trauma as the PNS is not protected by a ridged barrier and is therefore vulnerable to damage.<sup>104,105</sup> In fact, PNI is reported in nearly 3% of all trauma patients.<sup>106</sup> Unfortunately, the peripheral nervous system has a limited capacity to regenerate, and such injuries often lead to poor recovery and lifetime disabilities. Satisfactory outcomes from surgical intervention have been limited, with approximately 50% of patients reporting good to normal restoration

of function.<sup>107</sup> Tissue engineering and regenerative medicine offer an opportunity to address the limitations in current clinical approaches and improve functional recovery following nerve injury and repair.

### **Scaffolds**

Current methodologies for surgical intervention following nerve damage are dependent upon the type and degree of the injury.<sup>108</sup> End-to-end suturing can rejoin the perineurium in cases where the nerve is transected, and there is no gap present. For gaps of 1 cm or less, a biological or synthetic nerve conduit can be put in place to guide regeneration; however, for gaps greater than 1 cm, autologous nerve grafting is still the most common approach.<sup>109</sup> In general, a section of the sural nerve is harvested from the patient's calf region and implanted at the site of the injury.<sup>110</sup> Autologous nerve grafting has several limitations, however, including the need for multiple surgeries. Alternatively, cadaveric or donor tissue allografts can be employed; however, this approach requires systemic immunosuppression for extensive periods of time.<sup>111–113</sup> Decellularization of allogenic nerve grafts can eliminate the potential for immunogenic constituents while still retaining extracellular matrix elements and structural architecture to support tissue regeneration.<sup>114</sup> Decellularization of graft material and development of bioengineered artificial conduits are promising tools in the field of PNS regeneration that may reduce or even replace the necessity for autologous nerve grafts.<sup>115</sup> The essential goal of these scaffolds is to decrease the gap of the PNI.<sup>116</sup> In order to achieve appropriate recovery, bioengineered conduits should provide structural and growth support, guiding regenerating neurons and promoting migration of cells, such as glia into the graft.<sup>117</sup> Some of these engineering strategies include intraluminal guidance structures, multichannel conduits, and microgrooved designs with hydrogel fillers that promote structural support and topographical guidance for regenerating axons and migrating Schwann cells.<sup>118</sup> A similar strategy involves using nanofibrous conduits.<sup>119</sup> Nerve guidance conduits (NGC) can be fabricated from natural or synthetic material. The material used, however, should conform to certain criteria including biocompatibility and biodegradability, while maintaining mechanical stability during regeneration. The material should also be resistant to tears from sutures and exhibit flexibility as well as compliance in order to prevent compression of regenerating axons. An NGC should provide guidance cues (physical and/or chemical) for the neurite extension while concurrently preventing fibrous tissue ingrowth, and support exchange of nutrients and growth factors with the extracellular environment. Lastly, an NGC material must be able to tolerate production requirements such as sterilization, storage, and surgical manipulation.<sup>120</sup>

### **Cell source**

The direct movement of cells plays an important role in inflammatory and wound repair processes *in vivo*. Following PNI, Schwann cells (SCs) migrate to the site of injury, assist in removing debris, release growth factor, organize the basement membrane, and myelinate the regenerating nerve.<sup>7,121</sup> Given the critical role of Schwann cells in nerve regeneration, these cells are generally targeted in PNI investigation<sup>122</sup> and have been the focal point of clinical safety and efficacy studies addressing augmentation of autologous nerve grafts after severe PNI.<sup>123</sup> *In vitro* investigations have also included cell types such as nerve stem cells, PC12 and SH-SY5Y,<sup>124–127</sup> induced pluripotent stem cells (iPSCs), and mesenchymal stem cells derived from bone or adipose tissue, as these cells possess the potential to differentiate into neural cells and neurons.<sup>128–131</sup>

## Preclinical, clinical, and FDA-approved hollow tube conduits

Nerve growth conduits are being used increasingly as an alternate treatment to nerve autografts; to date, there are several NGC products with regulatory approval.<sup>132</sup> One of the initial NGC synthetic devices that obtained FDA approval, Salubria™, is a permanent flexible tubular sheath made of polyvinyl alcohol hydrogel. This material provides a mechanically stable structure that provides a protective environment for nerve repair after injury; however, non-resorption leads to nerve compression and tension related issues after the regeneration has occurred.<sup>132</sup> Synthetic devices with resorbable properties have incorporated materials such as polyglycolic acid (Neurotube™) and polylactate with poly-ε-caprolactone (Neurolac™). Polyglycolic acid has favorable degradation and mechanical properties, and in clinical studies has been shown to be efficient for gaps up to 20 mm; nevertheless, the acid degradation and low solubility of byproducts remains a limitation.<sup>133</sup> Similarly, the polylactate and poly-ε-caprolactone copolymer which has been extensively used in preclinical and clinical studies may have limitations due to its degradation process.<sup>134,135</sup> Natural material devices such as Avance® Nerve Graft (allogenic) and AxoGuard® Nerve Protector (xenogeneic) provide an acellular graft that preserves the inherent structural and mechanical characteristics of the tissue.<sup>136</sup> AxoGuard® is indicated for sutureless neurorrhaphy but is contraindicated in gap management, unless used in conjunction with allografts.<sup>137</sup> Collagen Type I is the most commonly used natural, resorbable material used for NGC (Neuragen®, Neuroflex®, NeuroMatrix®, NeuraWrap®, and NeuroMend™)<sup>138,139</sup> as it is biocompatible and has been shown *in vivo* to support nerve tissue regeneration.<sup>140</sup> Alternative naturally occurring materials that are currently in use for NGC devices include chitosan (Neuroshield and Reaxon® Plus)<sup>141,142</sup> and a combination hydrogel containing polysaccharides alginate and hyaluronic acid (Versawrap Nerve Protector).<sup>143</sup> Additionally, ongoing clinical phase studies include a minimally processed human umbilical cord membrane tissue (Axogen®) and a combination product of decellularized processed peripheral nerve allografts with autologous bone marrow aspirate concentrate (Avance®).

## Conclusions and future trend

With expansion of an aging population, the incident rate of peripheral nerve injury continues to rise while the accessibility of nerve autografts has become limiting. Bioengineered NGCs will become an increasingly critical component in PNI repair. Combinations of natural and/or synthetic materials are being considered in fabrication methodologies such as electrospinning, micropatterning, and 3D printing. Advances in these approaches will benefit the availability of customized scaffolds/conduits that will target and individualize the regenerative potential of the patient by introducing the appropriate cells and neurotropic factors to the site of injury.

## Skeletal Muscle Tissue Engineering

Skeletal muscle represents about 40% of the total human body mass.<sup>144</sup> It has an innate mechanism to self-heal wounds; however, the regeneration of large defects is limited.<sup>145</sup> Volumetric muscle loss (VML) occurrences due to skeletal muscle injuries from trauma or tumor ablation often result in deficiencies of extracellular matrix (ECM), muscle fibers, and satellite cells resulting in limitations in muscle regeneration.<sup>146</sup> Every year in the United States, approximately 4.5 million patients undergo reconstructive procedures to

restore normal soft-tissue function, but in many cases, the extensive muscle defect results in the impairment of tissue function and physical deformity.<sup>147</sup> Standard surgical intervention for these types of injuries include autologous muscle tissue transfer from adjacent regions (also known as muscle flap or graft transplantation).<sup>148</sup> While this option can provide lifesaving outcomes, limitations on functional restorations and challenges such as muscle tissue availability and donor site morbidity exist. In addition, infection and necrosis lead to failure in approximately 10% of the cases.<sup>10</sup> Approaches that are grounded in volumetric replacement though tissue engineering are therefore needed to increase functionality and restore the native skeletal muscle.<sup>149</sup>

Skeletal muscle contains satellite cells that are located underneath the basal sarcolemma membrane and are stimulated to divide when the tissue is injured, fusing with damaged muscle fibers to regenerate and restore function.<sup>8,150</sup> This mechanism for repair is limited, however, to mild injuries because skeletal muscle fibers cannot divide themselves; consequently, in VML the muscle cannot remodel and the function remains impaired after ablation or denervation.<sup>151</sup> Additionally, following VML fibroblasts deposit scar tissue more quickly than myoblast fusion and regeneration can occur, thereby contributing to the impediment of muscle regeneration within the defect size and increasing the functional deficit.<sup>152</sup> Tissue engineering strategies therefore target the restoration of muscle tissue volume as well as the regeneration of vascularized and innervated muscle for full functional recovery of an injured site.

## Scaffolds

Many tissue engineering approaches have been investigated for achieving functional recovery following VML. These include strategies for decellularized ECM (dECM), synthetic and natural polymer scaffolds, 3D-bioprinted constructs, and myogenic-inducing factor delivery systems.<sup>153</sup> An appropriate bioengineered scaffold needs to provide adequate porosity and interconnection in order to distribute growth factors and support vascularization.<sup>154</sup> Common natural scaffold materials include fibrin, hyaluronic acid, laminin, collagen, chitosan, and gelatin. These materials are generally biocompatible, and when combined with the right fabrication method have been shown to facilitate muscle, vascular, and neural regeneration.<sup>155</sup> Decellularized ECM scaffolds, which contain proteins such as collagen, laminin, and fibronectin, retain the architectural environment of the native tissue and contain residual growth factors and cytokines that promote tissue restoration. Studies have shown that myogenic factors, such as insulin growth factor-1 (IGF-1), can also be incorporated into the scaffold to improve the regeneration process by facilitating progenitor cell migration, proliferation, and differentiation.<sup>10,156</sup> The decellularization process can also diminish the potential for immune response rejection by removing antigenic cellular elements and therefore prevent allograft failure.<sup>10</sup>

Another promising approach for addressing skeletal muscle tissue replacement is minced muscle grafts, also known as muscle fiber therapy. The minced muscle tissue can be further processed into uniform structures to allow for rapid reassembly and engraftment within the defect site.<sup>157,158</sup> This strategy has been tested in several preclinical models to address muscle atrophy, VML, and urinary incontinence.<sup>159</sup> Developing organized muscle constructs composed of prealigned muscle fibers within multilayered myofiber bundles has also been targeted through recent advances in 3D bioprinting. Preclinical data demonstrate greater muscle functionality post-VML following the incorporation of human promyogenic cell bioink, such as muscle progenitor cells,

compared with acellular constructs; this approach promoted the regeneration of a highly mature and organized tissue with vascular and neural integrity.<sup>160,161</sup>

Synthetic materials commonly applied in skeletal muscle tissue engineering include polypropylene, poly-(lactic acid), poly-(glycolic acid), poly- $\epsilon$ -caprolactone, polyurethane, and polyethylene glycol.<sup>147</sup> These materials offer the advantage of tunable mechanical properties such that the degree of hardness, the fracture strength, or the durability of the scaffold, for example, can be adjusted to match the properties of the tissue to be replaced. While synthetic materials may be challenged with biocompatibility and degradation issues, they offer the benefit of increased batch-to-batch reproducibility, a feature that does not always exist with natural materials.

Tissue-engineered muscle grafts have been implanted in a variety of preclinical VML models; however, the lack of standardization in muscle type and location for this model still exists. Additionally, universal consensus on the definition of a critical sized defect in the model has not been achieved.<sup>144</sup> Consequently, a broad range of pre-clinical studies with mixed results support the efficacy of cell-based therapies, and acellular and cell seeded scaffolds for the treatment of VML defects.

### Cell sources

Skeletal muscle tissue engineering generally involves satellite cells or muscle-derived precursor cells; however, alternative sources of stem cells such as perivascular, bone marrow, umbilical cord, or adipose-derived stem cells may also be considered, and recently induced pluripotent stem cells have been targeted as a potential cell source.<sup>162–166</sup> Considerations for myogenic cell sources include expansion capacity, immunogenicity, translatability, differentiation potential, and engraftment potential *in vivo*. Cell-sheet-derived 3D constructs have been developed by culturing muscle-derived cells in monolayers until confluent and then forming a cylindrical construct with the cells to implant into the defect site.<sup>167</sup> This approach has produced promising results in small VML models; however, there is limited ability for scale up to clinically relevant sizes, and the lack of a supporting scaffold causes difficulties in manipulation.<sup>168</sup> Repair of sizeable tissue defects requires the implantation of either large tissue-engineered constructs or a substantial dose of cells. Oxygen diffusion to implanted cells under these conditions is difficult, however, which can impede the dissemination of vascularization cues.<sup>169</sup> Another issue is inefficient cell grafting within the implanted tissue. Delivery of human muscle progenitor cells in multiple doses has been shown to sustain cellular viability through the initial stages of vascularization and thereby improve muscle function in a rodent model of VML.<sup>169</sup> Translation of this technology into the clinical settings will require optimization of areas such as the cell injection parameters and the choice of a biomaterial for cell delivery that can maintain the implant volume for an appropriate period of time.<sup>170</sup> In general, cell-based approaches for repairing skeletal muscle injuries have gained attention; however, improvements in cell sourcing and expansion prior to implantation are still under investigation.

### Conclusions and future trend

Several Phase I clinical trials have addressed the regenerative potential of acellular dECM for mitigating human VML defects. These include the utilization of acellular non-crosslinked porcine collagen scaffolds, XenMatrix, and MatriStem UBM.<sup>171–174</sup> Subjects demonstrated average improvement in strength and range-of-motion

compared with preoperative performance, providing early evidence of feasibility for the use of bioscaffolding as a viable treatment for VML.

Although extensive work has been done to demonstrate the practicality of bioengineering scaffolds and cell therapies to treat skeletal muscle defects, challenges remain in clinical translation. Going forward it will be necessary to evaluate these approaches in relevant preclinical muscle injury models that standardized the critical defect size for VML. Evidence of the long-term contributions each technique provides to restoring muscle function will be essential given the highly organized and complex vascular and neural components that support native skeletal muscle function.

### Gastrointestinal Tissue Engineering

The gastrointestinal (GI) tract is a continuous hollow organ with structural complexities that include two layers of musculature, which regulate peristalsis and propulsion through coordinated interactions of the enteric nervous system and interstitial cells of Cajal, and a specialized epithelium that controls the secretion and absorption of nutrients.<sup>175–177</sup> Lymphatic and microbiome systems add an additional layer of complexity to this system creating, overall, a challenging prospect for tissue engineering.<sup>178</sup>

Multiple diseases, including cancer and congenital disorders, can adversely impact the function of the GI system.<sup>176</sup> Treatments generally include surgical intervention and pharmacological management of symptoms, but these are often temporary solutions and do not necessarily address the root cause of the pathology. Regeneration of the tissue is a promising approach, provided a functional bioengineered gut can be engineered.<sup>179</sup> Recent work has focused on regenerative alternatives through the use of cells and scaffolds, organoids, and organ-on-a-chip.<sup>180</sup>

### Scaffolds

Tissue engineering segments of the gut requires scaffold materials that promote cell-cell interactions, support the mechanical behavior of each tissue layer, and allow for adequate vascularization. Porosity and physical modifications, such as a micropatterned surface, are some of the strategies used to guide circumferential cell alignment and improved functionality.<sup>181</sup> Some common natural biomaterials utilized in GI scaffolds include collagen, silk fibers, and chitosan.<sup>182–184</sup> Natural materials generally have the advantage of biocompatibility and easily support cell growth and survival; however, these substances often possess weak mechanical properties and may therefore require reinforcement with synthetic materials. Decellularized small intestine submucosal (SIS) extracellular matrix tissue formed the basis for one of the first approaches in GI tissue reconstruction. This acellular material was shown to support both epithelial and muscle tissue regeneration in small intestinal repair.<sup>185</sup> Similarly, acellular porcine small intestine and urinary bladder matrix scaffolds have been used for esophageal reconstruction.<sup>186</sup> Natural intestinal scaffolds that retain their native architecture and connective tissue components are generated by detergent-enzyme treatments and used not only for drug testing and biological assays *in vitro* but have also shown similarly support for the regeneration of intestinal tissues in clinical applications.<sup>11</sup>

Utilization of synthetic scaffold materials, such as poly- $\epsilon$ -caprolactone alone or in combination with lactic acid and polyglycolic acid, has been investigated in esophageal indications.<sup>187</sup> Synthetic materials are often used as coating material in order to

strengthen the mechanical properties of natural biomaterial scaffolds and regulate degradation rates. Chemical modifications can be used to adjust the biocompatibility of these materials and enable modifications for incorporating bioactive molecules, such as growth factors that support vascularization of the implant.<sup>188,189</sup> Like natural material, scaffolds derived from synthetic substances are expected to promote infiltration of native cells, encouraging tissue remodeling and regeneration. In certain cases, wrapping the engineered organ in donor omentum or amniotic membrane facilitates metabolic exchange and vasculogenesis to support organ tissue restoration.<sup>190,191</sup>

### Cell sources

Composed of several layers, each segment of the GI track has a cellular complexity and specific architecture that is essential to its physiological function. In order to recreate a functional replacement, it is essential to consider the unique roles of the cells that normally populate a particular segment.<sup>192</sup> Various cell sources have been considered for tissue engineering portions of the gut, including primary cells isolated from biopsy, expanded in vitro and subsequently seeded onto a scaffold for implant.<sup>193–195</sup> While this approach has been shown to be promising for regenerating innervated musculature of the gut in preclinical studies, expanding these cells to clinically relevant numbers and maintaining phenotype is often proving to be a challenge.<sup>176</sup> Mesenchymal stem cells and other progenitor cells have also been shown to promote the regeneration of muscular components when stimulated with soluble factors to direct their differentiation.<sup>179,196</sup> Regeneration of the epithelial component of the gut, however, still faces complications regarding the initial in vitro expansion of precursor cells, and the downstream survival and differentiation when matured on scaffolds.<sup>197</sup> Importantly, induced pluripotent stem (iPS) cells have exhibited promising characteristics and abilities to differentiate into the various cells that populate the gut; this has been particularly encouraging for generating cells of epithelial lineage.<sup>198</sup>

### Alternative systems

Organoid systems are another promising approach for understanding the cellular complexities associated with engineering GI structures.<sup>199</sup> These small functional units mimic the original organ, essentially recapitulating its biological intricacy and function.<sup>200</sup> Recent preclinical work has demonstrated the capacity for stomach organoids to differentiate into rudimentary stomach-like structures.<sup>201</sup> A majority of the work associated with GI organoid systems has focused on the regeneration of epithelial components; however, models that successfully achieved contractility and immune functions and integration of the enteric neural system have also been reported.<sup>202</sup> Organ-on-a-chip is another emerging technology that will be instrumental in evaluating GI pathology and repair. This system uses microfluidic devices to generate and maintain organoid units and has been used to support drug discovery, toxicology, and pharmacokinetics.<sup>203</sup> Notably, investigations employing organ-on-a-chip technology for small intestine and colon biopsies have demonstrated spontaneous peristalsis like motion.<sup>204</sup> 3D printing technology has been used to create scaffold-free structures for the regeneration of esophageal tissue, composed of multicellular spheroids containing cell types such as human dermal fibroblasts, smooth muscle cells, and mesenchymal stem cells, which could then be transplanted into the injured site.<sup>205</sup> Studies to optimize these systems will facilitate advances in the field of GI regenerative medicine.

### Clinical data

Promising work that targets tissue engineering techniques for the regeneration of GI tissue has been performed in preclinical models; however, limited results have been reported from clinical trials. Esophageal reconstruction has been attempted using urinary bladder matrix (MatriStem™, ACell) to augment and restore damaged esophageal tissue in four patients with varying etiologies including progressive dysphagia, esophageal perforation, and mediastinitis, caustic ingestion, and adenocarcinoma.<sup>206</sup> A second trial (single patient) used an acellular dehydrated scaffold sprayed with autologous platelet-rich plasma to support esophageal regeneration and function.<sup>207</sup> Additionally, porcine small intestine ECM scaffolds (Surgisis®, Cook) were used in five patients for esophageal preservation following endoscopic resection,<sup>208</sup> and an extracellular matrix scaffold composed of collagen and natural proteins is currently been investigated in clinical pilot study for similar purposes.<sup>209</sup> In Japan, two separate studies have used endoscopic transplantation of tissue-engineered autologous oral mucosal epithelial sheets to prevent the formation of strictures after endoscopic submucosal dissection.<sup>210,211</sup> Finally, allogenic mesenchymal stem cells and endothelial cells derived from human umbilical vein are being used in separate studies as cell therapy for the treatment of conditions from Crohn's Disease.<sup>212,213</sup>

### Conclusions and future trends

From stem cell therapies, biomaterials and scaffolds, to organoids and organ-on-a-chip, the regeneration of digestive organs through tissue engineering is making promising progress. Most of these techniques, however, have only been observed in animal models; additional work will be necessary in order for many of these technologies to reach clinical applications. The GI system is a complex multilayer structure with uniquely functional segments driven by the integration of multiple cell types. To achieve tissue regeneration at this level of complexity, it will be important to develop effective strategies for incorporating and sustaining these unique components.

### Renal Tissue Engineering

Kidney disease is one of the leading causes of death globally; with acute kidney injury (AKI), chronic kidney disease (CKD) and end-stage renal disease (ESRD) representing the most common forms the disease. Medical therapies for AKI and CKD primarily involve hemodialysis or kidney transplantation. Currently, kidney transplantation is the only solution for resolving ESRD; the availability of transplantable donor kidneys, however, is less than 20% of the demand.<sup>214</sup> Regenerative medicine-based approaches employing cellular and tissue-engineered therapies present a promising alternative.

### Scaffolds

Cultured renal cells are generally seeded onto scaffold material where the cell population expands and matures into renal structures to form a 3D kidney construct. Currently, scaffold systems for engineering renal constructs include natural and synthetic polymers, and decellularized native tissue matrices. Natural polymers have biocompatible characteristics that can support cell proliferation, migration, and differentiation, thereby providing a potential option for regenerating native tissues. Collagen and collagen-based materials have been extensively used to produce scaffolds for kidney regeneration. Glomerular epithelial and mesangial cells isolated

from renal tissues have, for example, been used with collagen-vitrigel mixed membrane scaffolds,<sup>215</sup> and collagen Matrigel was used to reconstruct 3D renal tissues in vitro with neonatal rat renal cells.<sup>216</sup> The cells within the 3D collagen-Matrigel hydrogel self-assembled into tubule and glomeruli-like structures. Hyaluronic acid (HA), which exhibits the appropriate mechanical properties and plays a vital role in mammalian development, has also been utilized for fabricating 3D renal-like tissues in vitro where it supported ureteric bud branching, promoted mesenchymal-to-epithelial transformation, and stimulated differentiation of the metanephric mesenchyme and ureteric buds.<sup>217</sup> Natural polymers such as chitosan, fibrin, and alginate have also been studied for use in kidney tissue engineering. These materials have successfully supported the formation of hollow tubules, providing a platform for further renal tissue regeneration.<sup>218–220</sup> Decellularized native tissue is also highly attractive as scaffold material because it maintains the complex architecture of the kidney as well as many extracellular matrix components. Optimization of decellularization/recellularization processes has supported progress toward bioengineering whole kidneys in vitro. When transplanted orthotopically, these bioengineered grafts were ultimately perfused by the recipient's circulation and produced urine through the ureteral conduit.<sup>221</sup>

In order to improve the mechanical strength of scaffolds, synthetic biodegradable polymers such as poly(lacticacid) (PLA), poly(glycolic acid) (PGA), and poly(lactic-*co*-glycolic acid) (PLGA) have been evaluated for use as renal scaffold sources. These biopolymers are nontoxic, simple to fabricate, and have well-controlled degradation. Kidney-like constructs engineered from renal cells seeded onto PGA scaffolds, and subcutaneously implanted into athymic nude mice recapitulated nephron-like segments and tubule-like structures in the implant.<sup>222,223</sup> A PLGA scaffold containing kidney-derived ECM has also been shown to be an effective matrix for repair and reconstitution of glomeruli and blood vessels, in partially nephrectomized mouse model.<sup>224</sup>

### Cell sources

Studies indicate that it is possible to isolate renal stem and progenitor cells from the Bowman's capsule, papilla, and tubular sections of the kidney.<sup>225</sup> Progenitor cells derived from the Bowman's capsule and injected into a preclinical model of renal injury resulted in morphological and functional improvements such as reduced proteinuria and glomerular damage.<sup>226,227</sup> Notably, stem cells from non-renal tissues, including embryonic stem cells (ESC), induced pluripotent stem cells (iPSC),<sup>228–231</sup> amniotic fluid-derived stem cells (AFSC),<sup>232–235</sup> bone-marrow-derived mesenchymal stem cells (BM-MSCs),<sup>236–239</sup> adipose-derived stem cells (ADSC),<sup>240–243</sup> have also been evaluated as potential cellular sources for treating renal failure and demonstrated elements of renal regeneration in each case. Additionally, clinical studies applying intravenous administration of autologous MSC to improve the outcomes of renal transplantation have established that this therapeutic approach is safe, results in a lower incidence of acute rejection, provides a decreased risk for opportunistic infection, and supports better estimated renal function after one year.<sup>244,245</sup>

Primary kidney cells also represent a conceivable source of cells for regenerative therapies as these cells can be grown in three-dimensional culture systems to generate tubule structures that are positive for proximal, distal tubule, and collecting duct markers in vitro.<sup>246</sup> Administration of in vitro cultured human renal cells has been shown to result in functional and structural improvements in a

rat chronic kidney injury model.<sup>247</sup> Importantly, a Renal Autologous Cell Therapy (REACT), consisting of progenitor cells isolated from a patient's renal tissue, has been developed to delay or prevent renal replacement therapy, and is currently in clinical trials in the United States for safety and efficacy (clinicaltrials.gov, NCT04115345).

### Acellular therapy

Acellular approaches to kidney repair and regeneration include injection of extracellular vesicles (EV), biomolecules, and biomaterials. Administration of bone morphogenic protein-7 (BMP-7), a member of the TGF- $\beta$ 1 family that is highly expressed in the kidney, has been shown to be closely associated with reversal of chronic renal injury through epithelial-to-mesenchymal transition.<sup>248</sup> Naturally secreted extracellular vesicles derived from MSCs is one of the most studied methods for therapeutic use of EV. Mesenchymal stem cells have a distinct capability for renal repair as MSC-derived EVs reproduce the immunomodulatory and cytoprotective activities of their parent cells.<sup>249</sup> Adipose tissue-derived MSC-EVs, for example, were found to be enriched with IL-10 mRNA, an anti-inflammatory cytokine. Notably, a single intrarenal delivery of MSC-derived EVs ameliorated renal injury in chronical experimental model of pigs with metabolic syndrome.<sup>250</sup> Increasing evidence from various experimental models of acute kidney injury suggests that delivery of MSC EVs can restore renal structure and function by reducing inflammation, improving renal microvasculature, medullary oxygenation, and resident cell viability and proliferation.<sup>250–253</sup>

Besides working as a scaffolding system, naturally occurring extracellular matrices have also been used to promote *in situ* renal regeneration. For example, polymerized collagen (P-collagen), a mixture of pepsinized porcine Type I collagen and polyvinylpyrrolidone, has been used to reduce renal pathology and improve anatomic and functional renal properties in a model of nephrotoxicity.<sup>254</sup>

### Bioprinting

Three-dimensional bioprinting may also provide a potential solution to the widening gap between need and availability of transplantable kidney organs. This process constitutes engineering 3D functional living macrotissues and organ constructs through layer-by-layer robotic biofabrication with cells and bioink as building blocks.<sup>255</sup> Challenges include the hierarchical architecture and functional complexity of the native tissue/organ, which make it difficult to replicate on a large scale using current techniques.<sup>256</sup> Reported strategies include creating 3D renal proximal tubules in vitro that are fully embedded within an extracellular matrix and housed in perfusable tissue chips;<sup>257</sup> taking a microfluidics approach to build a 3D renal kidney tubulointerstitium using primary tubular cells suspended in a polysaccharide biomaterial ink solutions;<sup>258</sup> and engaging a cellular extrusion technique to produce 3D kidney organoids from iPSCs in bioink, for use in drug screening, disease modeling, and ultimately kidney organ engineering.<sup>259</sup>

### Conclusions and future development

Much research has been performed to develop strategies for repairing and regenerating tissue damage due to kidney failure. Various approaches, including cell therapies with autologous or "off-the-shelf" allogeneic cell sources and engineered acellular or cell-based kidney constructs, have been proposed. Improvements in 3D tissue and organ printing will support the generation of precise, customizable, and complex structures; current techniques, however, do not

yet provide a viable solution for addressing the shortage of organs for kidney transplantation. A combination of the strategies described in this section may be necessary in order to satisfy the heterogeneity, hierarchical structure, and complex functionality of this multidimensional organ as the field moves forward.

### Bone Tissue Engineering Update

Worldwide there is an increased need for bone grafting; it is the second most frequent tissue transplantation after blood transfusion.<sup>260</sup> The restoration of large bone defects is a critical issue that can negatively impact the quality of life and consequently have significant socioeconomic repercussions, particularly with increasing age expectancies.<sup>261</sup> Until recently, bone repairs were primarily done with autologous bone tissue or tissue from compatible donors.<sup>262</sup> This approach has had several limitations however, including shortages in tissue supply, donor site morbidity, and tissue incompatibility, all of which have led to greater focus on tissue engineering methods to address these insufficiencies. Significant progress has been made in the last two decades with respect to new materials and new fabrication methods.<sup>263</sup> While initial efforts focused mainly on mimicking the macrostructure of bones, recent work has targeted fabrication of scaffolds that create an ideal condition for tissue regeneration.<sup>264</sup> Advances in medical imaging and software have made it possible to design patient-specific scaffolds with high resolution, which can be shaped and targeted for a specific tissue.<sup>265</sup> New technologies focused on personalized medicine tailored to meet patient requirements, together with improvements in scaffold manufacturing techniques such as 3D printing, promise to improve outcomes. In addition to fine control over scaffold geometry, newer fabrication methods also refine scaffold pore size to facilitate cell infiltration, mechanical properties, structural characteristics, and degradation rates.

Developments in the area of biomaterials for bone-tissue engineering must integrate properties such as biocompatibility, biodegradation, cell attachment, differentiation, and early stage mineralization.<sup>266</sup> Efforts to mimic the composition and structure of native bone tissue generally involve a range of polymers, bioceramics, metals, and composites. Most of these materials require physiochemical modifications, however, such as incorporating osteoconductive factors, such as hydroxyapatite or bioglass, and bioactive factors such as vascular endothelial growth factor (VEGF) and osteogenic growth peptide (OGP) to improve vascularization and osteogenesis.<sup>267–270</sup>

Despite advances in the area of bone tissue engineering, most have yet to be tested in preclinical studies and only a few have been translated into clinical trials. A single-patient trial using a 3D-printed bioresorbable scaffold made of polycaprolactone (PCL) has been tested for periodontal repair. A limitation of the study was determined to be the material of choice; it was suggested that a more rapidly resorbing material with a porous structure would improve tissue ingrowth and vascularization for this specific application.<sup>271</sup> In a pilot clinical trial, PCL scaffolds were used for ridge preservation for dental implant placement following tooth extraction and reportedly supported better outcomes when compared to control group.<sup>272</sup> Additionally, several trials for the treatment of mandibular fractures with customized 3D-printed scaffolds have been initiated,<sup>273–275</sup> and EpiBone®, a decellularized bovine trabecular bone scaffold, is in clinical trial as a potential treatment for ramus continuity defects in the mandible. The EpiBone® scaffold can be crafted into the defect shape and seeded with autologous

adipose-derived stromal/stem cells, which have the capacity for osteogenic differentiation.<sup>276,277</sup>

Tissue engineering holds great promise for the treatment of bone defects due in part to advances in stem cell biology, development of novel biomaterials, and optimization of 3D bioprinting. Successful translation into a clinical setting will depend however, on whether these new approaches can outperform current treatments and overcome quality, safety, and regulatory challenges.<sup>278</sup> Decisions on dosage and the route of application will require an understanding of the underlying cellular and molecular mechanisms associated with stem cell action in bone regeneration.<sup>261</sup> The future of the field relies on combining cell therapy with biodegradable osteo-conductive scaffolds that exhibit suitable mechanical properties.

### Vascular Tissue Engineering Update

The clinical demand for safe and effective vascular grafts is significant. Synthetic vascular grafts constructed from expanded polytetrafluoroethylene (ePTFE) or polyethylene terephthalate (Dacron) have been widely applied in the clinic over the past four decades.<sup>279,280</sup> High risk of infection, thrombosis, and other complications however have limited the application of synthetic grafts in small diameter blood vessels. Vascular tissue engineering has progressed substantially, generating biological grafts that incorporate the functionality of structural layers, withstand physiologic stressors of the cardiovascular system, and effectively integrate into the tissue without serious immunologic rejection.<sup>281</sup> Several approaches for small vessel engineering have progressed to clinical trials, including vascular grafts from Cytograft, Gunze Limited, and Humacyte.<sup>282–284</sup> Humacyte has reported results for their bioengineered human acellular vessels tested in end-stage renal disease patients in a Phase II clinical trial; the patency results at 6 and 12 month intervals demonstrated safe and functional hemodialysis access, warranting further evaluation in randomized controlled trials.<sup>285</sup> Humacyte also achieved favorable results in a clinical trial for peripheral arterial bypass surgery. These engineered blood vessels were shown to be safe, with acceptable patency and a low incidence of infection.<sup>284</sup>

Various 3D-printing techniques, including laser-assisted, droplet-based, and extrusion-based manufacturing processes, are being considered for bioprinting cellular and acellular vascular grafts.<sup>286,287</sup> Studies in which small-diameter biomimetic blood vessels with two distinct proliferating cell layers (vascular endothelial cell [VECs] and vascular smooth muscle cell [VSMCs]) were created using an advanced coaxial 3D-bioplotter platform and demonstrated the feasibility of this approach in generating biocompatible small-diameter blood vessel replacements.<sup>288</sup> At the other end of the spectrum, the fabrication of scaffold-free tubular tissue grafts from multicellular spheroids composed of 40% human umbilical vein endothelial cells, 10% human aortic smooth muscle cells, and 50% normal human dermal fibroblasts resulted in patent tubular tissues that exhibited remodeling (enlargement of the lumen area and thinning of the wall).<sup>289</sup> Results from the “Ring Stacking Method,” in which bioprinted tissue rings are stacked to create a tubular construct mimicking the natural form of a blood vessel, also support the potential for manufacturing artificial blood vessels in a variety of dimensions and lengths that will meet the specific needs of the clinic and patient.<sup>290</sup> Recent developments in microfluidics and 3D-bioprinting technologies have also enabled the generation of highly controlled and reproducible patterns of microvasculature.<sup>291,292</sup> In one format, a densely cell-populated 3D vascular

network was produced by printing carbohydrate glass filaments into lattices surrounded by extracellular matrix. Dissolution of the glass filaments resulted in a 3D tubular network that could be perfused and loaded with cells.<sup>292</sup> Patterned vascular structures have also been created by lithography, using hydrogel micropatterning to form a microvascular network when bonded to another flat layer of hydrogel.<sup>293</sup>

Recent progress in tissue engineering and bioprinting techniques such as these suggests that we are entering a new era in treating vascular diseases, an era in which regenerative alternatives will play a key role in treating and replacing vascular tissues that are no longer functioning at optimal capacity.

### **Cardiac Tissue Engineering Update**

The restricted regenerative capacity of cardiomyocytes, coupled with limited supplies of heart tissue for transplantation, has produced challenges in treatment options for cardiovascular diseases, which are the leading cause of death in the world.<sup>294</sup> Alternative approaches to myocardial regeneration through tissue engineering and regenerative medicine technologies may provide a solution to this current deficit. Cell types such as MSCs, ESCs, and iPSC-derived cells, in addition to cardiomyocytes, have been transplanted into injured myocardium or used to investigate new methods for myocardial tissue engineering. Compared with other cell types, autologous MSCs are a prime candidate for cardiovascular tissue engineering as they are generally plentiful, easy to expand, do not require immunosuppressive therapies, and do not incur ethical issues.<sup>295</sup> MSCs derived from a variety of tissues have been utilized in preclinical as well as clinical studies. Several Phase I and II clinical trials have established safety and efficacy following intravenous (IV) administration of MSCs, demonstrating improvements in left ventricular ejection fraction, heart function, and quality of life.<sup>296–298</sup> A major limitation to this approach, however, is the low survival rate for MSC engraftment in the ischemic lesion. Strategies to address the poor longevity of engrafted MSCs following IV administration include tissue engineering with cell-biomaterial combinations that produce tissue-like architecture, such as a cardiac patch or 3D-printed cardiac structure. In preclinical studies, bioengineered cardiac patches constructed from stem cells (MSCs, cardiac progenitor cells, and endothelial progenitor cells) seeded on a multilayered scaffold and applied to the ligated left coronary artery (LCA) resulted in improved heart function that was accompanied by increased levels of several angiogenic cytokines (bFGF, vWF, and PDGF-B) and cardioprotective factors.<sup>299</sup> Recently, an MSC and scaffold-based therapy was approved for use in a clinical trial to treat infarcted myocardial tissue (ClinicalTrials.gov Identifier: NCT03798353). This cell-matrix construct, comprised of human pericardial matrix (PeriCord) together with allogeneic MSCs derived from Wharton's jelly, will be used to treat areas of ischemia that are noncandidate for revascularization by surgical intervention. The applications of 3D-bioprinting technology in the field of cardiovascular repair and regeneration have achieved significant progress in the past few decades.<sup>300–302</sup> Patterns engineered to mimic the original structure of cardiac tissue types can be generated through precise printing and crosslinking of cell-loaded cardiac bioinks. Structures including a neonatal-scale human heart,<sup>303</sup> an adult-scale trileaflet heart valve,<sup>303</sup> simplified anatomic heart and aorta tissues,<sup>304</sup> a cardiac chamber with large vessel extensions,<sup>305</sup> and microvascularized holistic engineered heart tissue<sup>306</sup> have been investigated through bioprinting techniques. Importantly, these

advanced 3D-bioprinted cardiac tissues have demonstrated elements of cardiac functions that include expression of cardiac-specific biomarkers,<sup>307</sup> spontaneous muscle-like contraction,<sup>305</sup> electrical function measured via calcium transients,<sup>308</sup> and cardiomyocyte-like depolarization–repolarization action potentials.<sup>309</sup> Additionally, the transplantation of a 3D-patterned cardiac patch into the infarcted heart tissue of immune-deficient rats was shown to greatly reduce the infarction area, significantly increase neovascularization in the tissue, and statistically improve both the ejection fraction and cardiac output of the animal.<sup>310,311</sup> Currently, preclinical studies in large animal models, as well as early clinical studies, continue to be indispensable for determining the safety and efficacy of bioprinted cardiac tissue as an acceptable therapeutic option for cardiovascular pathologies going forward.

### **Cartilage Tissue Engineering Update**

Cartilage defects caused by traumatic injury, disease, and aging are major problems requiring orthopedic surgery and a common cause of disability.<sup>312</sup> Due to the lack of vascularization, nutrient supply and cell (chondrocyte) density, cartilage has a limited capacity for self-repair.<sup>313</sup> Traditional treatments for cartilage repair include moisacplasty (transplantation of cylindrical plugs of bone and cartilage from less weight-bearing areas to the defect site), microfracture, osteochondral allograft, and autologous chondrocyte implantation.<sup>314</sup> These traditional treatments may have satisfactory outcomes initially, but often have poor long-term results, frequently leading to problems such as fibrocartilage formation. Various tissue engineering approaches have been engaged to address the challenges associated with cartilage regeneration. Among cell-based therapies, technologies for fabricating scaffolds and developing biomaterials that mimic the extracellular matrix facilitate cell growth, proliferation, and differentiation at the site of the defect.<sup>315</sup>

While autologous chondrocyte products such as MACI® (autologous cultured chondrocytes on porcine collagen membrane) are commonly used for the treatment of traumatic cartilage lesions, this type of approach has been known to experience limitations in cell sourcing, effectiveness in aged patients, and donor site recovery.<sup>316,317</sup> Clinical trials using Neocartilage Implant/DeNovo (RevaFlex) cartilage disks derived from juvenile donor chondrocytes report a low immunologic response, supporting the use of this therapeutic approach as an alternative for restoring cartilage in older individuals.<sup>318</sup> Stem-cell-based therapies also offer a promising approach for cartilage replacement as many of these cells exhibit a strong chondrogenic potential.<sup>319</sup> In addition to mesenchymal stem cells, induced pluripotent stem cells (iPSCs), peripheral blood mononuclear cells (PBMCs), and human umbilical cord blood-derived mesenchymal stem cells have also been used in cartilage engineering.<sup>320–322</sup> Recently, adipose tissue-derived stem cells (aMSCs) have become the second most common source of stem cells for cartilage applications as large numbers of cells can be isolated with minimal manipulation and subsequently expanded in vitro without losing chondrogenic potential.<sup>323</sup> In preclinical and clinical studies, this method was shown to block the progression of osteoarthritis and promote cartilage regeneration.<sup>324–326</sup> Recently, a clinical study reported high patient satisfaction following treatments composed of microfractures (creation of small fractures that penetrates the subchondral bone allowing for influx of pluripotent stem cells and growth factors) in combination with an aMSC-laden hydrogel scaffold (polyglucosamine/glucosamine carbonate).<sup>327</sup> The presence of aMSCs in the hydrogel proved to be

a significant factor leading to an improved chondrogenic environment. Approaches to enhance cartilage regeneration have also been linked with immunotherapy.<sup>328</sup> A cell-based combination therapy using aMSC- and TH1-specific autoimmune lymphocytes (effector cells) has been shown to improve cartilage repair in an experimental model of grade 3–4 osteoarthritis.<sup>329</sup>

Hydrogels are a versatile biomaterial often used in cartilage tissue engineering due to its similarity with natural tissue extracellular matrix.<sup>330</sup> The structure, composition, biochemical, and mechanical properties can be engineered to suit the application requirements. Hydrogels can also influence cellular attributes and behaviors such as morphology, proliferation, and differentiation at the defect site. Hydrogels populated with cells and chondrogenic promoting factors have the potential to address some of the challenges encountered with full-thickness cartilage regeneration.<sup>331</sup> Natural hydrogels products based on hyaluronic acid (Hyalograft C, no longer in the market) and collagen (GelrinC, current in clinical trial) have been evaluated clinically for efficacy in cartilage repair. While they are biocompatible and exhibit chondrogenic potential in vitro, the mechanical properties are weak; consequently, a role for these molecules in the regeneration of cartilage may be limited.<sup>332–335</sup> Hybrid hydrogels that combine natural and synthetic materials are designed to ensure adequate mechanical strength for shape maintenance and load bearing capacity.<sup>336</sup> Currently, only a few hybrid scaffolds have undergone clinical trials; these are mainly used to augment existing treatment strategies, such as microfracture surgery.<sup>337,338</sup>

Advanced biofabrication techniques enable the production of scaffolds with precise spatial control over the internal structure to facilitate the appropriate mechanical properties.<sup>339</sup> With 3D printing/bioprinting capabilities, it is now possible to create scaffolds that will match the defective area precisely and in combination with cells generate a living template that can develop into functional artificial tissue.<sup>337</sup> Several commonly used 3D printing technologies are used in the medical field; these include fused deposition modeling, extrusion, and inkjet.<sup>340</sup> Recently, a method called low-temperature deposition manufacturing has been introduced, which preserves the bioactive status of the material because of its nonheating feature. This allows for printing 3D structures containing natural biopolymers.<sup>341</sup>

Despite advances, many of the newer tissue-engineered technologies for cartilage repair have failed to demonstrate significant clinical benefits over older approaches.<sup>342</sup> Most existing methodologies have been unable to treat large defects or those associated with advanced osteoarthritis. Additionally, none of the current technologies have shown the potential for reconstructing an entire structural unit such as a joint head.<sup>316</sup> The research community is making efforts to determine how new and advanced technologies can be translated into measurable clinical gains by considering safe, standardized, cost-effective, and good manufacturing practice-compliant production. Some challenges remain with cell-based therapies, such as dedifferentiation of chondrocytes when expanded *in vitro* and limited stem cell chondrogenesis that can lead to fibrocartilage.<sup>336</sup> From a materials perspective, matching the rate of scaffold degradation with neocartilage formation has been problematic, and the bioadhesive properties of the material need to be considered in order to facilitate the integration of scaffold and newly formed cartilage to the host tissue.<sup>337</sup> Efforts to address these issues will make it possible to target the optimum conditions for producing tissue-engineered therapies that can promote human cartilage regeneration and repair.

## Skin Tissue Engineering Update

Significant progress has been made over the past few decades in the development of *in vitro* engineered substitutes that mimic human skin, either as grafts for the replacement of lost skin or for the establishment of *in vitro* human skin models. Several acellular (Biobrane®, AlloDerm®, and Integra®), allogenic (Apligraf®, Dermagraft®, and OrCel®), and autologous (Epicel®) skin substitutes have been approved by FDA.<sup>343</sup> This section primarily focuses on bioprinting as the next wave in tissue-engineered skin substitutes.

Cells used for the successful construction of an *in-vitro* epidermal-dermal skin-equivalent include keratinocytes, Langerhans cells, fibroblasts, endothelial cells, and mesenchymal stem cells.<sup>344</sup> A variety of natural hydrogels (fibrin, collagen, chitosan, etc.) and synthetic hydrogels (polyethylene glycol, polyacrylic acid, polyvinyl alcohol, etc.) have been investigated as potential sources for scaffolds.<sup>345,346</sup>

Recent advances in 3D printing (bioprinting) technologies have been explored in order to engineer cell-loaded scaffolds that match the structural complexity of native skin tissue.<sup>347</sup> Laser-assisted bioprinting, droplet-based bioprinting, and extrusion bioprinting are all commonly used bioprinting techniques for constructing skin tissue equivalents.<sup>344</sup> Bioprinting platforms support the programmed deposition of multiple skin cell types and allow for the utilization of various matrix biomaterials and processes that are lacking in conventional skin tissue engineering approaches. Fabrication of skin constructs using bioprinting techniques carries several advantages compared to conventional tissue engineering strategies including the potential for automation and standardization for clinical production. Autologous skin cells such as dermal fibroblasts and epidermal keratinocytes can be directly layered onto the wound area<sup>348</sup> or into a stabilized matrix such as collagen.<sup>349,350</sup> Alternatively, stem cells such as those derived from amniotic fluid or bone marrow may successfully support applications in skin tissue bioprinting. Stem-cell-driven wound closure and re-epithelialization, for example, have been shown to be significantly greater compared to wound closure and re-epithelialization with fibrin–collagen gel treatment alone.<sup>351</sup> Regeneration of fully functional skin substitutes using bioprinting methodologies is a complex process, requiring a variety of functionally discreet cell types. Recent studies targeting the incorporation of specialty cells such as melanocytes and epithelia that form sweat glands have had promising results.<sup>9,10,11</sup> Coprinting melanocytes and keratinocytes on top of a dermal layer resulted in producing freckle-like pigmentation in terminally differentiated keratinocytes,<sup>352</sup> and bioprinted sweat glands fabricated with epidermal progenitor cells in a composite hydrogel formulated with gelatin and sodium alginate supported functional restoration of sweat glands in burned mice.<sup>353</sup>

In general, technological limitations, including requirements for large numbers of specialized cells, high printing resolution, vascularization, and versatility in culture media, need to be overcome in order to achieve clinical translation for bioprinting skin.<sup>354</sup>

## Urology Tissue Engineering Updates—Urethra

Urethral stricture is a scar of the subepithelial tissue of the corpus spongiosum that constricts the urethral lumen, restricting the flow of urine and causing incomplete bladder emptying that can lead to urinary tract infection.<sup>355</sup> This condition affects approximately 1% of males over the age of 55.<sup>356</sup> Surgical intervention is available to repair the urethral defects; however, the management of long segment strictures represents an ongoing challenge due to the potential

for stricture recurrence following urethrotomy or urethral dilatation.<sup>357</sup> Autologous tissue transfer to support urethroplasty augmentation generally involves harvesting tissue from areas of the buccal mucosa, nonhairy skin, bladder, or colonic mucosa. These procedures often have associated limitations, including the availability of adequate tissue, the necessity for multiple surgeries, and the development of comorbidities.<sup>358</sup> Tissue engineering aims to reduce these limitations by through approaches such as seeding cells into damaged urethral tissue, implantation of acellular matrices, and implantation of a tissue-engineered substitute.<sup>359</sup>

Initial attempts at urethral reconstruction focused on nondegradable materials; this approach, however, resulted in complications such as tissue calcification and fistulae;<sup>360</sup> consequently, technologies to improve urethral tissue engineering currently rely on biodegradable scaffold materials with sufficient mechanical support to maintain an open lumen until newly forming tissue can fully regenerate.<sup>361</sup> Natural polymers such as collagen or gelatin cannot be used independently for urethral reconstruction due to their weak mechanical properties and rapidly biodegradability; however, natural biomaterials that may incorporate these polymers, such as acellular matrices obtained from cadaveric or animal organs, are an alternative source for scaffold material following decellularization.<sup>362,363</sup> Homologous transplantation of organ specific urethral acellular matrix from a donor has been shown to support complete epithelialization and regeneration of smooth muscle bundles.<sup>364</sup> Xenogenic small intestine submucosa (SIS), for example, has been tested clinically for repairing urethral strictures. While initial studies demonstrated a tendency for recurrent strictures,<sup>365</sup> recent studies have indicated that SIS may in fact function effectively in reconstructive procedures for ureters. Overall safety and long-term efficacy still require additional investigation however.<sup>366</sup> Decellularized cadaveric human bladder submucosa matrix has also been used clinically in urethral structure repair, but has demonstrated a lower success rate compared to utilization of buccal mucosa for recurrent and complicated strictures.<sup>367</sup> Nevertheless, preclinical studies using bladder submucosa as a matrix has produced promising results with a variety of cell types, including adipose-derived and urinary-derived stem cells, autologous bladder smooth muscle and urothelial cells, and autologous minced urethral tissue.<sup>368–370</sup> In general, various cell lines have been used in preclinical and clinical studies for urethral reconstruction, including human progenitor cells (epithelial cells and smooth muscle cells), adipose derived stem cells, fibroblasts, and keratinocytes.<sup>356</sup> Recently, urinary-derived stem cells have gained attention in tissue engineering due to the capacity for proliferation, adhesion, and differentiation into smooth muscle and urothelial cell lines.<sup>371</sup> Importantly, these cells can be obtained without the need of invasive procedures.<sup>372,373</sup>

Synthetic scaffolds for urethral tissue engineering include biodegradable poly(glycolic acid) (PGA), poly lactic-co-glycolic acid (PLGA), and poly(caprolactone) (PCL).<sup>374</sup> Scaffolds of PGA:PLGA containing autologous bladder smooth muscle and urothelial cells from bladder biopsies have been used clinically, in an observational study, to successfully bridge 4–6 cm urethral defects, in five patients.<sup>375</sup> This approach has currently been approved for a Phase I

clinical trial for safety and feasibility. Natural and synthetic materials are increasingly being used in combination to produce hybrid materials that meet desired properties on mechanical strength, porosity, cell affinity, biocompatibility, and biodegradability. Matching the scaffold degradation rate with the regrowth of healthy tissues is an important consideration. These materials can also be enriched with cytokines and growth factors that promote vascularization of the graft and tissue restoration.<sup>376,377</sup> Newly created functional and smart polymeric materials together with advances in composite biomaterials for 3D bioprinting will facilitate the optimization of tissue engineering solutions to meet clinical performance requirements in the future.<sup>378–380</sup>

## Concluding Remarks

In the last few decades, the field of regenerative medicine has made incredible strides in targeting tangible therapies for patients in need.<sup>381</sup> A variety of approaches involving diverse cellular, material and biochemical factor configurations have been explored for their capacity to support tissue regeneration. Complex tissues consisting of heterogeneous structure/function, such as skin, bone, cartilage, lung, kidney, and reproductive tissues, have been the object of innovative technologies such as programed bioprinting, which can mimic spatial architecture by depositing biological materials and cells in a well-controlled manner.<sup>382</sup> Platforms such as gene-editing using SiRNA<sup>383</sup> or CRISPR technology<sup>384</sup> also offer exciting pathways for regenerative and tissue-engineered inspired applications. Currently, clinical trials are investigating both cellular and acellular solutions for a variety of regenerative treatments.<sup>385</sup> Recent scientific advancements and clinical discoveries continue to inspire progress toward effective, clinically translatable therapies that will be amenable to commercialization in the near future, and thereby increase the availability of tissue-engineered products. Developments such as these will make it possible to achieve easily distributed, patient-specific regenerative treatments that will target a wide range of health issues going forward.

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## CHAPTER 58

# Human tissue allografts: responsibilities in understanding the path from donor to recipient

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### Introduction

A growing number of patients benefit from donated tissues of human origin. Regulated as human cells, tissues, and cellular- and tissue-based products (HCT/Ps), FDA requires that HCT/Ps be minimally manipulated (or processed) and retain homologous use of graft function as in the donor. Allografts promoted for use today include bone, cartilage, and tendons used to reconstruct joints and other orthopedic structures; skin to treat burns (split thickness graft) and to support underlying structures (acellular dermal matrix); adipose to provide cushioning and aesthetic improvement; veins, arteries, and heart valves for cardiovascular repair; placenta as a wound covering; cornea to restore sight; and reproductive tissue to treat infertility. The most commonly transplanted allografts are bone, musculoskeletal tissue, and dermis (Table 58.1).<sup>1,2</sup>

Tissue may be “fresh,” with minimal processing, in whole grafts and sections, such as osteochondral and osteoarticular types, or may be ground, morselized, or shaped for needed function, such as gels, pastes, or strips in specific applications. Further processing may strip the graft of cells, creating an acellular matrix. Storage parameters may also vary, either requiring room temperature, refrigeration, or freezing, for days, months, or years. Even when meeting criteria as HCT/Ps, these grafts may bear little resemblance to their original form, transplanted in a variety of medical and dental applications, but it is important that each tissue form be recognized as of human origin, with appropriate tracking and consent.

Tissues may be recovered from the same person as the recipient (autograft) or from a different donor than the recipient (allograft). Allografts are recovered from either living (placenta and gametes) or deceased (bone, cardiovascular, and cornea) donors.

Some grafts thought of as “tissue” are not covered in this chapter, including stem cells for infusion, bone marrow for transplantation, solid organs, and vascularized composite allografts (e.g., face, limbs, and uterus).

The total number and range of tissues collected, processed, and transplanted in the United States are difficult to determine, in the absence of a national reporting system or systematic survey approach that otherwise exist for organs and blood. Surveys

are conducted sporadically by AATB with support from US HHS.<sup>3</sup> In 1999, tissue banks accredited by the American Association of Tissue Banks (AATB) distributed approximately 750,000 allografts for transplantation. By 2003, the figure had more than doubled. By 2007, more tissue banks became accredited and greater than 2,000,000 units were distributed. As of 2015, AATB members recovered from more than 39,000 donors resulting in more than 3,200,000 units distributed.<sup>3</sup> Eye banks are accredited differently, and corneal transplants numbered >85,000 from over 68,000 donors recovered by 69 EBAA accredited banks in 2019.<sup>4</sup>

In addition to such tissues from deceased donors, many tissues for clinical use are derived from living donors, including semen and oocytes for use in artificial insemination and assisted reproductive technology procedures. A major increase in tissue recovery from living donors is attributable to placentas donated from delivery mothers for grafts derived from the amniotic/chorionic membrane. Tissue from living donors is also considered by some to include human milk. The US Food and Drug Administration (FDA), however, does not subject human milk to regulations that apply to human allograft tissue, and thus it is also not covered in this chapter.

### General principles of tissue preservation, storage, and clinical use

Allograft transplantation is increasing in the United States partially because of the successful clinical application of tissue allografts from living donors (e.g., delivery mothers donating the placenta for grafts derived from the amniotic/chorionic membranes). Further, a myriad of surgical specialties use human tissue allografts: orthopedic surgery, neurosurgery, cardiothoracic surgery, plastic surgery, vascular surgery, urology, ophthalmology, burn and other skin wound care, podiatry, sports medicine, trauma, and cranio/maxillofacial surgery. Additionally, increasingly refined and innovative HCT/Ps are being developed by tissue suppliers to meet the clinically diverse needs of patients.<sup>2</sup>

**Table 58.1** Some Common Human Allograft Applications, by Clinical Specialty

Specialty	Procedure/Application	Allograft Tissue Type
General orthopedics	Trauma/fracture repair Osseous defect repair Acetabular repair Total joint revision/arthroplasty	Femoral head; femoral condyle; whole, proximal, or distal bone shaft (femur, tibia, and humerus); hemipelvis; cancellous bone; corticocancellous bone; cortical strut/screw/pin; bicortical strip; tricortical wedge; whole joint* (knee, ankle, shoulder, and elbow); osteoarticular graft*; osteochondral graft*; DBM; osteobiologics
Sports medicine	Tendons, anterior cruciate ligament, posterior cruciate ligament, other knee ligament repair; meniscus repair/replacement; osteochondral defects; rotator cuff repair; ankle/tendon ligament repair; hand/wrist repair	Patellar ligament; Achilles tendon; tibialis tendon; semitendinosus tendon; gracilis tendon; peroneus longus tendon; fascia lata; rotator cuff; meniscus; meniscus w/tibial plateau; osteochondral plug*; femoral hemicondyle*; acellular dermal matrix
Craniofacial/maxillofacial	Cranial reconstruction; maxillary/mandibular reconstruction; facial palsy repair	Mandible; dura mater*; pericardium; fascia lata; acellular dermal matrix; bicortical strip; tricortical wedge; DBM; osteobiologics; sclera
Dental	Alveolar ridge augmentation for dental implant placement; onlay grafting; sinus elevations/augmentation; socket/ridge preservation; intrabony defect repair	DBM; osteobiologics; sclera; acellular dermal matrix; particulate and structural cortical and cancellous grafts and combinations
Ophthalmology	Postcataract corneal edema repair; Fuchs dystrophy repair; glaucoma drainage valve implantation; corneo-scleral fistula repair; keratoconus correction; phaco burn repair; orbital reconstruction following enucleation; eyelid ectropion repair; eyelid reconstruction	Cornea*; sclera*; pericardium
Neurosurgical	Cervical/lumbar interbody fusion; intermedullary rod placement; dura replacement	Dura mater*; fascia lata; pericardium; cancellous bone; corticocancellous bone; cortical strut; bicortical strip; various machined and constructed proprietary bone forms*; amniotic membrane*; acellular dermal matrix
Burn treatment	Wound covering*	Fresh skin*; cryopreserved skin*; freeze-dried skin*; acellular dermal matrix; amniotic/chorionic membrane* Fascia lata; pericardium; lyophilized skin*; acellular dermal matrix; adipose tissue
General surgery	Urologic incontinence procedure; pelvic floor reconstruction; herniorrhaphy; breast reconstruction HIV lipodystrophy	
Plastic surgery	Anatomic defects for aesthetic improvement Plastic surgery reconstruction	adipose tissue; acellular dermal matrix DBM
Cardiac	Congenital anomaly repair (both valve and outflow tract major vessel repair/replacement*); cardiac valve and vessel repair/replacement; major vessel blood "shunting" procedures	Aortic valve*; pulmonary valve*; various conduit-use-only grafts* from the ascending aorta or thoracic aorta or from the pulmonary artery trunk or its branches

**Table 58.1** (Continued)

Specialty	Procedure/Application	Allograft Tissue Type
Vascular	Vaso-occlusive disease (peripheral, abdominal, and thoracic); cardiac artery bypass grafting; arteriovenous shunt insertion; muscle-flap or organ transplant vascular bed extensions; replacement of infected prosthetic devices	Greater saphenous vein*; aorto-iliac artery*; iliac vein*; iliac artery*; femoral vein*; femoral artery*

\* Not sterilized.

† Can be lifesaving.

DBM: demineralized bone matrix.

Source: Modified from Eisenbrey *et al.* (2008)<sup>1</sup> and Powers and Linden (2015)<sup>2</sup>.**Table 58.2** Human Tissue Storage Conditions

Human Tissue	Storage Condition*	Temperature
Musculoskeletal	Frozen ( <i>cryopreserved and noncryopreserved</i> ) long term Frozen ( <i>cryopreserved and noncryopreserved</i> ) temporary storage for six months or less Refrigerated, short term	-40 °C or colder -40 to -20 °C
Skin	Lyophilized, long term Refrigerated, short term Frozen, long term	1–10 °C Ambient temperature** 1–10 °C
Cornea	Lyophilized, long term Refrigerated, short term (defined by method)	-40 °C or colder Ambient temperature** 2–8 °C
Semen	Frozen, long term	Liquid nitrogen (liquid or vapor phase)
Cardiac, vascular	Frozen, <i>cryopreserved</i>	-100 °C or colder
Dura mater	Lyophilized, long term	Ambient temperature**

\* Or as defined and validated by the processor.

\*\* Ambient temperature monitoring not required for lyophilized tissue.

Source: Adapted from Osborne JC *et al.* (2017).<sup>5</sup> and Trainor and Reik (2014).<sup>6</sup>

The most common storage condition for tissue preservation is at cold temperatures, either by refrigeration or freezing. Bone most often is lyophilized (freeze dried). (Table 58.2).<sup>5,6</sup> Short-term storage usually requires refrigeration, 4 °C, whereas longer-term storage generally requires a frozen state, -40 or -80 °C, or even at liquid nitrogen (liquid or vapor phase) temperatures if the graft is not lyophilized. Several types of tissues can be preserved by multiple methods depending upon their use. Bone, dura mater, and amnion can be cryopreserved or lyophilized. For tissues that will provide a structural role in the recipient, the maintenance of cell viability is not necessary. Tissues of this type, which include dermis and some forms of placenta, are composed of an extracellular matrix (such as collagen) with few or no viable cells present to support the matrix after transplantation, although they can contribute growth factors or proteins to facilitate remodeling. Even when the processing method used is intended to preserve cell viability, the donor cells typically will die within hours or days following transplantation. The extracellular matrix, whether transplanted containing viable cells or devoid of them, is repopulated through the in-growth of metabolically active recipient cells. Gradually, depending in part on the size and type of the allograft implanted, remodeling occurs, and the transplanted structure may eventually be entirely replaced by host cells.<sup>7</sup> The transplantation of allograft heart valves and cardiac conduit tissues provided in an acellular matrix form has been studied to determine the rate and extent of repopulation with recipient cells.<sup>2,8</sup>

In some tissues, such as cornea, a single layer of viable donor cells is important, and this requirement necessitates the maintenance of

the tissue in culture medium at refrigerated temperature.<sup>9</sup> Other human tissues, such as marrow, skin, and gametes, are stored by either refrigeration or cryopreservation. In the latter, a controlled rate freezing process and a cryoprotectant remove water from the cells while maintaining viability. The usefulness of tissues requiring post-transplantation cell viability depends on their maintenance of not only metabolic activity, but also capacity to synthesize protein, proliferate, or differentiate.<sup>2</sup>

### Donor-recipient matching: is it necessary?

Donor-recipient HLA matching is not necessary for most tissues and is rarely performed. Tissues such as bone, fascia, tendon, cartilage, and dura mater are not preserved or transplanted in a viable state for donor cells; rather, they serve as a support or matrix that the recipient's own cells can enter and gradually replace. Immunologic rejection, therefore, is not a significant concern, and matching of blood group or HLA antigens is considered unnecessary. There are a few exceptions where immunologic graft rejection can occur. In patients who have received a repeat cornea graft, efforts are made to use HLA-matched corneas in subsequent transplants.<sup>10</sup> HLA sensitization has also been reported in recipients of vascular allografts or allograft heart valves.<sup>11</sup>

The ABO antigens are a significant consideration in organ transplantation because they constitute strong histocompatibility antigens that are expressed on vascular endothelium. A major ABO mismatch can cause rapid graft rejection resulting from endothelial

damage by ABO antibodies and subsequent widespread thrombosis within the graft. Therefore, ABO matching is important to the success of vascularized organ grafts (i.e., kidney, heart, liver, and pancreas). However, ABO matching is not critical for a successful outcome when using most tissue grafts (i.e., fascia, bone, heart valves, skin, and cornea). Rarely, hypersensitivity to antigens expressed by fresh or cryopreserved donor tissue can occur and appears to be dependent on an ill-defined immune response by the recipient.<sup>2</sup>

Alloimmunization to minor red blood cell antigens, RhD, Fy<sup>a</sup>, and Jk<sup>b</sup>, following transplantation of frozen unprocessed bone has been reported.<sup>12,13</sup> Consequently, frozen unprocessed bone allografts usually are matched with the donor for the D antigen if the recipient is a female of childbearing potential, in addition to being matched for ABO group.<sup>2</sup>

### Clinical uses of allografts

#### Bone—frozen and lyophilized

Bone allografts have many uses, including provision of acetabular and proximal femoral support for replacement of failed prosthetic hip joints, packing of benign bone cysts, fusion of the cervical or lumbar spine to correct disk disease or scoliosis, restoration of alveolar bone in periodontal pockets, reconstruction of maxillofacial deficits, and replacement of bone that has been resected because of a bone malignancy, such as osteosarcoma (Table 58.1). Bone malignancy surgical procedures are accomplished with large osteochondral allografts that permit tumor resection and achievement of a cure and are limb sparing, avoiding amputation.

Modern technology allows the cutting, machining, and piecing together of allografts via precision instrumentation, and has resulted in stronger and more versatile grafts that can withstand the challenges of new surgical techniques. Linear grooves, notches, or crosshatchings may be incised into bone surfaces to make the bone graft less likely to slip or become dislodged after placement. Many bone allografts, especially those used in neurosurgical applications, are now placed using precision instrumentation that not only ensures exact placement but also enhances stability. Allografts can be cut or shaped to precise angles that accommodate, for instance, lordosis of the cervical and lumbar spine. Advanced processing methods are being developed to improve availability while retaining or improving function.<sup>2</sup>

Fresh autograft can be taken from the patient's own iliac crest intraoperatively, but this practice is becoming less common.<sup>7</sup> Fresh bone autograft is preferred by some surgeons; however, preserved allografts are practical and accepted alternatives that approximate the results obtained with fresh bone autograft.<sup>14</sup> In some patients, an autograft is not an option because sufficient high-quality bone is not available. Other advantages of bone allografts versus autografts are a reduction in operating room time, and the number of operative sites, leading to a reduction in morbidity and surgical costs. The use of bone allograft does carry a minimal risk of donor-to-recipient transmission of infectious disease,<sup>15</sup> although this risk is mitigated through careful donor selection and testing, coupled with disinfection and sterilization of tissue during processing as described earlier.

#### Frozen bone

Frozen bone, collected under aseptic conditions and then frozen or cryopreserved, is available in a wide variety of shapes and sizes from deceased donors, or obtained from a living donor recovered as a femoral head or tibial plateau undergoing total joint replacement in

an operating room (an uncommon practice in the United States, but continuing in Canada, Australia, and Europe). Frozen bone carries an increased risk of disease transmission compared with processed tissue. Diseases known to have been transmitted by unprocessed bone include HIV, HCV, HTLV, tuberculosis, and hepatitis.<sup>15</sup>

Frozen bone can cause alloimmunization from exposure to antigens on the attached connective tissues, marrow, and blood, although such alloimmunization apparently does not affect the graft's efficacy. Detailed reviews addressing the role of histocompatibility and the immune response in bone allograft transplantation have been published.<sup>16</sup> Antibodies to histocompatibility antigens,<sup>17,18</sup> blood group antigens,<sup>12,13</sup> and bone matrix proteins have been induced by transplanted frozen bone. In order to avoid Rh alloimmunization, bone from an Rh-negative donor is usually selected when using bone that has not been processed to remove red cells, and the recipient is an Rh-negative female of childbearing potential.

Frozen bone allografts are available as whole bones or cut into usable shapes and sizes. Frozen bone can generally be stored up to five years at -40 °C or colder, but the maximal storage duration and expiration date may vary based on processing and storage methods, as validated by the tissue bank. There is no evidence that the biomechanical or osteoinductive properties decline during frozen storage. However, in the absence of cryopreservation, frozen bone does not maintain cellular viability. Thus, frozen bone is used for structural support that depends on an intact calcified extracellular matrix or is used as filler to promote new bone formation.

#### Lyophilized bone

Following aseptic recovery, deceased donor bone can be maintained frozen at -40 °C or colder, and then can later be sent to a tissue processor. Alternatively, immediately after recovery, the tissue can be placed on wet ice and expedited for transport directly to the processing tissue bank where, within hours of recovery, it is frozen at -40 °C or colder until processing. Such processing includes removal of surface tissues and internal fat, blood, and marrow by means of mechanical agitation, high-pressure water jets, or alcohol soaks. It can also include detergents or other solutions as part of a proprietary process. Then, the bone is milled into clinically useful shapes and sizes. This may include computer-guided milling and use of assemblies that result in complex mechanical structures. Conventional allografts include corticocancellous strips, wedges, and dowels; cortical struts and rings; and cancellous and corticocancellous cubes and chips.

DeminerIALIZED (i.e., extraction of calcium) freeze-dried bone allografts (DBM) is obtained from cortical bone and maybe found as specific granule or particle sizes, as a powder, or in entangled, twisted fiber configurations. DBM products are available in combination products in the form of gels, pastes, putties, and flexible strips or sheets. DBM primarily provides growth factors, but accompanying collagen can help play a structural role as a scaffold for future bone growth. The combining of DBM with approved polymer carriers results in moldable grafts that are user friendly for the surgeon, do not migrate after placement, and whose bone content does not dissolve following transplantation. Such grafts can readily be applied to completely fill bony defects and to act as a scaffold for ingrowth of the recipient's own cells, or they can be used to enhance other structural repair devices, such as dental implants, vertebral body spacers and cages, or support devices such as rods, screws, and plates.<sup>1</sup> Potency can be evaluated by using assays for osteoinductive capacity and biomechanical properties, but these

analyses are usually conducted only when there is a change in the production process. Lyophilized bone is brittle unless fully rehydrated before use. Lyophilized bone can usually be stored at ambient temperature for up to five years if the graft's package integrity and its vacuum are maintained, depending on validation performed by the tissue processor.

While the purpose of lyophilization is to allow for convenience, other preservation methods exist that also allow for ambient temperature storage of grafts. Some tissues can be dehydrated via chemicals (such as acetone), some kept in saline (e.g., costal cartilage), and some are packaged with a humectant (such as glycerol). The last two examples are considered prehydrated and are "ready to use" off the shelf. Expiration dates for all of these alternate methods are established by validation of the packaging by the tissue processor.

Bone collected aseptically in an operating room and processed aseptically can be lyophilized without use of a sterilant. In aseptic processing, bone is cultured extensively before release, and final or terminal sterilization with  $\gamma$ -irradiation may not be applied. In addition, bone grafts with "viable cells" have become more popular. Although the bone should be free of bacteria that can be cultured through routine microbiological methods, it still has the potential to transmit disease, including viruses, mycobacteria, and some fungi. Despite this risk, some physicians have preferred aseptically processed, nonsterilized lyophilized bone because it was thought to have better osteoinductive capacity than sterilized bone. However, controlled dose low-temperature radiation has been found to have no significant effect on osteoinductive capacity.<sup>2,14</sup>

## Connective tissues

### Cartilage and meniscus

Human cartilage can be transplanted at weight-bearing or non-weight-bearing sites. For non-weight-bearing uses such as nasal reconstruction and mandibular or orbital rim augmentation, the graft provides structural support and need not be viable. *Costal cartilage* can be recovered for this purpose. Gamma irradiation can be used to sterilize the cartilage and then stored in saline at refrigerated temperatures, or it can be lyophilized and stored at ambient temperature.

*Articular cartilage* can be transplanted to weight-bearing articular surfaces to replace focal cartilage defects caused by trauma or degenerative disease, particularly in the knee. Cartilage in an osteochondral or osteoarticular allograft can be obtained as a femoral hemicondyle, a tibial plateau or fragment, or a measured segment removed with a template cutter that can be press fitted into a similarly cut area in the recipient. Osteochondral allografts avoid autograft site morbidity and are advantageous when the focal articular cartilage defects being repaired are large (>2.5 cm).<sup>19</sup> It has been assumed that, in weight-bearing applications, chondrocytes must survive the collection and preservation process and remain viable, producing normal cartilage matrix to maintain mechanical properties. It appears that chondrocytes deep within the cartilage matrix resist cell-mediated immune responses by the recipient and, if kept viable during storage, are able to survive after transplantation. Cartilage grafts from histo-incompatible donors, stored <24 hours at 4 °C, have survived for as long as seven years after transplantation, if the grafts developed a sound union and if conditions for correct biomechanical functioning were present.<sup>20</sup> Articular cartilage collected in a sterile manner can be stored at 4 °C in antibiotic containing saline or electrolyte solution, with or without 10% fetal (bovine) calf serum.<sup>21</sup> For solutions containing calf serum,

screening procedures are now in place to reduce the risk of source animals that may have bovine spongiform encephalopathy (BSE). Osteoarticular and osteochondral allografts can be stored refrigerated for up to 28 days with successful clinical outcomes. If such grafts have been cryopreserved, expiry for these allografts can be extended to one year.

The use of a large osteochondral allograft, such as the femur with the articular cartilage attached, is thought to require preservation of cartilage viability in order to maintain biomechanical properties. To accomplish this, grafts have been stored at refrigerated temperatures in electrolyte solutions for up to one month, or have been frozen in 10% glycerol or 15% DMSO and stored at -70 °C or colder.<sup>22</sup> Following transplantation in humans and animals, the surface of the articular cartilage allograft undergoes degenerative changes within a few years. These grafts carry the same risk of disease transmission as other fresh tissue allografts.

*Menisci* are C-shaped disks of fibrocartilage interposed between the femoral condyle and tibia. The presence and integrity of the meniscus are essential for knee mechanics and biochemical functions. Loss or disruption of the meniscus is associated with pain, joint laxity, and degenerative arthritis. Meniscal transplantation has been proposed as a method of providing a biologically and biomechanically acceptable structure to replace a damaged or removed meniscus, with a goal of relieving pain, decreasing stress on the anterior cruciate ligament, and preventing late arthritis, although evidence of allograft tissue being chondroprotective is lacking. Although there have been unpublished reports of successful transplantation of menisci stored <24 hours at 4 °C, fresh menisci are not usually available. Cryopreserved menisci are used successfully, with good outcomes (including reduced pain and increased knee function) reported.<sup>2,23,24</sup>

### Tendon and ligament

The knee is the joint most frequently involved in sports-related injury. Arthroscopic methods for replacing the anterior or posterior cruciate ligaments with autografts, allografts, or artificial tendons and ligaments are frequently used. Despite the attendant need for sacrifice or weakening of normal structures, the use of autografts appears to have a high success rate and low incidence of complications. However, allografts may be indicated for multiple ligament knee injuries, anterior cruciate ligament revisions, or posterior cruciate ligament reconstruction, and when extensor mechanisms are impaired (as with previous tendon tears). It is also sometimes preferable to avoid the morbidity associated with autograft. In addition, there are occasions when sources of adequate autograft tissue are not available.<sup>25</sup> Allografts used to replace the injured anterior cruciate ligament are usually derived from deceased donor patellar ligaments, tendons of the leg (e.g., tibialis, semitendinosus, gracilis, and peroneus longus), or Achilles tendons. Ligament and tendon allografts are usually stored frozen, but some are stored lyophilized. In vitro biomechanical properties of tendons do not seem to be greatly affected by freezing, lyophilizing, or ethylene oxide sterilization.<sup>26</sup> However, many surgeons avoid lyophilized tendon allografts because of experiences with clinical failure. Frozen tendon allografts are commonly sterilized by  $\gamma$ -irradiation, although this can reduce their mechanical strength, particularly if performed at room temperature or if the dose exceeds 20 kGy.<sup>27</sup> There is no evidence that maintenance of cellular viability during processing and storage is important to clinical effectiveness, although some growth and other biologic factors may be important. The effect of irradiation on the biomechanical properties of human tissue has been explored

extensively, with inconsistent results. This is probably because the studies failed to use uniform irradiation methods and comparable study designs. A key study found a difference in average stress at failure between nonirradiated and  $\gamma$ -irradiated tendons; that difference is likely a consequence of the free radicals generated, which can cause minor crosslinking of collagen fibers and alteration of the tendon's material properties.<sup>28</sup> In order to eliminate the potential for elongation of irradiated grafts after implantation, the authors encouraged "pretensioning" (e.g., stretching to a desired uniform length range before packaging) of grafts before insertion.

### Fascia lata

The fascia lata is a broad fibrous membrane surrounding the thigh muscles. The thick lateral portion acts as a flattened tendon, and its muscular insertions serving to maintain the trunk in an erect posture. Fascia lata can be removed and transplanted as an autograft or allograft. As an allograft, fascia lata has been used to suspend the upper eyelid to correct ptosis, as a covering for bone grafts in dental surgery, to replace injured anterior cruciate ligaments, to provide support for bladder suspension, and to repair ankle, hip, and shoulder suspensions (e.g., repair of a ruptured shoulder rotator cuff). Fascia lata is usually preserved by lyophilization, resulting in a residual moisture of <6% or 8% (depending on measurement method), the graft is then sterilized by  $\gamma$ -irradiation and stored for up to five years at ambient temperature. After rehydration, the graft's biomechanical properties equal those of fresh frozen fascia lata. The use of fascia lata has become less popular because of the availability of alternative products, such as decellularized skin.<sup>2</sup>

### Dura mater

Dura mater is the outermost, toughest, and most fibrous of the three meningeal membranes covering the brain and spinal cord. The intracranial portion is collected, processed, stored, and distributed for several clinical applications; the most common use is the closure of dural defects caused by resection of tumor or the repair of traumatic injury. Human dura allograft is most commonly preserved by lyophilization. Ethylene oxide and  $\gamma$ -irradiation are effective in preventing transmission of viruses and bacteria; however, Creutzfeldt-Jakob disease (CJD) has been transmitted by dura mater treated by these methods. Following findings by Brown and coworkers,<sup>29,30</sup> in 1986 The Committee on Health Care Issues of the American Neurological Association recommended using 1N NaOH for one hour or steam autoclaving for one hour at 132 °C as standard sterilization procedures for CJD-infected tissue or contaminated materials. Donors with a history of clinical dementia or other central nervous system disorders are not accepted as donors. Lyophilization and sterilization treatments do not lessen the effectiveness of dura mater allografts. Reconstituted freeze-dried dura mater is thick and strong, holds suture well, and is incorporated into normal surrounding tissue without rejection.<sup>2</sup>

### Skin for burn

Human skin allograft is the dressing of choice for temporary grafting onto deep burn wounds whenever sufficient amounts of autograft skin are unavailable. Early excision of burned tissue and covering of the wound with deceased donor skin allograft has shortened hospitalization and decreased mortality more than has any other treatment.<sup>31</sup> A skin allograft provides temporary coverage and acts as a barrier against loss of water, electrolytes, protein, and heat. It reduces opportunities for the invasion of bacteria and speeds re-epithelialization. Skin allografts are replaced periodically until

the patient's vascular bed is reestablished. Skin allografts are also used for unhealed skin defects (decubitus ulcers, autograft skin sites, pedicle flap sites, and traumatically denuded areas).

After collection, fresh skin can be stored in medium at 1–10 °C for up to 14 days,<sup>5</sup> but fresh skin is seldom used today. Skin also can be frozen using a method that retains cell viability in order to improve availability. Because cell viability declines during refrigerated storage, results are best when cryopreservation is performed within 2–3 days after recovery. Cryopreserved skin can be prepared as strips (often 3-inch by 8-inch sections), either unmeshed or meshed (most commonly with a 1:1.5 expansion ratio, which triples the area that can be covered). The skin is then covered in fine-mesh gauze and laid flat, packaged, and then cryopreserved with glycerol or DMSO at a concentration of 10% or 15% as a cryoprotectant. Cryogenic damage is minimized by controlling the rate of freezing to between -1 and -5 °C/minute. Many tissue banks use a "heat sink" freezing method rather than one that employs computer-controlled freezing chambers. Heat sinks involve aluminum plates combined with styrofoam-insulated boxes; these are placed directly into a -70 °C mechanical freezer. This simple process provides a slow, controlled freezing rate that is acceptable for skin and that also maintains cellular viability.<sup>32</sup> AATB standards permit frozen storage in a mechanical freezer at -40 °C or colder, in the vapor phase of liquid nitrogen, or submerged in liquid nitrogen.<sup>5</sup> Cryopreserved skin allograft is usually transported from the tissue bank to the hospital with dry ice in order to maintain a frozen environment until use.

Skin for use in burn applications is generally not preserved by lyophilization because this method decreases clinical efficacy.

### Acellular dermal matrix

Although skin has historically been used only as a covering, decellularized skin offers the opportunities for use of a collagen matrix that can be implanted and remodeled within the site with the recipient's own cells, and can be either processed aseptically using chemicals or terminally sterilized using irradiation.<sup>33,34</sup> It is used for such applications as bladder suspension surgery, tendon repair, post-mastectomy breast reconstruction, oral reconstruction, and repair of large defects, such as postoperative hernias and dehisced wounds. Lyophilized (and prehydrated) acellular dermal matrix is available in several thicknesses for different applications, and can serve as a natural biological matrix for soft tissue augmentation in soft tissue defects and in periodontal peri-implant soft tissue management. Lyophilized skin is sometimes used by oral surgeons to cover oral mucous membrane defects and to speed re-epithelialization. Following hydration, lyophilized skin has multidirectional strength and can adapt to surface contours, and it is then resorbed over 4–6 months, depending on the site, defect size, patient age and health status, and the biomechanical load on the graft. Depending on processing method and packaging configuration, lyophilized skin can be stored as long as five years at ambient temperature or it may require refrigeration.<sup>2</sup>

### Ocular tissue

Cornea is one of the most frequently transplanted tissues; > 85,000 corneas were transplanted in the United States in 2019.<sup>4</sup> Corneal transplantation has become highly effective because of improvements in suture materials, surgical instruments and techniques, and medications to prevent and reverse rejection. It is considered a standard therapy for a variety of conditions. In the last 10 years, there has been a paradigm shift from full thickness keratoplasty to selective keratoplasty, where only the diseased layers of the cornea

are replaced. One such selective keratoplasty techniques is Descemet's stripping endothelial keratoplasty (DSEK), which transplants only the innermost portion of the cornea; the tissue adheres to the host cornea with the use of an air bubble. The benefits of this technique are a smaller, stronger wound with minimal disruption of the interior curvature of the cornea, resulting in faster recovery improved visual acuity, as well as a reduction in the occurrence of adverse effects such as graft rejection and vision threatening intraoperative and postoperative complications. As a consequence, DSEK for Fuchs endothelial dystrophy has become the preferred surgical therapy at much earlier stages of the disease, resulting in a resurgence of demand for cornea allografts. Currently, the most common indications for corneal transplantation are keratoconus, Fuchs dystrophy, postcataract surgery corneal edema, and corneal regrafting. Donor cells in the avascular full-thickness cornea graft enjoy long-term survival without the aid of histocompatibility matching because the recipient site is also almost completely avascular. Because of the avascularity of the cornea, routine immunosuppression is accomplished with topical corticosteroids. However, systemic immunosuppression may be used in conjunction with topical agents for high-risk cases. Some experts believe that the failure rate of 5–10% might be improved by HLA matching; recipients known to be sensitized to HLA antigens have rejection rates higher than nonsensitized recipients.<sup>35</sup> The possibility of alloimmunization is of particular concern in patients who are undergoing repeat grafting procedures because of graft failure or who have ocular infections, as the corneal rim may become quite vascularized. Sclera may be used in the repair of ocular defects, in orbital reconstruction following enucleation, and in some dental applications.<sup>36</sup>

Ocular tissue can be recovered by enucleation or by *in situ* excision of the cornea, with a rim of sclera. It is preferable that recovery be performed within 10 hours after death. The most common method of storage for the cornea today is with attached rim of sclera, at 4 °C in a modified tissue culture medium, based on that developed in 1974 by McCarey and Kaufman.<sup>37</sup> One example commonly used is Optisol-GS (Bausch & Lomb, Irvine, CA), which contains dextran (as an osmotic agent), chondroitin sulfate, gentamicin, and streptomycin. Storage of corneas in the medium, at 2–8 °C, can maintain endothelial viability for as long as 14 days and can maintain functional integrity for eutopic graft applications not requiring visual acuity for even longer storage periods.<sup>38</sup> Grafts are usually used within seven days. Although they have been treated with antibiotics, allograft corneas are not considered sterile. Rarely, corneas are frozen with cryoprotectants. Sclera are usually preserved in ≥70% ethyl alcohol; such a method yields a shelf life as long as two years.

### **Cardiac and vascular tissue**

Cardiac and vascular tissue includes heart valves, patches, and non-valved outflow tract arteries and vessels that can be used as conduits. Donor medical history requirements differ, so AATB has established separate standards for cardiac tissues and for vascular tissues.<sup>5</sup> Since their introduction decades ago, human heart valve allografts have been shown to be an alternative for patients needing heart valve replacement for whom mechanical and xenograft valves are contraindicated. Human heart valve allografts do not require recipient anticoagulation, have a lower incidence of thromboembolism, and appear relatively resistant to infection. After valve allograft transplantation, donor endothelium is not maintained, but donor fibroblasts may remain for an undetermined period. Because anticoagulation is unnecessary, human valve allografts are the graft

of choice for children, females of childbearing potential, and patients with cardiac infection in the aortic root. The use of allograft valves has been slowed, however, because implantation is more technically difficult compared to modern versions of stented prosthetic valves. In addition, their availability is limited, especially for pediatric use. Additionally, clinical results with transplantation of xenograft tissue valves have improved, although these are not available in the small sizes required by many pediatric patients.

Technical impediments have made it impossible to successfully produce man-made (either completely artificial or modified xenograft) replacement heart valves for use in neonates and other pediatric patients who require very small grafts. Only donated human heart valves from newborns or small children offer unobstructed blood flow through such a small annulus. Also, the tissue's pliability renders human allografts adaptable to the ingenuity of cardiothoracic surgeons who repair congenital defects by using allografts to replace underdeveloped or otherwise defective valves or outflow tracts, or to construct valves and tracts that may be absent.<sup>39</sup> Complex repairs may need to be staged over many years or may be only palliative. Demand for clinical use of nonvalved conduit sections of cardiac allografts (mostly from the main pulmonary artery and/or its branches) has shown superior results in use as treatment of defects of the right ventricular outflow tract.<sup>40</sup> On a global scale, the availability of cryopreserved pediatric allograft heart valves has historically been low and unable to meet demand.

To obtain cardiac allografts, hearts are recovered aseptically, immersed in a sterile isotonic solution within a sterile container, placed on wet ice, and transported expeditiously to a tissue processing facility. The pulmonic and aortic valves, along with their intact outflow tracts and/or small pieces of these conduits, are dissected free of the heart within 48 hours of donor asystole and then placed in tissue culture medium amended with a low-dose antibiotic cocktail. Studies demonstrate that cryopreservation of heart valves allow successful banking of valves of various sizes and types while retaining the intact matrix and having a low clinical incidence of valve degeneration, rupture, leaflet perforation, and valve-related death. For these reasons, human heart valves are generally cryopreserved,<sup>41</sup> with a method that includes an initial exposure to antibiotic solutions for 12–24 hours. Cryopreservation then follows, using a 10% DMSO solution tissue culture medium that is often amended with 10% fetal calf serum. Freezing is accomplished using a computer-assisted controlled rate of -1 °C/minute to -40 °C. Valves are generally stored in the vapor phase of liquid nitrogen. Theoretically, heart valves can be stored indefinitely in liquid nitrogen, although the nature of any deterioration during storage is not well characterized. The viability of cryopreserved connective tissue matrix cells is maintained, but at a lower level than that of fresh valves, and endothelial viability is lost. In addition, noncellular matrix elements are maintained.

The aorta and iliac arteries can be preserved using the same methods applied to heart valves. Frequently, the aortic arch is preserved with the aortic valve intact; such grafts are intended for transplantation as a valved conduit. Preservation and storage methods are similar to those for valves. Synthetic grafts are often the graft of choice, but such grafts may be less effective in an infected field. Aortoiliac arteries are used successfully as conduits in mycotic aneurysm repairs, when synthetic grafts have become infected, and for aortoenteric fistulas in an infected field.<sup>42</sup>

Arterial or venous segments of vascular organs may be recovered in order to provide a source of vascular "conduits" for use in organ transplants when the organ's attached vessels are damaged or

inadequate. Vascular conduits that have been recovered and transplanted under these conditions are not considered tissues under the FDA's human cells, tissues, or cellular or tissue-based products (HCT/Ps) rules, but they are regulated as organs under 42 CFR Part 121. Donor screening and testing, as well as labeling and storage requirements, are identical to those for donor organs specified in a federal contract with the Organ Procurement and Transplantation Network.

Autograft veins are used in cardiac and peripheral vascular bypass graft procedures whenever it is possible, but veins from deceased donors that have been recovered under aseptic conditions may be used for revascularization when autologous vein grafts are not available. Cryopreservation of allograft vessels is similar to that of cardiac allografts. Well-established tissue bank procedures are designed to retain venous endothelial cells during recovery, processing, and preservation, but these cells are rapidly sloughed off the lumen after the vein is transplanted into the high-pressure arterial system. Retention of endothelial cells during recovery and processing does aid, however, in reduction of the risk of thrombosis or failure after implantation through protection of the integrity of the vessel's basement membrane and acellular matrix.<sup>39</sup>

Although not proven to be necessary for successful clinical outcome or to prevent alloimmunization, ABO- and Rh-compatible allograft valves and vessel conduits are usually requested. One recent case series, involving limb salvage utilizing allograft saphenous veins, showed significantly better results in cases with ABO blood-type compatibility.<sup>43</sup> Some studies have shown that the use of these tissue allografts carry a risk of HLA antigen sensitization.<sup>44</sup>

### **Peripheral nerves**

Fresh autografts of peripheral sensory nerves are used in nerve repair, but this practice is hampered by collection morbidity and resulting limitations on the amount of autologous nerve tissue that can be made available. Although allografts ideally might repair peripheral nerve defects without requiring the sacrifice of the patient's own nerve, frozen, irradiated, and lyophilized allografts have not functioned well. New animal studies using nerve allografts cold-preserved for seven weeks have shown promising results, as have cultured Schwann cells added into synthetic conduits.<sup>45</sup> Axogen, Inc. (Alachua, FL), has developed a thermally decellularized nerve allograft scaffold called Avance that is treated with chondroitinase in order to degrade chondroitin sulfate proteoglycan. Such grafts have been shown to inhibit both aberrant growth and retrograde regeneration in the absence of any immunosuppressive therapy. Animal studies employing such an approach demonstrated enhancement of nerve regeneration. The first human Avance nerve allograft was implanted in 2007 into a 38-year-old man who had suffered a traumatic facial nerve injury; a single nerve allograft was used to connect the severed facial nerve root to three nerve branches. The surgeons informally reported that the graft's handling characteristics were superior to those of autograft tissue.<sup>2</sup>

### **Extraembryonic tissue preservation and transplantation**

Extraembryonic tissues that have been used occasionally for transplantation include the amnion and the umbilical vein. Amnion, which is the smooth, slippery, glistening membrane lining the fluid-filled space surrounding the fetus, has been used as a covering for nonhealing chronic leg ulcers, burns, and raw surfaces following mastectomy, and in major oral cavity reconstruction and vaginoplasty. Amnion has also been used as a pelvic peritoneum substitute following pelvic exenteration and as a source of replacement enzymes

for infants with inborn errors of metabolism.<sup>46</sup> Most of the fetal amnion is covered on the maternal side by the chorion, a slightly roughened membrane. Amnion is collected under sterile conditions during cesarean section. The amnion's epithelium and basement membrane can be separated by blunt dissection from the underlying chorion immediately after collection or after temporary storage. The amnion is then cryopreserved or lyophilized. Human umbilical vein allografts previously were used occasionally as vascular substitutes to provide venous access for hemodialysis or as an arterial bypass graft, but such allografts proved to be inferior to saphenous vein autografts. Such umbilical vein grafts are no longer available, following application of FDA device manufacturing requirements to their recovery and processing.

### **Tissue autograft collection, storage, and use**

Tissue autografts, tissue recovered from a patient's own body, are utilized in many surgical reconstruction procedures. Autologous grafts have certain benefits and costs to the recipient when compared to use of tissue allografts. Some benefits ascribed to autografts include faster incorporation/healing and relative safety from transmission of viral disease or immunologic rejection.<sup>47</sup> Conversely, certain costs include morbidity associated with an additional surgical procedure, as well as pain and potential surgical-site infection. Further, the tissue integrity and quantity of autologous tissue may not be adequate for the intended use, and the removal of the patient's tissue may adversely affect function at the site of removal from which it was extracted.

FDA has exempted, with a few exceptions, the collection, storage, and use of autologous tissues. These require that the autograft (1) must be used in the *same surgical procedure* and (2) must be *minimally manipulated*.<sup>48</sup>

The same FDA rule applies to surgical procedures such as removal of the tissue and subsequent use within the same individual within a single operation or a staged follow-up surgery, where removal and implantation may be separated by several days but are technically considered part of the same surgical procedure. The following include examples of types of procedures: skin grafting, coronary artery bypass surgery utilizing autologous vessels, cranioplasty, and parathyroidectomy with implantation.<sup>47</sup>

Minimal manipulation allows exceptions for tissues that are maintained in their original form and include steps such as rinsing, cleansing, sizing, and shaping. A facility that removes the autologous tissue, which is intended to be shipped to a different facility for autologous use, does not qualify for the FDA exemption except under limited circumstances in order to accommodate the medical needs of an individual patient, which commonly occurs with cranial-flaps removed at one facility and the return of the flap to the patient occurs at another facility.<sup>48</sup> Contraindication to autograft use is in the context of patients with systemic infections or if the tissue is in close proximity to an area of infection. Autografts may be stored at the medical facility where they are collected or with same organization off-site.<sup>47</sup>

### **Oversight, tracking, and traceability of human tissues**

#### **Oversight**

With the rapid growth of all areas of tissue banking, there has been an increasing need for accountability and for measures that ensure that safe, quality tissues are recovered, processed, and available for

clinical use. Quality improvement can be affected through voluntary standards, and most tissue banks have incorporated the achievement of high standards into their goals. The American Association of Tissue Banks (AATB) has established comprehensive standards for donor screening, recovery, and processing of musculoskeletal, cardiac, vascular, and skin tissues, birth tissue, and reproductive cells.<sup>5</sup> Additionally, the standards contain institutional requirements; descriptions of required functional components of a tissue bank; requirements for construction and management of records and development of procedures; requirements for donor authorization and informed consent, tissue labeling, storage, and release; expectations for handling adverse outcomes, investigations, and tissue recalls; requirements for establishment of a quality program; specifications for equipment and facilities; and guidelines for tissue dispensing services and tissue distribution intermediaries. AATB's *Standards for Tissue Banking* are consulted not only by tissue bankers, but also by end-user health-care facilities, other standard-setting organizations, and regulators worldwide. Best practice for checking a tissue bank's accreditation status is to perform an accredited bank search on the AATB website ([www.aatb.org](http://www.aatb.org)).

AABB *Standards for Blood Banks and Transfusion Services*<sup>49</sup> address tissue inspection, handling, storage, preparation and dispensing, handling adverse events, and recordkeeping, which must provide traceability to each recipient or other disposition. The Joint Commission has standards for storage and issuance of tissue for hospitals and ambulatory surgery centers. These standards apply to bone, tendon, fascia, and cartilage, as well as cellular tissues of both human and animal (xenograft) origin. The standards address key functions, including the need to develop procedures for tissue acquisition and storage, recordkeeping and tracking, and follow-up of adverse events and suspected allograft-caused infections, which must be reported to the tissue bank from which the tissue was obtained. Similar to federal regulations and AATB *Standards*, the minimal record retention period is specified to be 10 years from the date of transplantation, distribution, other disposition, or expiration, whichever is latest. The College of American Pathologists' Laboratory Accreditation Program's Transfusion Medicine Checklist also includes several questions on storage and issuance of tissues, including accountability; procedures for proper storage, handling, in accordance with the source facility's directions; procedures for investigating recipient infections and adverse events, and handling look-back notifications from a supplier; and recordkeeping, which allow for tracking from donor to recipient and vice versa.

FDA authority to create and "enforce regulations necessary to prevent the introduction, transmission, or spread of communicable diseases between the States or from foreign countries into the States" under section 361(a) of the US Public Health Service Act (42 USC 264) applies to human tissue intended for transplantation. Formal enforcement policy and regulations did not exist until 14 December 1993 (codified in 21 CFR Parts 16 and 1270), when the "Interim Rule: Human Tissue Intended for Transplantation," which required donor screening, infectious disease testing and record-keeping "to prevent transmission of infectious diseases through human tissue used in transplantation," was adopted in response to reports of HIV transmission by human tissue and of potentially unsafe bone imported into the United States.<sup>50</sup>

These regulations were supplanted by a series of federal regulations, published in stages, first announced in the Proposed Approach to the Regulation of Cellular and Tissue-Based Products in March 1997.<sup>51</sup> A final rule, "Human Cells, Tissues, and Cellular and Tissue-Based Products: Establishment Registration and

Listing," published in January 2001, required organizations that are engaged in tissue recovery, donor qualification, tissue processing, and/or tissue-related laboratory testing to register as a tissue establishment with the FDA. The rule (21 CFR Part 1271) became effective for all tissue banks on 29 March 2004.

A final rule, "Eligibility Determination for Donors of Human Cells, Tissues, and Cellular and Tissue-Based Products," published on 25 May 2004, set forth donor eligibility requirements, including health history screening and laboratory testing. Another final rule, "Current Good Tissue Practice for Human Cell, Tissue and Cellular and Tissue-Based Product Establishments; Inspection and Enforcement," published on November 24, 2004, established elements of good tissue practice, analogous to good manufacturing practice for blood banks. Both rules became effective from 25 May 2005.<sup>48</sup>

In addition to requirements for establishment registration, donor eligibility screening and testing, and good tissue practice, the regulations set forth requirements for adverse reaction reporting and also define inspection and recall authority. To improve tissue safety and surveillance, the FDA Current Good Tissue Practice Rule, effective from 25 May 2005, requires that tissue establishments report infectious adverse events after allograft transplantation to the FDA through its MedWatch adverse event reporting system. More than half of the reports filed by tissue banks have been flagged to indicate possible recall of tissue(s). Of these flagged reports, the majority pertained to acceptance of ineligible donors for whom one or more components of the donor qualification process was not performed or was insufficiently documented. The FDA also encourages health-care professionals, patients, and consumers to voluntarily report tissue adverse reactions through the MedWatch system (see <http://www.fda.gov/Safety/MedWatch>).<sup>2</sup>

## Donation tracking

Each tissue bank assigns identification for each individual tissue, but assigned numbers are often unique only for the tissue bank issuing them. Because a typical numbering system is often based on year followed by sequence of recovery, numbers assigned between tissue banks may overlap so that tissues may have the same number from the same donor between tissue banks, which may create confusion and redundancy.<sup>52</sup> Systems using bar codes feature standardized approaches to numbering, may contain more details about the tissue (e.g., tissue processor, product description, and unique tissue identifier), and allow for health-care facilities to read the code. FDA requires use of coding to identify tissues but is agnostic on what coding system to use. Thus, coding used for tissues is fragmented in the United States. Apart from individual tissue bank coding, standardized systems include ISBT-128, GS-1, and HIBCC. However, ISBT-128 is the only major coding system designed specifically for biologics and is recognized by WHO as the sole global standard for the identification of medical products of human origin.<sup>53</sup> GS-1 is more commonly used in hospitals and more optimized for devices and other items with manufactured lots.<sup>54</sup>

The Veterans Affairs department has attempted to improve tissue tracking in their facilities, and now require their vendors to have ISBT-128 coding on tissues through the Strategic Acquisition Center. An OIG report outlines the challenges faced by tissue tracking in VA health-care facilities.<sup>55</sup>

The lack of donor identifier can be challenging in the event of a recall, when rapid confirmation on the location and implant status of tissue from the same donor or same processing period is paramount. This has been a barrier in past public health investigations.<sup>56</sup>

Currently, “tissue trace cards” are issued by processors to confirm that a tissue was implanted, with patient and surgeon information written onto the document and voluntarily sent back to the processor. However, there is no requirement that a standardized set of information be placed into the patient medical record.

An attempt was made to create a system for tracking and reporting of adverse events through a common donor identifier and monitoring of outcomes from the same donor (Transplantation Transmission Sentinel Network, a cooperative agreement between UNOS and CDC), but the project did not advance beyond the pilot phase.<sup>57</sup>

### Hospital-based tissue services

Hospital transfusion services have been greatly affected by transplantation, and have encountered new and increased demands for services. This stems from The Joint Commission requiring organizations to assign oversight responsibility for their tissue transplantation program, use standardized procedures in tissue handling, maintain traceability of all tissues, and have a process for investigating and reporting adverse events.<sup>58</sup>

Hence, the hospital transfusion service has become involved in transplantation in several ways, including (1) providing traditional blood components; (2) providing new or special blood components; (3) taking responsibility for tissue acquisition, storage, distribution, and tracking; and (4) providing specialized services. For organ transplants, the major demand is for traditional blood components, although special preparation may be required.

FDA regulations pertaining to tissue (see below) cover donor selection and testing, tissue recovery, processing, storage, labeling, and distribution to the “consignee.”<sup>48</sup> The consignee can be a distributor, a surgeon in a hospital operating room, a dentist in his or her office, or a designated individual or department in a hospital or other health-care institution. Tissue “banks” that are located in hospitals are not regulated by the FDA if they serve only to store and dispense tissues provided by comprehensive tissue banks or distributors. A hospital tissue service can be centralized in a support area for the operating suite, hospital central supply service, or hospital transfusion service.

Alternatively, tissues can be handled using a decentralized system and be ordered, received, and stored by each functional area of the hospital in which they are used. However, in the absence of centralization, records of storage and recipient identification may be inadequate. In one case involving an HIV-infected donor, the recipients of five of the tissues could not be identified from hospital records. Other examples of inadequate traceability exist.<sup>59</sup> The Joint Commission standard<sup>58</sup> on recordkeeping and traceability of tissues, College of American Pathologists Transfusion Medicine Checklist,<sup>60</sup> and AABB Standards for Blood Banks and Transfusion Services<sup>49</sup> require that the institutions’ records permit tracing of any tissue from the donor or source facility to all recipients or other final tissue disposition. However, the New York State is the only government regulatory agency that requires tracking of tissues to the recipients with records kept separate from patient charts.

The hospital transfusion service has the capacity, experience, and skills to act as a central depot and distribution point for all human tissue and to ensure that storage, issuance, and disposition records are maintained. Functions include allograft selection; vendor qualification and price negotiation; receipt of tissues, including inspection and accessioning; proper storage; inventory control; issuance; and recordkeeping, which must ensure traceability to recipient or other disposition; promotion of tissue recipient informed consent;

and assurance that allograft tissue preparation steps (i.e. instructions for use) are followed and documented.<sup>61</sup> Recordkeeping, especially if barcodes are used in the laboratory, is complicated by the fact that barcodes are not yet standardized among tissue banks, although adoption of ISBT 128 labeling standards has been suggested.<sup>62</sup> An additional challenge is that some tissues are produced in lots or batches, so each individual unit may not carry a unique identifier. Effective development of such tissue storage and distribution services takes time and relies on good relationships and communications with both operating room staff who will handle tissues and the surgeons who use them.<sup>61</sup>

A transfusion service operating as a central tissue repository and dispensing service may also be called upon to manage autologous tissues, such as calvaria (skull bone flaps), bone, skin, and parathyroid gland. Such tissues may require preparation and packaging before storage. Testing is not required, but careful labeling and recordkeeping are essential. Such tissues may ultimately be reimplanted in the original location (e.g., calvaria) or in a heterotopic location (e.g., limbs for parathyroid gland). It is prudent to establish time limits for storage either on an individual basis as specified by the surgeon or on a generalized basis because stored tissues may not be claimed if the tissue was not needed because the patient died or for other reasons. The tissue dispensing service may also be called upon to package tissues in an appropriate, qualified, properly labeled transport container for transport to another institution.<sup>61</sup>

### Quality and safety measures

Unlike the system implemented for organ procurement, tissue banking is not formally organized based on defined geographic areas, and there is direct competition between tissue banks for processing. Both non-profit and for-profit tissue organizations exist, including over 120 tissue establishments accredited by AATB.<sup>3</sup> The vast majority of tissue recovered in the US is performed by organ procurement organizations for distribution by tissue processors (referred to as tissue banks). Processors independently perform donor eligibility assessment, even if the recovery partner has already performed such steps. Eye banks are organized in a separate system, and approximately 69 organizations are accredited by the Eye Bank Association of America.<sup>4</sup> Although in the past a number of blood centers have provided tissue processing and banking, few have continued to offer these combined services.

Procedures to assure high-quality and safe allografts are guided by national professional standards set by organizations such as AATB, EBAA, AABB, and the American Society for Reproductive Medicine. Some states require additional standards.<sup>63</sup>

Tissue donors are carefully evaluated to assess for both safety and quality. This assessment is performed through information obtained (1) during interviews with family members, significant others, and health-care providers; (2) available medical records; (3) findings on physical assessment; (4) results of autopsy, including toxicology, if performed; and (5) results of laboratory tests for infectious diseases.

General donor eligibility criteria include the absence of significant infection or sepsis, and any evidence of risk factors for a list of conditions FDA terms “relevant communicable disease agents or diseases (RCDADs).<sup>64</sup> This requires evaluation for disease conditions such as HIV, hepatitis B, and hepatitis C, which have been known to be transmitted through tissue transplantation, and may be a risk despite laboratory test screening, particularly for acute infection. In an effort to improve the uniformity of the donor history, a standardized questionnaire has been developed by multiple

organizations collaboratively, led by AATB.<sup>65</sup> Physical assessment seeks evidence consistent with risk for infectious diseases or quality of the tissues being recovered.

Beyond FDA guidance and voluntary organizational standards, many tissue processors have criteria above and beyond the minimum to address both safety (e.g., history of malignancy) and quality (e.g., advanced age, history of autoimmune disease, organ failure, and long-term diabetes). Donors of reproductive tissue are additionally screened for inherited diseases. Placental tissues are specifically screened for history of genital infections such as HPV. Both placental and dermis tissues may be rejected if discolored by meconium or bilirubin, respectively.

Tissues are collected aseptically in an operating room, autopsy room, or other suitable locations where aseptic recovery can be performed. Recovery methods are described in AATB guidance.<sup>5</sup> Tissues are cultured by swabbing directly or through a sample of transport solution. There may also be additional cultures done during processing and after processing to provide evidence of sterility.

Infectious disease laboratory testing is performed where screening of serum or plasma samples has specifically been approved for cadaveric tissue donation (Table 58.3). Laboratory methods include the use of antibody, antigen, and nucleic acid testing. The list of required tests is relatively small, and some are done only seasonally (WNV), while screening may depend on travel history (Zika, CJD). Hemodilution may be an issue for laboratory sensitivity if the donor was heavily transfused. In addition, the testing of post-mortem samples may be complicated by hemolysis or presence of inhibiting factors that can cause false-positive results in some assays. Tests on living donors are subject to different issues and must be performed as close to recovery as possible to avoid false-negative results.

### Tissue processing

A variety of methods, including chemical treatments and irradiation, have been used to reduce or eliminate pathogens in tissue intended for transplantation.<sup>2</sup> Tissue sterilization is defined as the killing or elimination of all micro-organisms from allograft tissue, whereas disinfection refers to the removal of microbial contamination. Sterility assurance level (SAL) is the probability that an individual device, dose, or unit is nonsterile (i.e., one or more viable micro-organisms being present) after it has been exposed to a validated sterilization process. SAL is generally applied only to the level of possible contamination with bacteria or parasites, and does not include viruses or prions. Furthermore, standard terminal

sterilization does not substantially affect viability of prions. In contrast to log reduction of viruses determined in assessments of virus reduction methods, SAL is an absolute determined by the ability of the method to eradicate or reduce micro-organisms, the susceptibility of organisms that may be present to the sterilization method applied, and the maximal bioburden that could occur in the initial material. For example, an SAL of  $10^{-6}$  means that there is less than a 1 in 1,000,000 chance of a viable micro-organism remaining after the sterilization procedure. The FDA requires that medical devices be sterilized using a method validated to achieve an SAL of  $10^{-6}$ . A medical device derived from or that includes a biological product component must also meet an SAL of  $10^{-6}$  if it is to be labeled sterile. Human tissue is different from a device in terms of its ability to withstand harsh processing methods. The initial bioburden, which may be high in some tissues, also must be considered. Many tissues are unable to withstand the harsh treatment needed to achieve a more restrictive SAL without an impairment of tissue function. Such tissues may not then be labeled as sterile.

The complex physical structures and density of musculoskeletal tissues pose challenges for adequate penetration of antimicrobial agents to eradicate micro-organisms. Allografts will not tolerate methods usually applied to metal and plastic medical devices because such treatment would impair the mechanical and biologic properties necessary for clinical utility. As an alternative, sterilization of tissues has been accomplished by several methods, including heat, chemicals, ethylene oxide gas, supercritical CO<sub>2</sub>, and gamma or electron beam irradiation. However, not all of these methods have adequate tissue penetration. Some tissues are treated with antibiotics *in vitro* before storage, but this treatment decontaminates only the surface and may be effective against bacteria only.

A variety of methods, including chemical treatments and irradiation, have been used to reduce or eliminate pathogens in tissue intended for transplantation.

First introduced over 40 years ago,  $\gamma$ -irradiation of bone is still used widely, usually employing a cobalt-60 source. The  $\gamma$ -rays penetrate bone effectively and work by generating free radicals, which may have adverse effects on collagen and limit utility in soft tissues unless performed in a controlled dose fashion at ultralow temperature. The minimal bacteriocidal level of  $\gamma$ -irradiation is 10–20 kGy (1 kGy = 100,000 rad).

Concerns about pathogen transmission and the limitations of irradiation, especially for soft tissues, have prompted improvements in sterilization methods and in the validation of these methods. It should be noted that FDA does not have a standard for tissue processing or sterilization for HCT/Ps, but simply requires that tissue processors have a validated reproducible method for ensuring prevention of transmission of relevant communicable diseases as outlined in guidance.

A number of proprietary chemical-based processing methods have been developed with aims of effectively penetrating tissues and reducing, killing, or inactivating micro-organisms and viruses without unacceptable adverse effects on the tissue's biomechanical properties. Additionally, for use in transplantation, the agents must either be able to be effectively removed or be nontoxic. All methods in current use are applied only to tissue from donors who have met stringent criteria for medical history and behavioral risk assessment, as well as negative results on infectious disease marker testing.

### Need for surveillance

Despite a careful donor selection process, the risk of donor-to-recipient transmission of viral, bacterial, fungal, and prion diseases

**Table 58.3** Relevant Communicable Disease Agents or Diseases (RCDAD) for Human Cells, Tissues, and Cellular and Tissue-Based Products (HCT/Ps)

HIV*
Hepatitis B virus*
Hepatitis C virus*
Transmissible spongiform encephalopathies, including Creutzfeldt–Jakob disease
<i>Treponema pallidum</i> (syphilis)*
HTLV-I, II* (for leukocyte-rich cells or tissues only)
<i>Chlamydia trachomatis</i> * (for reproductive cells or tissues only)
<i>Neisseria gonorrhoeae</i> * (for reproductive cells or tissues only)
West Nile virus
Sepsis
Vaccinia

\* Laboratory screening required; cytomegalovirus not an RCDAD, but required to be tested for leukocyte rich cells or tissues.

**Table 58.4** Infectious Diseases Reportedly Transmitted by Deceased Donor-derived Allografts

Allografts	Infectious Disease
Bone	Hepatitis C Hepatitis, unspecified type Human immunodeficiency virus type 1 Bacteria Tuberculosis Fungus
Cardiovascular patch	Hepatitis C
Cornea	Hepatitis B Rabies Creutzfeldt–Jakob disease Cytomegalovirus (unconfirmed) Bacteria Fungus
Dura mater	Creutzfeldt–Jakob disease
Heart valve	Hepatitis B Tuberculosis
Skin (unprocessed)	Fungus Bacteria Cytomegalovirus (unconfirmed) HIV (unconfirmed)
Pericardium	Creutzfeldt–Jakob disease
Pancreatic islet	Bacteria Bacteria

Source: Adapted from Eastlund and Warwick (2012).<sup>15</sup>

cannot be completely eliminated (Table 58.4).<sup>15,66</sup> Because of the sheer number of tissues recovered from one donor, the consequences of transmission from an infected donor are amplified in this setting, similar to processed plasma donors, with many products recovered from a single donor. However, unlike plasma, tissues recovered from the same donor may be processed differently, leading to different risks of transmission. Transmitted infections can occur despite both a robust evaluation of donor history and laboratory testing, particularly for any grafts with minimal processing or containing viable cells.

Serology (antibody testing) was the first laboratory screening tool developed to detect donor infection, but has limited sensitivity in the early phase of incubation due to the “window period” in detection. HIV was the first well-publicized example of laboratory failure due to the window period of antibody. In 1985, 48 organ or tissue recipients received an organ or tissue from a single donor who, although without apparent risk for HIV infection according to medical history, proved to have been recently infected with HIV and in the window period before HIV-1 antibody could be detected by the assays in use at the time (October 1985).<sup>66</sup> All four organ recipients became infected with HIV, but the majority of tissue recipients did not. Whole unprocessed frozen bone did transmit HIV to three recipients, whereas bone from which the marrow had been removed did not; transplanted corneas, lyophilized soft tissue, and  $\gamma$ -irradiated dura mater also did not transmit the virus. In addition, this case additionally served to highlight vulnerabilities associated with inadequate disposition records, given that six recipients could not be identified from hospital records.

The window period of serology remained an ongoing challenge in the setting of acute infection of the donor. In 2002, tissue from a man with no identifiable infectious disease risk by history or physical examination, and a negative test for anti-HCV, was found to have transmitted HCV to recipients.<sup>67</sup> All organ recipients who could subsequently be tested were found to be infected with HCV. Among 32 tissue recipients, five probable cases occurred: one of two saphenous vein recipients, one of three tendon recipients, and

three of three recipients of tendon with bone allografts. No cases occurred in recipients of skin, cornea, or irradiated bone. All eight recipients whose infection was linked to the transplant were determined to be infected with the same HCV genotype. With the implementation of nucleic acid testing, first for HIV and HCV, and then for HBV, the current risk of viral transmission is thought to be exceedingly low.<sup>68,69</sup> A combination of stringent donor selection, testing strategies that now include both antibody and NAT, and processing methods now in use that reduce the risk for tissues, particularly for those forms not requiring viable cells, transmission risk is as low as it has ever been.

Since the implementation of NAT for tissue donor screening, there has been one high profile transmission of HCV in 2011. The HCV positive donor, which was not detected due to a testing error, transmitted the virus to two organ recipients (a third having been infected with HCV previously). A total of 44 tissue grafts were recovered, with 16 grafts, including 15 musculoskeletal tissues and 1 cardiovascular patch, transplanted. The cardiovascular patch transmitted HCV, which was the only tissue not treated with chemicals or by irradiation.<sup>70</sup>

West Nile virus has been transmitted through organ transplantation and blood transfusion,<sup>71</sup> but transmission through tissue has not been recognized. *Trypanosoma cruzi*, the etiologic agent of Chagas’ disease, has been transmitted through solid organs but transmission of parasitic diseases through vascular tissue allografts has not been reported, but is considered to be possible, particularly for minimally processed grafts.<sup>72</sup>

One poorly understood risk in the solid organ transplant setting is when arteries or veins are recovered from a different donor than a recovered organ, and may be used for a transplant, particularly liver, if the donor and/or recipient vessels are damaged or insufficient. Although such vessel grafts associated with organ transplantation are not considered regulated HCT/Ps by the FDA, such grafts caused documented transmission of rabies in 2004, and have been implicated in hepatitis B and C transmission.<sup>73,74</sup>

Transmission of malignancy via tissue transplantation has not been reported, but is thought to be possible, particularly in fresh, minimally processed grafts with viable cells, where infectious disease transmission has been recognized, such as fresh femoral head collected during hip arthroplasty.<sup>15</sup>

Although most disease transmission reported has involved deceased donors, infectious diseases can also be transmitted through transplantation of tissue from living donors. Cases are well documented in the semen banking arena with a variety of agents and diseases having been transmitted to semen recipients. Human immunodeficiency virus type 1 (HIV-1) has been transmitted both by unrelated donors and from husband to wife. This virus has the greatest number of reports of transmission. Hepatitis B virus (HBV), gonorrhea, *Ureaplasma urealyticum*, *Mycoplasma hominis*, *Trichomonas vaginalis*, *Chlamydia trachomatis*, group B *Streptococcus*, and herpes simplex virus type 2 (HSV-2) have also been reported. Transmission of HCV and human T-cell lymphotropic virus type 1 (HTLV-1) is likely, and the transmission of cytomegalovirus, human papilloma virus (HPV), and syphilis is a possibility.<sup>75</sup>

Transmission of bacteria is a known risk of tissue transplantation. Bacteria can be present in the donor, either as a normal occurrence or as the result of a disease process or medical intervention.<sup>15</sup> Resuscitation efforts can increase the dispersion of such organisms. In addition, “agonal bacteremia” is a well-known process whereby endogenous bacteria, such as normal intestinal flora, begin to

disperse throughout the body after cessation of cardiopulmonary functions, as the putrefaction process begins. This process is accelerated in persons with sepsis, rhabdomyolysis, or drug overdose before death.<sup>15</sup> Bacteria can be introduced during tissue recovery; during processing, whether through cross contamination, insufficient aseptic technique, or contaminated chemicals or solutions; or even during packaging. Some tissues, including ocular tissue, epidermis, and semen, are inherently not sterile even if collected aseptically.

At particular risk for transmission of bacteria are tendon allografts used in orthopedics because these cannot be subjected to extensive processing if their mechanical properties are to be preserved. In 2001, a 23-year-old man was found to have died from *Clostridium sordellii* sepsis following receipt of a femoral condyle allograft. An extensive investigation identified 14 patients who had received allografts between 1998 and 2002 and who developed postoperative infections with *Clostridium* species.<sup>15,76</sup> The tissues were derived from nine donors, but all were prepared by the same processor, and investigation identified several factors that could have contributed to the infections. Although the tissues were cultured, they had already been suspended in antimicrobial solutions, likely leading to false-negative results. Additionally, for two of the donors, including the donor of the tissue that was implicated in the fatal case, the interval between death and refrigeration of the body (19 hours for the donor in the fatal case) exceeded the industry's voluntary standards at the time of 15 hours maximum without refrigeration of the deceased donor. This likely permitted excessive bacterial proliferation prior to tissue recovery. In addition, tissue was processed aseptically without the use of terminal sterilization, and the processing methods used had not been validated. Human error can also lead to release of contaminated tissues. In one case, a technician failed to follow standard procedures, resulting in release of tissue labeled as having been subjected to irradiation when, in fact, no irradiation had occurred.<sup>77</sup> Although a wound infection in a tissue recipient, or even disseminated bacteremia, does not necessarily implicate the donor tissue, any unusual organism or cluster of infections associated with grafts recovered from the same donor should be investigated promptly. Site infections are a well-known risk of many surgical procedures and can result from the use of contaminated solutions or equipment insufficiently sterilized between procedures.<sup>78</sup>

Placental tissues are increasingly used in a variety of settings as a wound covering and undergo a wide range of processing, from minimal to sterilized through chemical or radiologic means. In 2017, *Mycoplasma hominis* was documented to be transmitted through a minimally processed amniotic product. After a report of a cluster of surgical site infections associated with the use of a common product, an investigation revealed *M. hominis* infections confirmed in 2 recipients of implants out of 14 patients, and 2 unopened vials of the amniotic product that were cultured also grew the organism. Sequences were identical in isolates from these two unopened vials and patient cultures. Also commented on was the lack of a standardized system to track tissue products in health-care facilities, limiting the ability of public health agencies to respond to outbreaks and investigate other adverse events associated with these products.<sup>79</sup>

### **Adverse event monitoring—role of the hospital-based tissue bank**

Inherent risk of human-derived medical products, such as tissue allografts, must be balanced with the clinical benefits when transplantation of tissue is undertaken. Rarely, human tissue allografts

have transmitted disease by bacteria, viruses, and fungi, and prion disease (dura mater only). Other risks are noninfectious in nature and may pertain to the integrity of the graft itself or processing done after recovery resulting in structural weakness that can lead to an unsuccessful outcome.

Hospital-based tissue services are required, by the TJC to have procedures to investigate, in an acceptable timeframe, any adverse outcome suspected to be caused by a tissue allograft. Furthermore, once an investigation begins at the hospital, it is incumbent upon the hospital, under TJC standards, to immediately report allograft-transmitted infections and other severe adverse events that may have occurred to the tissue supplier.

Surgeons and end users play a critical role in identifying allograft-associated adverse outcomes and must notify the hospital-based tissue service immediately when they suspect such events. Prompt adverse event reporting aids the hospital-based tissue service to investigate the root cause, report the issue to the tissue supplier, and implement corrective action, including quarantine of any other suspect allografts. In addition, tissue-associated adverse events may also be voluntarily reported via MedWatch to the FDA, but the reporting person should be aware that FDA regulation is focused on if an infection is suspected to have been caused by the tissue allograft not on a graft integrity adverse event. Cooperation is key between the hospital-based tissue service, clinicians, and the tissue supplier when investigating adverse events. Reporting the serial number of the graft with the report is critical for further investigation by the tissue bank. The hospital infection-control department or an infectious disease specialist should be linked via protocol and/or procedure to this process. The earlier the notification, the better this can help prevent complications in other potential recipients of allografts implicated by this same donor or other tissue donors.<sup>47</sup>

Another requirement is via state health departments that have lists of communicable diseases that must be reported when they are newly diagnosed. Examples consist of new diagnoses of HIV or viral hepatitis in a tissue allograft recipient where the allograft is suspected as a possible source. Additionally, it is suggested that an epidemiologic investigation may be necessary to trace the tissue allograft to determine the source of the recipient's infection.<sup>47</sup> One resource that contains a database of notable infectious disease transmission and process errors has been developed by the World Health Organization and the Italian National Transplant Centre as its collaborating center.<sup>80</sup> The website features a searchable website that allows for review of the spectrum of adverse events that can occur from transfusion and transplantation, including infectious diseases transmitted through tissue transplantation, and details on risk factors and outcomes.

### **Recalls and look-back investigations**

Suppliers notify hospital-based tissue banks about product recalls and market withdrawals (i.e., field action) when tissues are deemed, by their criteria, compromised. This may encompass, tissue suppliers sequestering tissue in inventory not yet distributed, recall of all tissues belonging to a specific donor or lot, or direct notification to a hospital of a specific affected allograft received. The recall nature usually dictates next steps, but customarily the hospital would first quarantine allografts in inventory (if not yet implanted), identify recipients (if already implanted), and/or notify the transplanting surgeon(s) of the hospital-based tissue service notification. The responsibility of the surgeon would be to evaluate the circumstances and notify, if appropriate, each recipient receiving a recalled or withdrawn tissue graft.

A look-back investigation is when a tissue donor is discovered after donation of tissue to have a communicable disease that is known to be transmitted by tissue graft transplantation. The type of infections include, but are not limited to, HIV, human T-cell lymphotropic virus type I or II, HBV, and HCV. Tissue graft infectious transmission is an uncommon occurrence, but requires investigation, or other communicable disease known to be transmitted by tissue grafts.<sup>47</sup>

### **Reimbursement**

Reimbursement for tissue transplantation is similar to that for blood transfusion. The tissue bank recovers expenses through a service fee (per tissue) billed to the hospital. This service fee includes such costs as services rendered by the organ/tissue recovery agency; recovery supplies and logistical support of the recovery agent that may be provided by the tissue processor; the tissue processor's operating costs associated with processing, storage, and distribution, as well as research and development; and overhead costs incurred with support of all operations. Health-care insurance carriers reimburse hospitals for most tissue service fees. Current procedural terminology codes specific to allograft transplantation procedures are available and used routinely.<sup>2</sup>

### **Conclusion**

Tissue donation is a gift, and those who donate must be honored for this act of altruism through responsible stewardship. Part of honoring and respecting donors is to ensure there is safety and quality applied to the process of tissue recovery, processing, packaging, and implantation of the various types of tissues to maximally benefit multiple recipients. From donor to recipient, the stewardship of this process lies in a path from the tissue suppliers to health-care providers, connected by the hospital-based tissue bank. Medical directors in both arenas must continue to ensure that processes, as described in this chapter, are in place to have reliable traceability and trackability from tissue recovery to implantation, allowing rapid feedback to the tissue supplier in case there is a problem with

that tissue. Infectious and noninfectious hazards are critically important to report and trace as they may impact multiple recipients, and the more robust the system of traceability, the more rapidly affected tissue can be located and assessed. Human tissue has the potential to save and heal millions of lives each year, when recovered, processed, tracked, and used in a responsible way.

### **Acknowledgments**

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# Index

5% serum albumin in saline 261–262  
A antigen 81  
A-transferase (GTA) 81, 83–84  
AABB *see* Association for the Advancement of Blood and Biotherapies  
AAV *see* ANCA-associated vasculitis  
ABC transporters 116  
ABC/EBA *see* America's Blood Centers/  
European Blood Alliance  
ABCC1 blood group system 117  
ABCC4 116–117  
ABH antibodies 82, 87  
ABH system *see* ABO system  
abnormal clotting tests absent of bleeding,  
prophylactic plasma 213–214  
ABO antibody titers 38–39  
ABO gene 83–84  
ABO system 81–87, 330  
  antibodies 82  
  biological roles 86–87  
  biosynthesis 81–82  
  chemistry 81–82  
  coagulation 86  
  donor testing 37–39, 38  
  epitopes 81–82  
  expression 81–82, 84, 85  
  hemolytic disease of the fetus and  
    newborn 85, 364–365  
  innate immunity 87  
  molecular biology 83–85  
  platelets 170–171, 484  
  secretor-positive individuals 81–82  
  serology 82  
  solid organ transplantation 85  
  stem cell transplantation 85–86  
  tissue-specific regulation 84  
  transfusion considerations 85  
  weak subtypes 82–83  
ABO typing  
  donors 37–39, 38  
  hematopoietic stem cells 620  
  human tissue allografts 672–673  
  plasma composition 202  
  recipients 124

absent iron stores (AIS) 57  
absenteeism, health emergencies 16  
absolute neutrophil count (ANC) 190–191  
accreditation, hospital transfusion services 73  
ACE *see* angiotensin-converting enzyme  
acellular dermal matrix 675  
acellular therapy, renal failure 655  
acid–citrate–dextrose (ACD)  
  solutions 8–9, 151, 152  
acidified serum test (HAM test) 360  
ACKR1 *see* atypical chemokine receptor 1  
acquired bleeding disorders 443–452  
acquired immune deficiency syndrome (AIDS)  
  initial cases 10  
  nucleic acid tests 31  
  *see also* human immunodeficiency virus  
acquired von Willebrand disease (AVWS)  
  451–452  
ACS *see* acute chest syndrome  
ACT *see* activated clotting time  
actin, megakaryocytes 160, 161  
actin depolymerizing factor (ADF) 161  
activated clotting time (ACT),  
  intraoperative use 459  
activated partial thromboplastin time (aPTT) 205–207, 457–458  
activated prothrombin complex concentrate (aPCC) 444–445  
  adverse reactions 445  
  dosing 445  
  indications 444  
  purification 227–228  
  storage 444  
activin receptor ligand traps 606  
acute adverse reactions  
  apheresis 264–266  
  blood collection 49–55  
    air embolism 54, 258  
    allergic 258  
    apheresis 53–55, 256–258  
    automated component collections 53  
    citrate reactions 53–54  
    citrate toxicity 257  
    delayed 50  
  donation frequency 54  
  granulocyte collection 54–55  
  hypotension 257  
  immediate 50  
  multiple component collection 54  
  phlebotomy-related 52–53  
  phlebotomy-related nerve injury 53  
  presyncopal 50, 257  
  risk factors 51, 53  
  syncopal 51–52  
  therapeutic plasma exchange 264–266  
acute autoimmune diseases,  
  immunoglobulin therapy 245–246  
acute chest syndrome (ACS), red cell exchange 279, 333  
acute disseminated encephalomyelitis (ADEM), apheresis 268  
acute hemolytic transfusion reactions (AHTR) 543–552  
  *see also* hemolytic transfusion reactions  
acute inflammatory diseases,  
  immunoglobulin therapy 245–246  
acute lung injury *see* transfusion-related acute lung injury  
acute multiorgan failure syndrome, red cell exchange 279–280  
acute myeloid leukemia, myeloid growth factors 610  
acute myocardial infarction, red blood cell administration 321–322  
acute nonvolemic hemodilution (ANH) 301–302, 462, 484  
acute phase reaction, plasma composition 202  
acute radiation syndrome, myeloid growth factors 611  
acute splenic sequestration crisis (ASSC) 334  
acute stroke  
  red cell exchange 279  
  sickle cell disease 332  
N-acylsphingosine 93  
adaptive mechanisms, anemia 316  
additive solutions  
  platelets 184–185  
  red blood cells 152–154

ADEM *see* acute disseminated encephalomyelitis  
adenosine 5'-triphosphate (ATP)  
platelets 181  
red blood cells 143, 145, 147–148,  
151, 156  
adenosine diphosphate (ADP) 145  
adenosine monophosphate (AMP)  
147–148  
ADF *see* actin depolymerizing factor administration  
acute nonvolemic hemodilution 301–302, 462, 484  
bedside pumps 313  
blood avoidance techniques 461–462  
blood group considerations  
ABO system 85  
Diego system 114  
Duffy protein 113  
I blood group 92  
Kell blood group 112  
Kidd system 113–114  
Lewis blood group 89  
MNS blood group 111  
P blood group 96  
Rh system 106–107  
Sd<sup>a</sup> antigen 97  
blood warming 313  
burn patients 478–481  
coagulation factor replacement 424–452  
component issue and release 307  
component modifications 309–310  
component preparation 309–311  
conservative practice 461  
cryoprecipitate 210–211, 214–215  
burn patients 480  
children 390–391  
hemophilia 426–427  
trauma patients 480  
donor screening 31–32  
factor VIII 427  
fetal 379–380, 420–421  
gene therapy 642–643  
granulocytes 195–198, 487  
hematopoietic stem cells 622  
hemovigilance 503–506  
hospital tissue services 679  
immunoglobulin G 240–248  
infusion flow rates 312  
intravenous immunoglobulin 241–248  
adverse reactions 242–243  
autoimmune mucocutaneous bullous diseases 247  
chronic inflammatory demyelinating polyradiculoneuropathy 246  
drug interactions 241  
Guillain–Barré syndrome 245–246  
hemolytic disease of the fetus and newborn 370  
heparin-induced thrombocytopenia 414–415

high-dose therapy 241  
hyperimmune globulins 247–248  
immune thrombocytopenic purpura 246, 407–408  
Kawasaki disease 245  
multifocal motor neuropathy 246–247  
neonatal alloimmune thrombocytopenia 420  
oncology patients 486  
posttransfusion purpura 421  
primary immune deficiencies 244–245  
secondary immune deficiencies 245  
transplantation 247  
vaccine-induced immune thrombotic thrombocytopenia 417  
warm autoimmune hemolytic anemia 350  
obstetrics 375–380  
perioperative practice 453–470  
plasma 209–215  
adverse reactions 215  
angiography 468–469  
bleeding treatment 212–213  
bronchoscopy 468  
burn patients 480  
central venous catheter insertions 466–467  
children 388–390  
clinical use 212  
combined factor V and factor VIII deficiency 438  
cryoprecipitate 214–215  
disseminated intravascular coagulation 213  
epidural anesthesia 468  
factor V deficiency 438  
hemolytic disease of the fetus and newborn 370–371  
hemophilia 426–427  
infants 388–390  
liver biopsy 467  
lumbar puncture 468  
neurosurgical procedures 468  
oncology patients 486  
paracentesis 468  
perioperative guidelines 464–466  
prophylactic 213–214  
prothrombin deficiency 437  
thoracentesis 468  
transbronchial lung biopsy 468  
trauma patients 480  
upper airway procedures 468  
platelets 395–400  
bronchoscopy 469  
cardiac surgery 470  
central venous catheter placement 469  
children 385–388  
dosing 397  
epidural anesthesia 470  
fetal 379  
infants 385–388  
liver biopsy 469  
lumbar puncture 470  
maternal 378  
neurosurgical procedures 470  
oncology patients 484–486  
paracentesis 469  
perioperative guidelines 464–466  
preoperative 397–398  
prophylactic 396–397  
refractoriness 398–399, 422  
therapeutic 398–400  
thoracentesis 469  
transbronchial lung biopsy 469  
upper airway procedures 469  
pretransfusion considerations 306–307  
prophylactic  
granulocytes 198  
hemophilia 427  
plasma 213–214  
platelets 396–397  
rapid infusion practices 313–314  
recipient management 129  
red blood cells  
acute myocardial infarction 321–322  
cardiac surgery 319–321  
children 322, 381–385  
chronic anemia 324  
clinical trials 318–332  
cold agglutinin disease 354  
complications 342–345  
critical care 319  
decision-making 323  
dosing 324  
fetal 379  
guidelines 322–324  
hemolytic disease of the fetus and newborn 370–371  
infants 381–385  
maternal 378  
microcirculatory effects 316–317  
monitoring 341–342  
oncology patients 322, 482–484  
orthopedic surgery 321  
paroxysmal cold hemoglobinuria 355  
paroxysmal nocturnal hemoglobinuria 362–363  
perioperative guidelines 464–466  
preterm infants 382–383  
protocols 339–341  
sickle cell disease 331–335, 332  
surgical outcomes 455–457  
targets 341–342  
transfusion thresholds 324  
upper gastrointestinal bleeding 321  
warm autoimmune hemolytic anemia 350–351  
Rh immune globulin 408  
sets and filters 311

- administration (*cont'd*)  
 transfusions 311–313  
 trauma patients 471–478, 479–481  
 venipuncture 307  
 whole blood 480
- ADP *see* adenosine diphosphate
- adult neutropenias 192–193
- adverse sequelae  
 activated prothrombin complex  
 concentrate 445  
 acute lung injury 569–579  
 allergic responses 558–561, 580  
 anaphylaxis 559–561  
 andexanet alfa 450  
 antifibrinolytics 448  
 apheresis 264–266  
 bacterial contamination 46, 487, 533–542  
 blood collection 49–55  
 air embolism 54, 258  
 apheresis 53–55, 256–258  
 automated component collections 53  
 citrate reactions 53–54  
 delayed 50  
 donation frequency 54  
 granulocyte collection 54–55  
 hypotension 257  
 immediate 50  
 multiple component collection 54  
 phlebotomy-related 52–53  
 phlebotomy-related nerve injury 53  
 presyncopal 50, 257  
 risk factors 51, 53  
 syncopal 51–52
- children 388
- chimeric antigen receptor T-cell therapy 488
- circulatory overload 563–564, 579–580
- citrate toxicity 257, 561–562
- cryoprecipitate transfusion 215
- desmopressin 448–449
- dimethyl sulfoxide toxicity 565–566
- dyspnea 580
- electrolyte disorders 562–563
- erythroid growth factors 604–605
- febrile nonhemolytic transfusion reactions 553–558
- gene therapy 644
- hematopoietic progenitor cells 487–488
- hemolytic transfusion reactions 242–243, 543–552
- hemovigilance 503–506
- hypothermia 563
- idarucizumab 450
- immunoglobulin transfusion 242–243
- iron overload 281, 344–345, 587–597
- massive transfusion protocols 561–564
- microaggregate debris 563
- myeloid growth factors 608–609
- nonhemolytic transfusion reactions 553–568
- oncology patients 487–488
- parasite infections 523–529
- plasma transfusion 215
- plastizer toxicity 565
- prion transmission 529–531
- protamine 449
- prothrombin complex concentrates 444
- rapid infusion practices 561–564
- recombinant activated factor VII 445–446
- red blood cell transfusion 342–345
- reporting 503–506
- respiratory 569–580
- therapeutic plasma exchange 264–266
- thrombopoietic growth factors 613–614
- transfusion-associated graft-versus-host disease 582–586
- transfusions, reporting 70–73, 129–130
- viral infections 507–522
- vitamin K 447
- see also* acute adverse reactions; chronic effects
- AF *see* atrial fibrillation
- affinity maturation 119–120
- afibrinogenemia 441
- A4GALT1 94, 95
- age, plasma composition 202
- agglutination  
 blood groups discovery 7  
 mechanism 120–122
- AHF *see* antihemophilic factor
- AHG *see* antihuman globulin
- aHUS *see* atypical hemolytic-uremic syndrome
- AIDS *see* acquired immune deficiency syndrome
- AIHA *see* autoantibody hemolytic anemia
- AIM-1 kinase (Aurora-B kinase) 159
- air embolism, apheresis 54, 258
- AIS *see* absent iron stores
- alanine transferase (ALT) 506
- albumin 200  
 pathogen reduction 496
- alemtuzumab, warm autoimmune hemolytic anemia 350
- aliquoting 310
- allergic responses, apheresis 54, 258, 264
- allergic transfusion reactions (ATR)  
 diagnosis 558–559  
 prevention 559  
 transfusions 558–561  
 treatment 559
- allergic transfusion reactions (ATRs) 580
- allografts 670–683  
 ABO typing 672–673  
 acellular dermal matrix 675  
 adverse event monitoring 682  
 bone 673–674  
 cardiac 676–677  
*rejection, extracorporeal photopheresis* 287–288  
 connective tissues 674–675
- donation tracking 678–679
- donor–recipient matching 672–673
- extraembryonic tissue 677
- hospital-based tissue services 679
- human tissue 670–683
- lung, rejection, extracorporeal photopheresis 288
- ocular tissue 675–676
- oversight 677–678
- peripheral nerves 677
- recalls and lookback 682–683
- reimbursement 683
- rejection, extracorporeal photopheresis 287–288
- skin 675
- surveillance 680–682
- tissue processing 680
- vascular tissue 676–677
- alloimmune thrombocytopenia 417–422  
 neonatal 395, 419–421  
 passive 421–422  
 post-transfusion purpura 421  
 transplantation-associated 422
- alloimmunization  
 febrile nonhemolytic reactions 555  
 granulocytes 487  
 pathogen reduction 498–499  
 platelets 171–177, 399–400, 485, 498–499  
 red blood cells 342–343, 483
- alpha thalassemia 328, 337
- alpha-1 antitrypsin 201
- alpha<sub>1</sub>-proteinase inhibitor (API), purification 228
- alphaviruses 518
- ALT *see* alanine transferase
- altered CE phenotype 103–104
- Alyx instrument 253
- amotosalen/UV-A pathogen reduction 490, 492, 495, 497, 498, 499
- American Association of Blood Banks *see* Association for the Advancement of Blood and Biotherapies
- America's Blood Centers/European Blood Alliance (ABC/EBA) 77–78
- ε-aminocaproic acid (EACA) 447–448  
 surgical patients 462
- ammonia transport 105
- AMP *see* adenosine monophosphate
- AMR *see* antibody-mediated rejection
- amustidine/glutathione pathogen reduction 492–493, 496, 499–500
- anamnestic response 119
- anaphylaxis  
 apheresis 258, 265  
 diagnosis 560  
 etiology 559–560  
 prevention 561  
 transfusions 559–561  
 treatment 560–561

- ANC *see* absolute neutrophil count  
 ANCA-associated vasculitis (AAV) 272  
 andexanet alfa 449–450  
 anemia 314–326
  - adaptive mechanisms 316
  - autoimmune hemolytic 346–358
  - biochemistry 138–141
  - children 383
  - chronic 324, 375
  - chronic inflammation 141
  - clinical outcomes 317–319
  - cobalamin deficiency 138–140
  - definition 297
  - folate deficiency 138–140
  - hemolytic disease of the fetus and newborn 371
  - infants 381–382
  - macrocytic 138–140
  - microcytic 56–64, 140–141
  - neurocognitive effects 60
  - pathophysiological interactions 137
  - perioperative practice 453–455
  - pica 60–61
  - postpartum 377–378
  - pregnancy 373–375
  - preoperative management 297–299, 454–455
  - restless legs syndrome 60
  - risk factors 317–319
  - surgical outcomes 454
  - thalassemia 141
  - therapeutic plasma exchange 271
  - warm autoimmune hemolytic 106–107, 346–351
 anesthesia
  - epidural
    - plasma administration 468
    - platelet administration 470
  - angiography, plasma
    - administration 468–469
  - angiotensin-converting enzyme (ACE) inhibitors 265
  - ANH *see* acute nonvolemic hemodilution
  - animal transfusions 4
  - animal-to-human transfusions 4–5, 7
  - anti-HBc antibody testing 44
  - antibodies
    - affinity maturation 119–120
    - blood groups 120–122, **121**
      - antigen interactions 120–122
      - physical properties 120
    - heavy chains 119
    - light chains 119
    - molecular structure 118–119, 120
    - Rh system 106, 107
    - thrombopoietic growth factors 614
    - transfusion-related acute lung injury 571, 574–576, 578
  - antibody testing
    - Chagas' disease
    - CMV 46


 HBV 44  
 HCV 44  
 HIV 43  
 HLA 47
  - human leukocyte antigens 630–632
  - human T-cell lymphotropic virus 44
  - identification principles 126–127
  - IgA deficiency screening 47
  - platelets 47
  - red blood cell antigens 38–39
  - syphilis 4–6
  - transfusion-related acute lung injury 578
 antibody titers
  - ABO system 38–39
  - IgA deficiency screening 47
 antibody-mediated rejection (AMR) 274  
 antibody-specific prediction
  - (ASP) 173–174
 anticoagulant-nutrient solutions, red blood cells 152  
 anticoagulants
  - acid–citrate–dextrose solutions, development 8–9
  - apheresis 251–252
  - Braxton-Hicks 6–7
  - paroxysmal nocturnal hemoglobinuria 360
  - perioperative practice 459–461
  - twentieth century development 8–9
 antifibrinolytics 303, 447–448
  - adverse reactions 448
  - dosing 448
  - hemophilia 432
  - indications 447–448
  - manufacturing 447
  - obstetrics 377
  - storage 447
 antigen receptors 118  
 antigen testing
  - ABO typing 37–39, **38**
  - HLA 47
  - RBC extended 47
  - Rh typing 37–38
 antigen-presenting cells (APCs) 119  
 antigens
  - ABCC1 system 117
  - ABO system 81–84
  - AUG system 117
  - Cartwright system 115
  - Chido/Rogers system 115
  - Colton system 114–115
  - CTL2 system 117
  - Diego system 114
  - Dombrock system 115–116
  - Gerbich system 115
  - GIL system 114–115
  - human leukocyte antigens 168–169, 625, 628
  - human platelet antigens 169–170
  - I blood group 90–92
  - Indian system 115
  - JR system 116
  - Kell blood group 112–113
  - Kidd system 113–114
  - Knops syste 115
  - LAN system 116
  - Lewis blood group 87
  - Lutheran blood group 111
  - LW system 107
  - MAM system 117
  - MNS blood group 109–111
  - OK system 116
  - P blood group system 93–94
  - PEL system 116–117
  - platelets 168–171
  - RAPH system 116
  - Scianna system 114
  - Vel system 117
  - antiglobulin tests 122, 124–125, 129
  - antihemophilic factor (AHF) 486
  - antihuman globulin (AHG) reagent 122
  - antineutrophil cytoplasmic antibodies *see* ANCA
  - antiphospholipid antibody syndrome (APS), pregnancy 375
  - antiplatelet therapies, perioperative 459–461
  - antiretroviral therapy (ART) 516
  - antithrombin III, purification 230
  - aPCC *see* activated prothrombin complex concentrate
  - APCs *see* antigen-presenting cells
  - apheresis 249–289
    - 5% serum albumin in saline 261–262
    - acute adverse reactions 53–55, 256–258, 264–266
    - acute disseminated encephalomyelitis 268
    - air embolism 54, 258
    - allergic responses 54, 258
    - allograft rejection 287–288
    - ANCA-associated vasculitis 272
    - angiotensin-converting enzyme inhibitors 265
    - antibody-mediated rejection 274
    - anticoagulants 251–252
    - autoantibody hemolytic anemia 271
    - babesiosis 282
    - bacterial contamination 536
    - biochemical changes 263–264
    - blood donation 252–258
    - catastrophic antiphospholipid syndrome 271
    - children 266
    - chronic effects 63–64
    - chronic inflammatory demyelinating polyradiculoneuropathy 267
    - citrate toxicity 257, 266
    - complement-mediated thrombotic microangiopathy 270
    - complications 256–258, 264–266

- apheresis (*cont'd*)  
 component separation 251  
 continuous renal replacement therapy 263  
 cryoglobulinemia 271–272  
 cryoprecipitate 262–263  
 cutaneous T-cell lymphoma 287  
 desensitization in transplant recipients 274  
 dilated cardiomyopathy 275–276  
 donor collection 33–34  
 erythrocytapheresis 282–285  
 essential thrombocythemia 285–286  
 exchange volumes 260–261  
 extracorporeal membrane oxygenation 263  
 extracorporeal photopheresis 287–289  
 familial hypercholesterolemia 276  
 ferritin monitoring 63  
 focal segmental glomerulosclerosis 272–273  
 fraction of the preprocedure cells remaining 278–279  
 fresh frozen plasma 262–263  
 Goodpasture syndrome 272  
 graft-versus-host disease 288–289  
 granulocyte collection 54–55, 195  
 Guillain–Barré syndrome 261, 266–267  
 Hashimoto's encephalography 269  
 hematologic disorders 269–272  
 hydroxyethyl starch 263  
 hypergammaglobulinemia 270–271  
 hyperleukocytosis 286  
 hypotension 257  
 immunoabsorption 275–276  
 immunoglobulin G 260–261  
 instrumentation 253–254, 259–260  
 iron overload prevention 281  
 Lambert–Eaton myasthenic syndrome 268  
 LDL removal 276  
 loiasis 289  
 malaria 281–282  
 N-methyl-D-aspartate receptor antibody encephalitis 267  
 multicomponent collections 253, 256  
 multiple sclerosis 268  
 mushroom poisoning 273  
 myasthenia gravis 267  
 myeloma cast nephropathy 273  
 neurological disorders 266–269  
 neuromyelitis optica spectrum disorder 268–269  
 nonhematopoietic cells 289  
 oral calcium supplementation 54  
 overdose 273  
 PANDAS 269  
 paraproteinemic demyelinating polyneuropathies 268  
 phytanic acid storage disease 269  
 plasma collection 254–255  
 platelet collection 255–256  
 pregnancy 281  
 principles 251–258  
 procedure requirements 254–256  
 product requirements 254–256  
 red cell exchange 278–282  
 rejection of transplants 274  
 replacement solutions 261–263, 265  
 secondary erythrocytosis 283–284  
 sickle cell disease 279–281  
 systemic lupus erythematosus 273  
 techniques 259–263  
 therapeutic phlebotomy 278, 282–285  
 therapeutic plasma exchange 251–252, 259–277  
 thrombocytosis 285–286  
 thrombotic microangiopathies 269–271  
 thrombotic thrombocytopenic purpura 261, 263, 270  
 thyroid storm 273  
 ticlopidine-associated thrombotic microangiopathy 270  
 transplantation 273–274  
 vascular access 252, 260, 265  
 vasculitis 272  
 vasculitis with hepatitis B-associated polyarteritis nodosa 272  
 voltage-gated potassium channel antibody diseases 269  
 Wilson Disease 273  
 API *see* alpha<sub>1</sub>-proteinase inhibitor  
 aplastic anemia 614–615  
 aplastic crisis, sickle cell disease 333–334  
 aPTT *see* activated partial thromboplastin time  
 Aranesp *see* darbepoetin alfa  
 argatroban 415–416  
 arginine metabolism, red blood cells 149  
 ART *see* antiretroviral therapy  
 arterial puncture, blood donation 52–53  
 arteriovenous anastomosis, development of method 7–8  
 ASP *see* antibody-specific prediction  
 ASPEN *see* association of sickle cell priapism, exchange transfusion and neurological events  
 aspirin, surgical protocols 460  
 ASSC *see* acute splenic sequestration crisis  
 Association for the Advancement of Blood and Biotherapies (AABB) 12  
 donor screening regulations 39–40, 41–42  
 iron depletion regulations 58–59  
 physical examination requirements 32  
 transfusion services regulations 70–71  
 association of sickle cell priapism, exchange transfusion and neurological events (ASPEN) 280  
 atherosclerosis, Lewis blood group 89–90  
 ATP *see* adenosine 5'-triphosphate  
 atrial fibrillation (AF) 460  
 atypical chemokine receptor 1 (ACKR1) 112–113  
 atypical drug-induced immune thrombocytopenia 412–413  
 atypical hemolytic-uremic syndrome (aHUS) 374–375  
 Augustine (AUG) blood group system 117  
 Aurora platform 253  
 Aurora-B kinase (AIM-1 kinase) 159  
 autografts 677  
 autoimmune diseases, immunoglobulin therapy 241, 245–247  
 autoimmune mucocutaneous bullous diseases 247  
 autoimmune neutropenia 192–193  
 autoimmune thyroiditis 269  
 autologous donations 29  
 autologous transfusions, nonhemolytic reactions 566  
 automated pretransfusion testing 125–127, 128–129  
 automated testing systems, infectious diseases 40  
 automimmune hemolytic anemias (AIHAs) 346–358  
 apheresis 271  
 cold 351–355  
 cold agglutinin disease 351–354  
 drug-induced 356–358  
 mixed 355–356  
 paroxysmal cold hemoglobinuria 354–355  
 pathophysiology 346–348  
 prevalence 346  
 warm 346–351  
 Autopheresis-C platform 253  
 avatrombopag (Doptelet) 613  
 B antigen 81  
 B-CAM/LU *see* Basal Cell Adhesion Molecule–Lutheran antigen  
 B-transferase (GTB) 81, 83–84  
 babesiosis 45, 282, 523–524  
 bacterial contamination 533–542  
 apheresis 536  
 cryoprecipitate 541  
 culture 536  
 detection 536–538  
 febrile nonhemolytic reactions 555–556  
 first aliquot diversion 535  
 hematopoietic progenitor cells 487  
 international comparisons 541–542  
 large volume delayed sampling 537  
 nucleic acid testing 538  
 pathogen reduction 538–539  
 plasma 541  
 platelets 46, 533–539  
 point of care immunoassays 537–538  
 post-transfusion sepsis prevention 535–540  
 red blood cells 540–541

- secondary culture 536–537  
skin disinfection 535  
bacterial culture 536–537  
barbell protoplatelets 161  
Basal Cell Adhesion Molecule–Lutheran (B-CAM/LU) antigen 111  
BasoEBs *see* basophilic erythroblasts  
basophilic erythroblasts (BasoEBs) 134  
BC method *see* buffy-coat method  
BCEs *see* blood collection establishments  
BCL11A targeted gene editing trials 645–646  
BECS *see* blood establishment computer software  
bedside blood pumps 313  
behavioral theories, blood donors 28  
beta thalassemia 328, 335–336  
BFU-Es *see* burst-forming units-erythroid  
BFU-MK *see* burst-forming units-megakaryocyte  
*B3GALNT1* 94, 95  
*B4GALNT2* 97–98  
*B4GALNT4* 98  
biochemistry  
  clotting 203  
  erythropoiesis 133–141  
  human leukocyte antigens 625–626  
  I blood group 91  
  Lewis blood group 87–88  
  macrocytic anemia 138–140  
  microcytic anemia 140–141  
  P blood group system 93–94  
  pretransfusion testing 118–122  
  red blood cell metabolism 143–150, 144  
  Sd<sup>a</sup> antigen 97  
  thalassemia 141  
  therapeutic plasma exchange effects 263–264  
biological agent attacks 16  
biological roles  
  ABO system 86–87  
  human leukocyte antigens 625–626  
  I blood group 92–93  
  Lewis blood group 89–90  
  LW system 107  
  P blood group system 96–97  
  Rh system 105  
  Sd<sup>a</sup> antigen 98  
biologically active lipids, transfusion-related acute lung injury 576  
biologics 39  
biophysical niche forces, megakaryopoiesis 164–165  
bioprinting  
  lung tissue engineering 649–650  
  renal tissue 655–656  
  reproductive system tissue 651  
bioreactors, lung tissue engineering 649  
biosynthesis  
  ABO system 81–82  
  hemoglobin 135  
Lewis blood group 87–88  
P blood group system 93–94  
  red blood cells 135, 148–149  
  Sd<sup>a</sup> antigen 97  
  **biovigilance** 129–130  
  **bivalirudin** 416  
  **bleeding, plasma transfusion** 212–213  
  **blood administration**  
    bedside pumps 313  
    component issue and release 307  
    component modifications 309–310  
    component preparation 309–311  
    cryoprecipitate 210–211, 214–215  
    donor screening 31–32  
    granulocytes 195–198  
    group considerations  
      ABO system 85  
      Diego system 114  
      Duffy protein 113  
      I blood group 92  
      Kell blood group 112  
      Kidd system 113–114  
      Lewis blood group 89  
      MNS blood group 111  
      P blood group 96  
      Rh system 106–107  
      Sd<sup>a</sup> antigen 97  
    immunoglobulin G 240–248  
    infusion flow rates 312  
    plasma 209–215  
      adverse reactions 215  
      bleeding treatment 212–213  
      clinical use 212  
      cryoprecipitate 214–215  
      disseminated intravascular coagulation 213  
      prophylactic 213–214  
    **pretransfusion considerations**  
      306–307  
      rapid infusion practices 313–314  
      recipient management 129  
      sets and filters 311  
      transfusions 311–313  
      venipuncture 307  
      warming 313  
      *see also* administration  
    **blood avoidance techniques, surgical patients** 461–462  
  **blood banks**  
    defective product reporting 72  
    European regulations 75–78  
    FDA regulation 71–72, 78  
    initial development of 9  
    World Health Organization programs 74–75  
  *see also* hospital transfusion services  
  **blood collection** 32–36  
    acute adverse reactions 49–55, 256–258  
    administering screening 31–32  
    air embolism 54  
    apheresis 33–34, 53–54, 252–258  
    chronic effects 56–64  
    iron depletion 56–64  
    mitigation 61–62  
    platelet donation 63  
    source plasma 63–64  
  **CJD/vCJD risk** 31  
  **component separation** 33  
  **donor screening** 29–32  
  **educational materials** 31  
  **European regulations** 75–78  
  **ferritin testing** 61–62, 63  
  **frequency limitations** 61  
  **frequency-related adverse reactions** 54, 56–64  
  **good manufacturing practices** 67–68  
  **granulocytes** 54–55, 487  
  **informed consent** 31–32  
  **iron supplementation** 61  
  **limiting blood loss** 304  
  **men who have sex with men** 31  
  **multiple component collection complications** 54  
  **patient needs** 36  
  **phlebotomy-related adverse events** 52–53  
  **phlebotomy-related nerve injury** 53  
  **physical examination** 32, 34–35  
  **postdonation care** 32–33  
  **presyncopal reactions** 50  
  **pretransfusion testing** 123–124  
  **skin disinfection** 535  
  **source plasma** 34–36  
  **syncopal reactions** 51–52  
  **whole blood** 32  
  **World Health Organization programs** 74–75  
  **blood collection establishments (BCEs)**  
    **computer software** 70  
    **device regulatory controls** 70  
    **educational materials** 31  
    **European regulations** 75–78  
    **FDA guidance documents** 68–69  
    **good manufacturing practices** 67–68  
    **inspections** 69  
    **physical examination** 32, 34–35  
    **postdonation care** 32–33  
    **reportable deviations** 69  
    **United States regulations** 66–70  
    **World Health Organization programs** 74–75  
  *see also* blood collection  
  **blood components** 131–248  
    adverse reaction reporting 70–73  
    apheresis collection 180, 251, 254–256  
    bacterial contamination 533–542  
    coadministration 311  
    current good manufacturing practices 67–68  
    defective products reporting 69, 72  
    disaster management 18  
    European regulation 75–78

- blood components (*cont'd*)  
 good manufacturing practices 67–68  
 granulocytes  
   collection 194–195  
   donor screening 194–195  
   pediatrics 198, 199  
   storage 195  
   transfusion 195–199  
 immunoglobulin products 236–248  
 initial usage 9  
 irradiation 155, 186, 221, 309–310  
 issue and release 307  
 leukocyte reduction 33, 155, 186, 309  
 lookback 42  
 manufacturing processes  
   activated prothrombin complex  
     concentrate 227–228  
   albumin 222–224  
   alpha<sub>1</sub>-proteinase inhibitor 228  
   antithrombin III 230  
   C1 inhibitor 229–230  
   factor IX 232  
   factor VIII 233–234  
   factor X 227  
   factor XI 228–229  
   factor XIII 227  
   fibrinogen 231  
   immunoglobulins 224–227  
   pathogen reduction 219–221  
   prion safety 222  
   prothrombin complex concentrate  
     231–232  
   validation of virus reduction/removal  
     219–220  
   virus filtration 221  
   virus inactivation 220–221  
   virus reduction 219–220  
   von Willebrand factor 232–233
- neutrophils 188–193  
 clearance 189  
 kinetics 188–190  
 migration 189  
 mobilization 188–189  
 NETosis 189–190  
 neutropenias 190–193  
 neutrophilia 190  
 production 188
- plasma 200–235  
 adverse reactions 215  
 alternate preparations 209–211  
 bleeding treatment 212–213  
 chromatographic separation  
   218–219, 222  
 clinical use 212  
 clotting factors 210–211  
 cold ethanol fractionation 217–218  
 cryoprecipitate 210–211, 214–215, 308  
 disseminated intravascular  
   coagulation 213  
 dried 209–210
- medicinal product manufacturing  
 processes 222–234  
 octanoic acid treatment 221  
 pathogen reduced 210, 219–221  
 pH 221  
 prion safety 222  
 prophylactic 213–214  
 protein purification 216–235  
 solvent and detergent treatment  
   220–221  
 storage 308  
 transfusion 209–215  
 ultraviolet treatment 221  
 virus filtration 221  
 virus inactivation 220–221  
 virus reduction 219–220  
 warfarin effect 214
- platelets 158–187  
 ABO system antigens 170–171  
 additive solutions 184–185  
 alloimmunization 171–177  
 alternate sources 181  
 antibody-specific prediction 173–174  
 antigens 168–171  
 apheresis-derived 180  
 buffy-coat method 180  
 centrifugation 186  
 circulatory survival 165  
 containers 183  
 crossmatching 174  
 cryopreservation 186  
 differentiation 158–165  
 HLA-matched transfusions 172–173  
 human leukocyte antigen 168–169  
 human platelet antigens 169–170  
 immunology 168–177  
 irradiation 186  
 kinetics 165–167  
 lesions 183–184  
 leukocyte reduction 186  
 lifespan 165–167  
 lyophilization 186–187  
 megakaryopoiesis regulation  
   162–165  
 metabolism in storage 181–183  
 modifications 185–186  
 novel storage techniques 186–187  
 pathologies of  
   megakaryopoiesis 161–162  
 pH 182–183  
 plasma removal 186  
 platelet-rich plasma 179  
 plateletpheresis-derivation 180  
 preparation 179–181  
 prevention of alloimmunization  
   176–177  
 production 158–165  
 refractory evaluation 174  
 respiratory capacity 183  
 sequestration 167
- storage 181–187, 307  
 storage containers 183  
 storage lesions 183–184  
 temperature 181–182, 186  
 volume reduction 186  
 washing 186
- preparation 309–311  
 red cells 133–157  
   additive solutions 152–154  
   alternate metabolic substrates  
     146–147  
   anemia of chronic inflammation 141  
   anticoagulant-nutrient solutions 152  
   biosynthesis 135, 148–149  
   effectiveness 155–156  
   eryptosis 150  
   erythropoiesis 133–142  
   erythropoietic therapies 141–142  
   expiration time 307  
   glucose metabolism 145–148  
   guanine nucleotides 148  
   irradiation 155  
   leukocyte reduction 155  
   macrocytic anemia 138–140  
   membrane metabolism 149–150  
   metabolic regulation 147–148  
   metabolism 143–150  
   microcytic anemia 140–141  
   pathogen reduction 155  
   preservation 150–157  
   quality evaluation 156–157  
   rejuvenation 156  
   separation 151–152  
   storage 154–155, 156, 307  
   temperature and time lapse 154  
   thalassemia 141  
   in vivo recovery 156–157  
   washing 155
- reportable deviations 69  
 returns 309  
 separation 33  
   apheresis 251  
 testing 37, 38, 46  
   *see also* blood testing
- transfusion-related deaths reporting 71  
 transport 309  
 United States safety regulations 65–73  
 white cells 188–248  
   granulocytes 193–199  
   neutrophils 188–193
- World Health Organization programs  
   74–75  
   *see also* administration
- blood derivatives  
 prerelease testing 46  
   *see also* blood testing, donors
- blood donation  
 acute adverse reactions 49–55, 256–258  
 apheresis 33–34, 252–258  
 autologous 29

- chronic effects 56–64  
 iron depletion 56–64  
 mitigation 61–62  
 plateletpheresis 63  
 source plasma 63–64  
 collection 32–36  
 component separation 33  
 demographics 27  
 development of 9  
 direct antiglobulin testing 39  
 directed 29  
 disasters 28–29  
 exception medical needs 29  
 ferritin testing 61–62, 63  
 frequency limitations 61  
 granulocytes 487  
 iron supplementation 61  
 operational chain 11  
 patient needs 36  
 physical examination 32, 34–35  
 postdonation care 32–33  
 recipient-specific 29  
 recruitment 27–29  
 screening 29–32, 37–48  
 source plasma 34–36  
 testing 37–48  
*see also* blood testing, donors  
 whole blood 32  
*see also* blood collection
- blood establishment computer software (BECS) 70
- blood groups 329–330  
 ABCC1 system 117  
 ABO system 81–87, 330  
 antibodies 120–122  
 antibody classes 121  
 AUG system 117  
 carbohydrate 81–99  
 Cartwright system 115  
 Chido/Rogers system 115  
 Colton system 114–115  
 compatibility testing 8  
 crossmatching 128–129  
 CTL2 system 117  
 Diego system 114  
 discovery 7  
 DNA-based typing 127–128  
 Dombrock system 115–116  
 Duffy system 112–113, 329  
 extended antigen typing 47  
 Gerbich system 115, 329  
 GIL system 114–115  
 I system 90–93  
 Indian system 115  
 inheritance discovery 8  
 JR system 116  
 Kell system 111–112  
 Kidd system 113–114  
 Knops system 115, 330  
 LAN system 116
- Lewis system 87–90, 330  
 Lutheran system 111  
 LW system 107  
 MAM system 117  
 MNS system 109–111, 330  
 OK system 116  
 P system 93–97  
 PEL system 116–117  
 pretransfusion testing 123–130  
 RAPH system 116  
 Rh system 100–107  
 Scianna system 114  
 Sd<sup>a</sup> 97–99  
 unit selection 128  
 Vel system 117  
 Xg system 114
- blood loss, pregnancy 373–375  
 blood recovery systems 299–301  
 Blood Regulators Network, WHO (BRN) 75
- blood safety  
 adverse reaction reporting 70–73  
 biovigilance 129–130  
 blood collection establishment regulation, US 66–70  
 defective products reporting 69, 72  
 donor screening 29–32  
 European regulations 75–78  
 hospital transfusion services regulation, US 70–73  
 initial concerns 10–11  
 reportable deviations 69  
 testing 37–48  
*see also* blood testing  
 transfusion-related deaths reporting 71  
 United States regulations 37, 38, 39–40, 41–42, 65–73  
 US accreditation 73  
 World Health Organization requirements 39, 74–75
- blood services  
 current system organization 11–13  
 operational chain 11  
 United States organizations 12  
 worldwide organization 12–13  
*see also* blood donation
- blood testing  
 compatibility testing 122–130  
 donors 37–48, 40  
 ABO antibody titers 38–39  
 ABO typing 37–39, 38  
 automated systems 40  
 babesiosis 45  
 blood derivatives 46  
 Chagas' disease 46  
 components 37, 38  
 cytomegalovirus 46  
 direct antiglobulin testing 39  
 extended blood group antigen typing 47
- false positives 39  
 hemoglobin S 47  
 hepatitis A 47  
 hepatitis B 43–44  
 hepatitis C 44  
 HIV 43  
 human leukocyte antigen 47  
 human T-cell lymphotropic virus 44  
 IgA deficiency 47  
 infectious diseases 37, 39–47  
 lookback 42  
 management of donors 41–42, 41  
 Parvovirus B19 47  
 platelet antigen typing 47  
 platelets 46  
 pooling 39, 43  
 product management 42  
 RBC antigens 38  
 recipient notification 42  
 reentry 41, 42–43  
 regulations 39–40, 41–42  
 resource-limited settings 40  
 Rh groups 37–38  
 Rh system 37–38  
 Rh typing 37–38  
 sequence 40  
 source plasma donors 38–41  
 syphilis 45–46  
 transfusion-transmitted infections 37, 39–47  
 United States regulations 37, 38, 39–40, 41–42  
 West Nile virus 45  
 World Health Organization requirements 39  
 Zika virus 45
- Blundell, J. 5–6  
 Bombay phenotype 83  
*para*-Bombay phenotype 83  
 bone allografts 673–674  
 bone marrow  
 collagen fibrosis 614  
 microenvironment 617–619  
 paroxysmal nocturnal hemoglobinuria 363  
 reticulin fibrosis 614  
 bone tissue engineering 656  
 bradykinin (BK) 265  
 Braxton-Hicks, J. 6–7  
 breast cancer, I blood group 93  
 breast milk, hemolytic disease of the fetus and newborn 371  
 BRN *see* Blood Regulators Network, WHO
- bronchoscopy  
 plasma administration 468  
 platelet administration 469
- bruises, blood donation 52  
 buffy-coat (BC) method 180

- burn patients 471, 478–481  
 coagulation factor replacement 480  
 cryoprecipitate 480  
 freeze-dried plasma 480  
 initial resuscitation 478  
 prothrombin complex concentrates 480  
 recombinant activated factor VII 480  
 skin allografts 675  
 tranexamic acid 479–480  
 transfusion therapy 478–479  
 whole blood 480
- burst-forming units-erythroid (BFU-Es) 134, 135–136
- burst-forming units-megakaryocyte (BFU-MK) 159
- bystander hemolysis 547
- C/c antigens, Rh system 102–103
- C/EBP $\alpha$  see CCAAT/enhancer binding protein  $\alpha$
- C1 inhibitor 201, 229–230
- CAD see cold agglutinin disease
- calcium supplementation, apheresis 54
- calculated panel-reactive antibody (CPRA) tests 630–631
- California maternal quality care collaboration (CMQCC) 376
- Campylobacter jejuni* 86–87
- Canadian Blood Services (CBS), physical examination requirements 32
- cancer
- ABO system 86
  - adverse reactions 487–488
  - chimeric antigen receptor T-cell therapy 488
  - erythroid growth factors 605
  - granulocyte transfusions 486–487
  - I blood group 93
  - Lewis blood group 89–90
  - neutropenia 192
  - P blood group system 95, 96
  - platelet transfusions 484–486
  - red blood cell administration 322, 482–484
  - Sd $^a$  antigen 98
  - T-cell immunotherapies 633, 636–637
  - thrombocytopenia 394
  - thrombopoietic growth factors 614
  - transfusion support 482–488
  - see also specific forms of cancer...
- CAP see College of American Pathologists
- caprylate see octanoic acid
- CAPS see catastrophic antiphospholipid syndrome
- CAR see chimeric antigen receptor
- carbohydrate blood groups 81–99
- ABO system 81–87
  - I system 90–93
  - Lewis system 87–90
  - P system 93–97
  - Sd $^a$  antigen 97–99
- carboxylic acids, red blood cell synthesis 148
- cardiac allografts 676–677
- rejection, extracorporeal photopheresis 287–288
- cardiac surgery
- platelet administration 470
  - red blood cell administration 319–321
- cardiac tissue engineering 657
- Carrel, A. 7
- cartilage, allografts 674
- cartilage tissue engineering 657–658
- Cartwright blood group system 115
- CASI see computer-assisted self-interview
- catastrophic antiphospholipid syndrome (CAPS), apheresis 271
- CBER see Center for Biologics Evaluation and Research
- CBS see Canadian Blood Services
- CCAAT/enhancer binding protein  $\alpha$  (C/EBP $\alpha$ ) 91–92
- CD17 see transferrin receptor
- CD40 ligand, transfusion-related acute lung injury 576
- CD105 see endoglin
- CDER see Center for Drug Evaluation and Research
- CD-P-TS see European Committee on Blood Transfusion
- CDRH see Center for Devices and Radiologic Health
- CDSS see clinical decision support systems
- cell salvage 299–301
- devices 312
  - obstetrics 377
- cell-based immunotherapies, storage 308
- Center for Biologics Evaluation and Research (CBER) 68
- Center for Devices and Radiologic Health (CDRH) 68
- Center for Drug Evaluation and Research (CDER) 67, 68
- central venous catheters (CVCs)
- insertion 466–467, 469
  - therapeutic plasma exchange 260
- centrifugation, platelets 186
- ceramide lipid tails 93
- CFR see US Code of Federal Regulations
- CFU-Es see
- colony-forming units-erythroid
- CFU-MK see colony-forming units-megakaryocyte
- cGMP see current good manufacturing practices
- Chagas disease 46, 524–525
- Chediak–Higashi syndrome 191
- chelation
- compliance 594–595
  - initiation 594
  - intensification 595
- iron overload 592–596
- monitoring 595–596
- chemical agent attacks 16
- chemistry, ABO system 81–82
- chemotherapeutics, cold agglutinin disease 353
- chemotherapy-induced neutropenia 609–610
- chemotherapy-induced thrombocytopenia (CIT) 615
- Chido/Rogers blood group system 115
- chikungunya virus (CHIKV) 518
- CHIKV see chikungunya virus
- children 381–391
- anemia 383
  - coagulation system development 388–389
  - cryoprecipitate transfusions 390–391
  - granulocyte transfusions 198, 199
  - I blood group 90–93
  - immune thrombocytopenic purpura 410–411
  - paroxysmal nocturnal hemoglobinuria 363
  - plasma transfusions 388–390
  - platelet transfusions 385–388
  - red blood cell administration 322
  - red blood cell transfusions 383–385
  - therapeutic plasma exchange 266
  - TRALI 573
  - see also infants; neonates
- chimeric antigen receptor (CAR) T-cell therapy 634, 635–637
- adverse reactions 488
- chlorpromazine 192
- CHMP see Committee for Medicinal products for human Use
- cholera, ABO system 86–87
- choline transporter-like protein-2 (CTL2) system 117
- chromatographic separation
- albumin 222–223
  - plasma 218–219, 222
- chromium-51 labeling 156–157
- chronic anemia
- maternal 375
  - red blood cell administration 324
- chronic autoimmune diseases, immunoglobulin therapy 246–247
- chronic effects
- donation-related 56–64
  - iron depletion 56–64
  - mitigation 61–62
  - neurocognitive 60
  - pica 60–61
  - platelet donation 63
  - pregnancy-related 60
  - restless legs syndrome 60
  - source plasma 63–64
  - vulnerable populations 61

- chronic inflammation  
anemia 141  
immunoglobulin therapy 246–247
- chronic inflammatory demyelinating polyradiculoneuropathy (CIDP)  
apheresis 267  
immunoglobulin therapy 246
- chronic liver disease, coagulation factor concentrates 451
- chronic renal failure, erythroid growth factors 605
- chronic transfusion therapy (CTT)  
sickle cell disease 280–281, 331–335, 332  
thalassemia 335–337
- CIDP *see* chronic inflammatory demyelinating polyradiculoneuropathy
- circular preplatelets 160–161
- circulation, discovery of 3
- circulatory overload 563–564
- circulatory survival, platelets 165
- CIT *see* chemotherapy-induced thrombocytopenia
- citrate anticoagulants 8–9, 53–54, 251, 257, 266, 561–562
- citrate phosphate dextrose adenine (CPDA-1) solutions 151, 152
- citrate phosphate dextrose (CPD) solutions 151, 152
- citrate phosphate double dextrose (CP2D) solutions 152
- citrate reactions 53–54
- citrate toxicity 257, 266, 561–562
- CJD *see* Creutzfeld–Jakob disease
- Clair Y g, purification 226
- classifications, von Willebrand disease 434–436
- clearance, neutrophils 189
- CLIA *see* Clinical Laboratory Improvement Act
- clinical decision support systems (CDSS) 295–297
- clinical features  
cold agglutinin disease 352–353  
combined factor V and factor VIII deficiency 438  
drug-induced immune hemolytic anemia 357  
factor V deficiency 437–438  
factor VII deficiency 438  
factor X deficiency 439  
factor XI deficiency 439–440  
factor XIII deficiency 440  
hemolytic transfusion reactions 547–550  
hemophilia 425  
immune thrombocytopenic purpura 406–407  
iron overload 590  
paroxysmal cold hemoglobinuria 354–355
- paroxysmal nocturnal hemoglobinuria 359–360  
prothrombin deficiency 437  
transfusion-related acute lung injury 569–570
- von Willebrand disease 434
- warm autoimmune hemolytic anemia 348–349
- Clinical Laboratory Improvement Act (CLIA) 70, 71
- clinical outcomes  
anemia 317–319  
cold agglutinin disease 354  
paroxysmal cold hemoglobinuria 355  
paroxysmal nocturnal hemoglobinuria 363  
red blood cell administration 318–322  
transfusion-related acute lung injury 573  
warm autoimmune hemolytic anemia 351
- clonal selection 120
- clonality 120
- clopidogrel, surgical protocols 460
- clotting factors 204  
in plasma 201–202, 203  
storage 308
- clozapine 192
- Clustered Regularly-Interspaced Short Palindromic Repeats (CRISPR) 644
- CM-TMA *see* complement-mediated thrombotic microangiopathy
- CMQCC *see* California maternal quality care collaboration
- CMV *see* cytomegalovirus
- coadministration  
fluids and components 311  
medications 311–312
- coagulation  
ABO system 86  
activation and regulation 203, 204  
screening tests 204–208, 457–458
- coagulation factor inhibitors, in plasma 201–202
- coagulation factors 424–442  
acquired bleeding disorders 443–452  
afibrinogenemia 441  
burn patients 480  
combined factor V and factor VIII deficiency 438  
dysfibrinogenemia 441–442  
factor V deficiency 438  
factor VII deficiency 438–439  
factor X deficiency 439  
factor XI deficiency 439–440  
factor XIII deficiency 440–441  
fibrinogen disorders 441  
hemophilia 426–432  
hypodysfibrinogenemia 441–442
- hypofibrinogenemia 441  
inherited bleeding disorders 424–442  
obstetrics 377  
in plasma 201–202, 203, 204–205  
prothrombin deficiency 437  
trauma patients 480  
von Willebrand disease 436  
*see also* factor-n...
- coagulopathies, trauma patients 474–475
- cobalamin deficiency, macrocytic anemia 138–140
- CoE *see* Council of Europe
- Coga, A. 4
- Cohn, E.J. 9
- Cohn process  
albumin 222–223  
 $\alpha_1$ -proteinase inhibitor 228  
immunoglobulins 224–226
- cold agglutinin disease (CAD)  
clinical features 352–353  
pathophysiology 351–352  
prognosis 354  
treatment 353–354
- cold autoimmune hemolytic anemia 351–355
- cold ethanol fractionation 9, 217–218  
albumin 222–224  
immunoglobulins 224–226
- collagen fibrosis 614
- College of American Pathologists (CAP) 17
- transfusion services regulations 70, 73
- colon cancer  
I blood group 93  
 $Sd^a$  antigen 98
- colony-forming units-erythroid (CFU-Es) 134–135
- colony-forming units-megakaryocyte (CFU-MK) 159
- Colton blood group system 114–115
- combined factor V and factor VIII deficiency 438
- Committee for Medicinal products for human Use (CHMP) 76–77
- communication, donor screening results 41–42
- compatibility testing 122–130  
donor testing 122–123  
granulocyte collection 194–195  
Ottenberg 8  
patient testing 123–130
- complement, hemolytic transfusion reactions 545–547
- complement scavenging, immunoglobulins 244
- complement-mediated thrombotic microangiopathy (CM-TMA), apheresis 270
- compliance, chelation 594–595

- complications  
 apheresis 256–258  
 hemolytic transfusion reactions 550  
 massive transfusion protocols 561–564  
 rapid infusion practices 561–564  
 red blood cell transfusion 342–345  
 therapeutic plasma exchange 264–266  
*see also* adverse reactions; adverse sequelae
- component therapy  
 disaster management 18  
 initial development of 9  
 irradiation 309–310  
 leukoreduction 309  
*see also* administration; blood components
- composition of plasma 200–208  
 albumin 200  
 alpha-1 antitrypsin 201  
 C1 inhibitor 201  
 clotting factors 201–202, 203  
 coagulation factor inhibitors 201, 203  
 factors influencing 202–203  
 fibrinolysis 203–208  
 immunoglobulins 201  
 von Willebrand factor 201–202
- computer-assisted self-interview (CASI) 32
- computerized physician order entry (CPOE) systems 295–296
- confirmatory testing, HIV 43
- congenital neutropenias 191–192
- connective tissue allografts 674–675
- consent, recipients 129
- containment, health emergencies 22
- contaminant levels, albumin 224
- continuity of operations plans (COOP) 17, 17
- continuous renal replacement therapy (CRRT) 263
- contraindications  
 cell salvage 301  
 heparin-induced thrombocytopenia 416–417  
 sickle cell disease 335
- convalescent plasma 23, 212
- conventional-dose therapy, subcutaneous therapy 240
- COOP *see* continuity of operations plans
- cord blood 621
- corticosteroids  
 immune thrombocytopenic purpura 407  
 warm autoimmune hemolytic anemia 350
- Council of Europe (CoE) 76–77
- COVID-19 14, 16, 18–23  
 ABO type 87
- cell-based therapies 650
- convalescent plasma 23
- hematopoietic stem cells 622–623  
 vaccine-induced immune thrombotic thrombocytopenia 417
- COX2 *see* cyclooxygenase 2
- CP2D *see* citrate phosphate double dextrose
- CPD *see* citrate phosphate dextrose
- CPDA-1 *see* citrate phosphate dextrose adenine
- CPOE *see* computerized physician order entry systems
- CPRA *see* calculated panel-reactive antibody tests
- Creutzfeld-Jakob disease (CJD) 11, 31, 529–531
- crisis standards of care 16–17
- CRISPR *see* Clustered Regularly-Interspaced Short Palindromic Repeats
- critical care  
 red blood cell administration 319  
 transfusion-related acute lung injury 577
- cross-reactive groups (CREGs), human leukocyte antigens 172–173
- crossmatching 128–129  
 platelets 174, 400, 484
- CRRT *see* continuous renal replacement therapy
- CRS *see* cytokine release syndrome
- cryoglobulinemia, apheresis 271–272
- cryoprecipitate  
 administration 210–211  
 burn patients 480  
 children 390–391  
 hemophilia 426–427  
 therapeutic plasma exchange 262–263  
 trauma patients 480  
 bacterial contamination 541  
 blood collection 32  
 clinical use 214–215  
 separation 33  
 storage 308
- cryopreservation, hematopoietic stem cells 620–621
- CTCL *see* cutaneous T-cell lymphoma
- CTL2 (choline transporter-like protein-2) system 117
- CTT *see* chronic transfusion therapy
- current good manufacturing practices (cGMP) 67–68
- cutaneous T-cell lymphoma (CTCL) 287
- CVCs *see* central venous catheters
- cyclic neutropenia 192
- cyclooxygenase 2 (COX2) inhibitors 432
- cysteine metabolism, red blood cells 148
- cytapheresis *see* therapeutic plasma exchange
- cytokine release syndrome (CRS) 488
- cytokines  
 megakaryopoiesis regulation 162–164  
 storage-generated 555
- cytomegalovirus (CMV) 519  
 neutropenia 192  
 testing 46
- cytotoxic agents, cold agglutinin disease 353
- D antigen, Rh system 101
- D antigen testing 37–38
- damage-associated pattern molecules (DAMPs) 474–475
- damage-control resuscitation 475–478
- DAMPs *see* damage-associated pattern molecules
- danazol 410
- darbepoetin alfa (Aranesp) 603–604
- DAT *see* direct antiglobulin testing
- DBCD *see* Division of Blood Components and Devices
- DC *see* dendritic cell
- DDAVP *see* desmopressin
- decision-making, red blood cell transfusions 323
- defective products, reporting 69, 72
- deferasirox (ICL670Q / Exjade / DFX) 593–594
- deferiprone (1,2-dimethyl-3-hydroxypyridin-4-one / DFP / L1) 593
- deferoxamine B mesylate (DFO / Desferal) 592–593
- deglycerolizing, red blood cells 156
- dehydrated hereditary stomatocytosis (DHSt) 339
- delayed acute adverse reactions, blood donation 50
- delayed hemolytic transfusion reactions (DHTRs) 343–344, 543–552  
*see also* hemolytic transfusion reactions
- DEM *see* Donor Educational Materials
- demarcation membrane system (DMS) 160
- demographics, blood donors 27
- denaparoid sodium 416
- dendritic cell (DC) immunotherapy 638
- dengue virus 517–518
- Denis, J-B. 4–5
- Department of Homeland Security (DHS) 15
- desensitization, therapeutic plasma exchange 274
- Desferal *see* deferoxamine B mesylate
- desmopressin (DDAVP) 203, 303–304, 432, 436, 448–449  
 adverse reactions 448–449  
 dosing 448  
 indications 448  
 manufacturing 448  
 oncology patients 486  
 storage 448
- DETTD *see* Division of Emerging and Transfusion Transmitted Diseases

- devices  
 administration sets and filters 311  
 donor apheresis 253–254  
 regulatory controls 70  
 therapeutic plasma exchange 259–260
- DFO *see* deferoxamine B mesylate
- DFP *see* deferiprone
- DFX *see* deferasirox
- DHQ *see* Donor History Questionnaire
- DHS *see* Department of Homeland Security
- DHSt *see* dehydrated hereditary stomatocytosis
- DHTRs *see* delayed hemolytic transfusion reactions
- diagnosis  
 allergic responses 558–559  
 anaphylaxis 560  
 babesiosis 524  
 Chagas disease 525  
 combined factor V and factor VIII deficiency 438  
 cytomegalovirus 519  
 dengue virus 517  
 dysfibrinogenemia 441  
 factor V deficiency 438  
 factor VII deficiency 438  
 factor X deficiency 439  
 factor XI deficiency 440  
 factor XIII deficiency 440  
 febrile nonhemolytic reactions 556  
 hemolytic transfusion reactions 547–550  
 hepatitis A 507–508  
 hepatitis B 510–511  
 hepatitis C 511  
 herpesviruses 519  
 hypodysfibrinogenemia 441  
 prothrombin deficiency 437  
 transfusion-associated graft-versus-host disease 584–585  
 transfusion-related acute lung injury 571–572  
 transfusion-related circulatory overload 580  
 von Willebrand disease 434–436  
 West Nile Virus 518
- diagnostic testing, health emergencies 22
- dialysis, thrombocytopenia 394
- DIC *see* disseminated intravascular coagulation
- Diego blood group 81, 110, 114
- differentiation  
 erythroid progenitor cells 133–134, 137–138  
 hematopoietic stem cells 133  
 megakaryocytes 159–165  
 platelets 158–165
- DIIHA *see* drug-induced immune hemolytic anemia
- dilated cardiomyopathy (DCM), apheresis 275–276
- dimethyl sulfoxide (DMSO)  
 toxicity 565–566
- 1,2-dimethyl-3-hydroxypyridin-4-one *see* deferiprone
- 2,3-diphosphoglycerate (2,3-DPG) 143, 145–146, 147–148, 151, 153, 155–156
- direct antiglobulin test (DAT) 39, 122
- paroxysmal cold hemoglobinuria 354–355
- warm autoimmune hemolytic anemia 347–349
- direct oral anticoagulants (DOACs), surgical protocols 460
- direct transfusion, development of method 7–8
- directed donations 29
- disaster management 14–20  
 blood components 18  
 continuity of operations plans 17, 17  
 COVID-19 14, 16, 18–23  
 extraordinary donation volumes 28–29  
 local plan development resources 15  
 mitigation 14  
 national authorities 15–16, 18  
 operations organization 15–16  
 preparedness 14–15, 16–17  
 records management 18  
 recovery 15  
 regulatory considerations 17–18  
 responses 15  
*see also* health emergencies
- disialogalactosylgloboside (DSGG) 94
- disseminated intravascular coagulation (DIC)  
 coagulation factor concentrates 450–451  
 plasma transfusion 213  
 pregnancy 375
- dithiothreitol (DTT) 107
- DITP *see* drug-induced immune thrombocytopenic purpura
- Division of Blood Components and Devices (DBCD) 68
- Division of Emerging and Transfusion Transmitted Diseases (DETTD) 68
- DMS *see* demarcation membrane system
- DMSO *see* dimethyl sulfoxide
- DNA-based blood group typing 127–128, 129
- DOACs *see* direct oral anticoagulants
- Dombrock blood group system 115–116
- donation tracking, allografts 678–679
- Donor Educational Materials (DEM) 31–32
- Donor History Questionnaire (DHQ) 30–31
- donor-recipient testing 128–129
- donors  
 acute adverse reactions 49–55  
 apheresis 53–55, 256–258  
 citrate reactions 53–54, 257  
 frequency-related 54  
 granulocyte collection 54–55  
 multiple component collection 54  
 phlebotomy-related 52–53  
 presyncopal 50, 257  
 risk factors 51, 53  
 syncopal 51–52  
 apheresis 252–258  
 complications 256–258  
 devices 253–254  
 procedure requirements 254–256  
 product requirements 254–256  
 autologous 29  
 blood collection 32–36, 252–258  
 chronic effects 56–64  
 blood collection 56–62  
 mitigation 61–62  
 plateletpheresis 63  
 source plasma 63–64  
 demographics 27  
 directed 29  
 exceptional medical needs 29  
 frequency limitations 61  
 granulocyte collection 194–195, 487  
 iron supplementation 61  
 management 41–42  
 transfusion-related acute lung injury 577–578  
 physical examination 32, 34–35  
 postdonation care 32–33  
 pregnancy 33  
 re-entry 42–43  
 recipient-specific 29  
 recruitment 27–29  
 behavioral theories 28  
 disasters 28–29  
 recipient-specific 29  
 retention 28  
 repeat 28  
 retention 28  
 screening 29–32, 37–48  
 ABO antibody titers 38–39  
 ABO system 37–39, 38  
 administration 31–32  
 automated systems 40  
 bacterial infections 535  
 CJD/vCJD risk 31  
 communicating results 41–42  
 direct antiglobulin testing 39  
 Donor History Questionnaire 30–31  
 educational materials 31  
 ferritin levels 61–62, 63  
 granulocyte collection 194–195  
 infectious diseases 37, 39–47  
 informed consent 31–32  
 lookback on discovery of disease 42  
 men who have sex with men 31

- donors (*cont'd*)
- RBC antibody detection 38
  - re-entry 42–43
  - Rh system 37–38
  - source plasma 34–35, 38–41
  - transfusion-related acute lung injury 577
  - vital sign measurement 32
  - see also* blood testing, donors; prerelease testing
  - source plasma 34–36, 38–41
- Doptelet *see* avatrombopag
- dosing
- activated prothrombin complex concentrate 445
  - andexanet alfa 450
  - antifibrinolytics 448
  - desmopressin 448
  - factor VIII 427
  - idarucizumab 450
  - immunoglobulin therapy 244–247
  - platelet transfusions 397
  - protamine 449
  - prothrombin complex concentrates 444
  - recombinant activated factor VII 445
  - red blood cell administration 324
  - vitamin K 446–447
- 2,3-DPG *see* 2,3-diphosphoglycerate
- 2,3-DPG shunt (Rapoport–Luebering shunt) 145–146, 147
- dried plasma 209–210
- drug interactions, immunoglobulin therapy 241
- drug-induced autoimmune thrombocytopenia 413
- drug-induced immune hemolytic anemia (DIIHA) 356–358
- drug-induced immune thrombocytopenias 411–417, **412**
- drug-induced immune thrombocytopenic purpura (DITP) 411–412
- drug-induced thrombotic microangiopathy 413
- dry heat, virus inactivation 220
- DSGG *see* disialogalactosylgloboside
- DTT *see* dithiothreitol
- du Mauroy, A. 5
- Duffy blood group system 112–113, 329
- dura mater allografts 675
- dysfibrinogenemia 441–442
- dyspnea 580
- E/e antigens, Rh system 102–103
- ebola virus 520–521
- EBV *see* Epstein–Barr virus
- ECBS *see* Expert Committee on Biological Standardization
- ECMO *see* extracorporeal membrane oxygenation
- ECP *see* extracorporeal photopheresis
- eculizumab
- cold agglutinin disease 353
  - paroxysmal nocturnal hemoglobinuria 361–362, 363
- EDQM *see* European Directorate for the Quality of Medicines
- education, physicians 295–297
- educational materials, donors 31
- effectiveness, red blood cells 155–156
- efficacy of pathogen reduction 493–495
- ELANE gene mutations 191
- electrolyte disorders 562–563
- eltrombopag (Promacta / Revolade) 613
- EMA *see* European Medicines Agency
- EMAs *see* emergency management agencies
- Embden–Meyerhof–Parnas pathway (EMP) 145
- embryonic development
- I blood group 93
  - P blood group system 96
- emergency management agencies (EMAs) 16–17
- emergency operations, organization 15–16
- emergency release 129
- emergency treatment, immune
- thrombocytopenic purpura 410
- EMP *see* Embden–Meyerhof–Parnas pathway
- endoglin (CD105) 134
- engineered T cell receptor expressing T cell therapy 634–637
- ENT1 *see* equilibrative nucleoside transporter 1
- enteric infections
- ABO system 86–87
  - Lewis blood group 89–90
  - P blood group 97
  - Sd<sup>a</sup> antigen 99
- enzymopathies, red blood cells 328–329, 337–338
- epidemiological surveillance, health emergencies 22
- epidemiology
- alloimmune thrombocytopenia 418–419
  - cold autoimmune hemolytic anemia 351
  - drug-induced immune hemolytic anemia 356
  - paroxysmal nocturnal hemoglobinuria 358
  - paroxysmal cold hemoglobinuria 354
- physical injury 472–473
- transfusion-related acute lung injury 572–573
- warm autoimmune hemolytic anemia 346
- see also* prevalence
- epidural anesthesia
- plasma administration 468
  - platelet administration 470
- epitopes
- ABO system 81–82
  - human leukocyte antigens 631
  - Kell blood group 112
  - P blood group system 93–94
- EPO *see* erythropoietin
- epoetin alfa (Epogen / Procrit) 603
- epoetin alfa-epbx (Retacrit) 603
- Epogen *see* epoetin alfa
- Epstein–Barr virus (EBV) 192, 519
- equilibrative nucleoside transporter 1 (ENT1) 117
- error reporting
- defective products 69, 72
  - transfusion services 71–72
- eryptosis 150
- erythrocytapheresis 282–285
- hereditary hemochromatosis 285
  - polycythemia vera 283
  - protocol 341
  - secondary erythrocytosis 284
- erythroid growth factors 603–606
- adverse effects 604–605
  - indications 605
- erythroid maturation agents 596
- erythroid progenitor cells 133–134, 137–138
- erythropoiesis 133–142
- anemia of chronic inflammation 141
  - extracellular requirements 135–136
  - hemoglobin synthesis 135
  - intracellular requirements 135
  - macrocytic anemia 138–140
  - microcytic anemia 140–141
  - nutritional requirements 138–142
  - stages 133–134
  - thalassemia 141
  - therapies 141–142
- erythropoietin (EPO) 136–138
- erythropoietin-stimulating agents (ESAs) 484
- ESAs *see* erythropoietin-stimulating agents
- Escherichia coli*, P blood group system 97
- essential thrombocythemia (ET)
- apheresis 285–286
  - platelet lifespan 165–166
- ET *see* essential thrombocythemia
- EUHASS *see* European Hemophilia Safety Surveillance
- European Committee on Blood Transfusion (CD-P-TS) 77
- European Directorate for the Quality of Medicines (EDQM) 76–77
- European Hemophilia Safety Surveillance (EUHASS) 78
- European Medicines Agency (EMA) 76–77

- European Pharmacopeia (PhEur) 77  
 European regulations 75–78  
   albumin 223–224  
   alpha<sub>1</sub>-proteinase inhibitor 228  
   antithrombin III 230  
   Council of Europe 76–77  
   European Union 76  
   factor IX 232  
   factor XI 228–229  
   fibrinogen preparations 231  
   immunoglobulins 226–227  
   medicines safety monitoring 77  
   plasma derivatives and recombinant products 77  
   prothrombin complex concentrate 232  
   von Willebrand factor 233  
 evidence-based guidelines, patient blood management 294–295  
 exceptional medical needs, blood donations 29  
 exchange volumes, therapeutic plasma exchange 260–261  
*Exjade* *see* deferasirox  
 Expert Committee on Biological Standardization (ECBS) 75  
 expiration time, red blood cells 307  
 expression  
   ABO system 81–82, 84, 85  
   I blood group 90–91, 92–93  
   Lewis blood group 88–89  
   LW system 107  
   P blood group system 93–94, 96  
   Rh system 101–104  
   Sd<sup>a</sup> antigen 97–98  
 extended blood group antigen typing 47  
 extended phenotype testing 127–128, 129  
 extracellular requirements, erythroid differentiation 135–136  
 extracorporeal membrane oxygenation (ECMO) 263  
 extracorporeal photopheresis (ECP) 287–289  
   allograft rejection 287–288  
   cutaneous T-cell lymphoma 287  
   graft-versus-host disease 288–289  
 extraembryonic tissue preservation/transplantation 677  
 extrinsic pathway 204  
  
 F-actin, megakaryocytes 160, 161  
 factor II (prothrombin) 201, 437  
 factor IX 201, 204–205  
   hemophilia 431–432  
   purification 232  
 factor V 201  
   deficiency 437–438  
 factor VII deficiency 438–439  
 factor VIII 201, **428–430**  
   cryoprecipitate transfusion 210–211  
   hemophilia 427  
   purification 233–234  
   von Willebrand disease 436–437  
 factor VIII inhibitor bypassing activity (FEIBA) 227–228  
 factor X 201, 204–205  
   deficiency 439  
   purification 227  
 factor XI 201, 204–205  
   deficiency 439–440  
   purification 228–229  
 factor XII 201, 204–205  
 factor XIII  
   cryoprecipitate transfusion 210–211  
   deficiency 440–441  
   purification 227  
 factor-*n*, *see also* recombinant...  
 false positives, infectious disease screening 39  
 familial hypercholesterolemia (FH), apheresis 276  
 fascia lata allografts 675  
 FC *see* fibrinogen concentrates  
 FCR *see* fraction of the preprocedure cells remaining  
 FDA *see* United States Food and Drug Administration  
 febrile nonhemolytic transfusion reactions (FNHTRs) 553–558  
   alloimmunization 555  
   bacterial contamination 555–556  
   diagnosis 556  
   etiology 554  
   leukocyte reduction 557–558  
   prevention 557–558  
   storage-generated cytokines 555  
   treatment 556–557  
 Federal Emergency Management Agency (FEMA) 15  
 FEIBA *see* factor VIII inhibitor bypassing activity  
 Felty syndrome 192  
 FEMA *see* Federal Emergency Management Agency  
 Fenwal Amicus instrument 253  
 ferritin  
   depletion  
     absent iron stores 57  
     adverse outcomes 59–61  
     blood donors 56–62  
     iron-deficient erythropoiesis 57  
     mitigation 61–62  
     monitoring 61–62, 63  
     neurocognitive effects 60  
     pica 60–61  
     pregnancy-related outcomes 60  
     restless legs syndrome 60  
     source plasma donation 63–64  
     vulnerable populations 61  
     monitoring 61–62, 63  
 fetal development  
   I blood group 93  
   P blood group system 96  
 fetal hemoglobin (HbF) inducers 596  
 fetal transfusions 379–380, 420–421  
 fetal typing 105, 379  
 fetal/neonatal alloimmune thrombocytopenia (FNAIT) 379–380  
 FFP *see* fresh frozen plasma  
 FH *see* familial hypercholesterolemia  
 fibrin clot formation 203  
 fibrin stabilization 203  
 fibrinogen 201  
   purification 231  
   viral-inactivated purified concentrate 211  
 fibrinogen concentrates (FC) 211, 304  
   obstetrics 377  
   oncology patients 486  
 fibrinogen disorders 441  
 fibrinolysis 203–208  
   screening tests 204–208  
 Fibrogammaglobulin, purification 227  
 FII *see* prothrombin  
 Filamin A 161  
 filgrastim (Neupogen) 607  
 filgrastim-aafi (Nivestym) 607–608  
 filgrastim-sndz (Zarxio) 607–608  
 filters 311  
 filtration, viruses 221  
 first aliquot diversion 535  
 flaviviruses 517–518  
 Flebogamma DIF, purification 224–225  
 FLI1-related thrombocytopenia 161  
 Flossdorf, E.W. 9  
 flow cytometry, paroxysmal nocturnal hemoglobinuria 360  
 fluids, coadministration 311  
 FNAIT *see* fetal/neonatal alloimmune thrombocytopenia  
 FNHTRs *see* febrile nonhemolytic transfusion reactions  
 focal segmental glomerulosclerosis (FSGS), apheresis 272–273  
 folate deficiencies, macrocytic anemia 138–140  
 fondaparinux 416  
 Food, Drug, and Cosmetic Act (FDCA) 66  
   device regulatory controls 70  
   hospital transfusion services 71  
 Forssman phenotype 95–96  
 Forssman synthase (GBGT1) 96  
 fostamatinib 409  
 fraction of the preprocedure cells remaining (FCR) 278–279  
 frequency-related adverse reactions, blood collection 54

- fresh frozen plasma (FFP)  
 adverse reactions 215  
 bleeding treatment 212–213  
 burn patients 480  
 clinical uses 212  
 combined factor V and factor VIII deficiency 438  
 disseminated intravascular coagulation 213  
 factor V deficiency 438  
 hemophilia 426–427  
 pathogen reduction 495–496  
 preparation 33  
 prophylactic use 213–214  
 prothrombin deficiency 437  
 storage 308  
 therapeutic plasma exchange 214, 262–263  
 trauma patients 480  
 warfarin effect reversal 214
- frozen bone 673
- frozen storage  
 plasma composition effects 203  
 platelets 186  
 red blood cells 156
- FSGS *see* focal segmental glomerulosclerosis
- Fulphilia *see* pegfilgratim-jmdb
- FUT1* 83, 84–85
- FUT2* 81, 82, 89
- FUT3* 88–89
- FV/FVIII *see* combined factor V and factor VIII deficiency
- G proteins, red blood cells 148
- G6PD *see* glucose-6-phosphate dehydrogenase
- galectins 93
- Gammimune N, purification 226
- Gammaguard, purification 224
- Gammunex, purification 224
- gastrointestinal (GI) disorders, Lewis blood group 89–90
- gastrointestinal (GI) infections  
 ABO system 86–87  
 P blood group system 97  
 Sd<sup>a</sup> antigen 99
- gastrointestinal (GI) tissue engineering 653–654
- GATA-binding factor 1 (GATA1) 159, 164
- GATA1 133, 135
- GATA1 *see* GATA-binding factor 1
- GBS *see* Guillain–Barré syndrome
- GBV-C (hepatitis G) 515
- GCNT2 (IGNT) 91–92
- GEF-H1 *see* guanine nucleotide exchange factor H1
- gel column testing 125
- gender, plasma composition 202
- gene editing 644
- gene therapy 642–647  
 administration 642–643  
 adverse effects 644  
 gene editing 644  
 hemophilia 434  
 risks 643–644  
 target selection and insertion 642, 643  
 trials 644–646  
 vaccinations 644  
 vectors 643–644
- genetic regulation, megakaryopoiesis 164
- genetically engineered T cells 634
- genetics  
 hemophilia 424–425  
 I blood group 91–92  
 LW system 107  
 P blood group system 95–96  
 Rh system 101–106
- genotyping  
 human leukocyte antigens 629  
 Rh system 105–106
- Gerbich blood group system 115, 329
- gestational thrombocytopenia (GT) 373–375
- GFI1 *see* growth factor interdependent 1
- GI *see* gastrointestinal
- GIL blood group system 114–115
- Glanzmann thrombasthenia 161
- GLOB 93
- glucose metabolism, red blood cells 145–148
- glucose-6-phosphate dehydrogenase (G6PD) deficiency 328–329, 337–338
- glutaminolysis, red blood cells 148
- glutathione, red blood cells 143, 148
- glycerolizing, red blood cells 156
- glycolysis 145
- glycophorin A and B *see* MNS blood group system
- glycophorin C and D *see* Gerbich blood group system
- glycosphingolipids (GSLs), P system 93–97
- glycosylphosphatidylinositol (GPI)-anchored blood groups 115–116
- good manufacturing practices 67–68
- Goodpasture syndrome (GPS), apheresis 272
- GPI *see* glycosylphosphatidylinositol
- GPS *see* Goodpasture syndrome
- graft-versus-host disease (GVHD), extracorporeal photopheresis 288–289
- Granix *see* TBO-filgrastim
- granulocytes 195–199  
 administration, oncology patients 487  
 adverse reactions to collection 54–55  
 alloimmunization 487  
 apheresis 195  
 clinical recommendations 199
- collection 194–195, 487  
 donor selection 194–195  
 indications 486  
 modification 487  
 nonhemolytic transfusion reactions 566  
 pediatric transfusions 198, 199  
 prophylactic transfusions 198  
 stimulation 195  
 storage 195, 308, 487  
 transfusion 195–198
- Group A *see* ABO system
- Group B *see* ABO system
- Group O, discovery of universality 8
- growth factor interdependent 1 (GFI1) 164
- GSLs *see* glycosphingolipids
- GT *see* gestational thrombocytopenia
- GTA *see* A-transferase
- GTB *see* B-transferase
- guanine nucleotide exchange factor H1 (GEF-H1) 164
- guanine nucleotides, red blood cells 148
- guidance documents, FDA, blood collection establishments 68–69
- guidelines  
 perioperative practice 464–466  
 red blood cell transfusions 322–324
- Guillain–Barré syndrome (GBS)  
 apheresis 261, 266–267  
 immunoglobulin therapy 245–246
- GVHD *see* graft-versus-host disease
- H antigen 81
- H gene 84–85
- H. pylori* *see* *Helicobacter pylori*
- H5N1 pandemic influenza 521
- HAA *see* hospital-acquired anemia
- Haemonetics MCS+ instrument 254
- Haemonetics PCS-2 instrument 254
- HAM test (acidified serum test) 360
- haplotypes, human leukocyte antigens 629
- Harvey, W. 3
- Hashimoto's encephalopathy, apheresis 269
- Hasse, O. 7
- HAV *see* hepatitis A
- HBV *see* hepatitis B
- HCV *see* hepatitis C
- HDFN *see* hemolytic disease of the fetus and newborn
- HDIVIG *see* high-dose intravenous immunoglobulin
- HDV *see* hepatitis D
- HE *see* hereditary elliptocytosis
- health emergencies 14–23  
 absenteeism 16  
 containment 22  
 continuity of operations plans 17, 17
- COVID-19 14, 16, 18–23

- diagnostic testing 22  
 epidemiological surveillance 22  
 mitigation 22  
 operations organization 15–16  
 pandemic management 21–23  
 preparedness 14–15, 16–17  
 records management 18  
 regulatory issues 17–18, 21–23  
 therapeutics 23  
 vaccines 23  
*see also* disaster management
- heat treatment, virus inactivation 220  
 heavy chains 119  
*Helicobacter pylori* (*H. pylori*) 90  
**HELLP syndrome** *see* hemolysis, elevated liver enzymes, and low platelets (HELLP) syndrome
- hemagglutinins, levels allowable in immunoglobulin 226  
**hemapheresis** *see* apheresis; blood collection; blood components; plasma; source plasma  
**hematologic disorders, therapeutic plasma exchange** 269–272  
**hematomas, blood donation** 52  
**hematopoiesis**  
 acute radiation syndrome 611  
 Duffy protein 113  
**hematopoietic development, I blood group** 93  
**hematopoietic growth factors** 601–606, 601–616  
 erythroid 603–606  
 general principles 601–603  
 myeloid 606–611  
 thrombopoietic 611–616  
**hematopoietic progenitor cells (HPC), adverse reactions** 487–488  
**hematopoietic stem cell transplants (HSCT)**  
 oncology patients 482–483, 487–488  
 paroxysmal nocturnal hemoglobinuria 362  
 thalassemia 336  
**hematopoietic stem cells (HSC)** 617–623  
 ABO typing 620  
 collection 620–621  
 cord blood 621  
 COVID-19 622–623  
 cryopreservation 620–621  
 differentiation 133  
 dimethyl sulfoxide toxicity 565–566  
 erythropoiesis 133–134  
 infusion 622  
 megakaryopoiesis 158–160  
 microenvironment 617–619  
 mobilization 619–620  
 peripheral blood 620  
 regulations 623  
 storage 308  
 thawing 621–622
- hemoglobin**  
 biosynthesis 135  
 gene editing 645  
 structural mutations 327–328  
**hemoglobin C** 327–328  
**hemoglobin E** 328  
**hemoglobin S** 47, 327  
**hemolysis, mechanism** 122  
**hemolysis, elevated liver enzymes, and low platelets (HELLP) syndrome** 373–374  
**hemolytic disease of the fetus and newborn (HDFN)** 106, 364–372  
 ABO system 85, 364–365  
 anemia 371  
 breast milk 371  
 care of newborn 368–371  
 care of sensitized pregnancies 368  
 D alloimmunization prevention 367–368  
 exchange transfusions 370–371  
 high-dose intravenous immunoglobulin 370, 378  
 hyperbilirubinemia 368–369  
 I blood group 92  
 immunology 365  
 Lutheran blood group 111  
 monitoring and management 365–368  
 necrotizing enterocolitis 371  
 phototherapy 369–370  
 primary prevention 367  
 simple transfusion 371  
 unexpected red blood cell antibodies 365  
**hemolytic transfusion reactions** 543–552  
 bystander hemolysis 547  
 causes 544–545  
 clinical features 547–550  
 complement 545–547  
 complications 550  
 diagnosis 547–550  
 immunoglobulin therapy 242–243  
 incidence 543–544  
 macrophages 547  
 pathophysiology 545–547  
 prevention 551–552  
 reticulocyte suppression/destruction 547  
**hemolytic-uremic syndrome (HUS)** 374–375  
 P blood group 97  
**hemophilia** 424–434  
 antifibrinolytics 432  
 clinical features 425  
 cryoprecipitate 426–427  
 cyclooxygenase 2 inhibitors 432  
 desmopressin 432  
 factor IX 431–432  
 factor VIII 427, **428–430**  
 gene editing 645  
**gene therapy** 434  
**genetics** 424–425  
**immune tolerance induction** 433–434  
**inhibitory antibodies** 432–433  
**management** 426–434  
**prophylaxis** 427  
**vaccinations** 432  
**hemorrhage protocols, obstetric** 376–377  
**hemostasis**  
 patient blood management 302–304  
 plasma 203  
**hemostatic testing, preoperative** 457–459  
**hemovigilance** 503–506  
 data analysis and assessment 504–506  
 events not captured 505  
 reporting systems 530–534  
**heparin** 251  
**heparin re-exposure** 417  
**heparin-induced thrombocytopenia (HIT)** 394, 413–417  
 argatroban 415–416  
 bivalirudin 416  
 contraindications 416–417  
 denaparoid sodium 416  
 fondaparinux 416  
 high-dose intravenous immunoglobulin 414–415  
 iceberg model 414  
 management 414–417  
**hepatic toxicity, thrombopoietic growth factors** 614  
**hepatitis A (HAV)** 507–508  
 discovery 9–10  
 neutropenia 192  
 testing 47  
**hepatitis B (HBV)** 508–511  
 diagnosis 510–511  
 discovery 9–10  
 neutropenia 192  
 pathogenesis 506–507  
 polyarteritis nodosa 272  
 testing 43–44  
 transmission 509–510  
 treatment 511  
**Hepatitis B surface antigen (HBsAg)** 44  
**hepatitis C (HCV)** 511–513  
 testing 44  
 thrombopoietic growth factors 614  
 treatment 511–513  
**hepatitis D (HDV)** 513–514  
**hepatitis E (HEV)** 514  
**hepatitis F** 515  
**hepatitis G (GBV-C)** 515  
**hepcidin mimetics** 596  
**hereditary elliptocytosis (HE)** 339  
**hereditary hemochromatosis (HH)**,  
 apheresis 284–285  
**hereditary neutropenias** 191–192  
**hereditary pyropoikilocytosis (HPP)** 339  
**hereditary spherocytosis** 338–339

- hereditary stomatocytoses 339  
 hereditary xerocytosis (HX) 339  
*HES* *see* hydroxyethyl starch  
*HEV* *see* hepatitis E  
 hexose monophosphate shunt (pentose shunt) 146, 147  
*HH* *see* hereditary hemochromatosis  
*HHVs* *see* human herpesviruses  
*HIFs* *see* hypoxia-inducible transcription factors  
 high-dose intravenous immunoglobulin (HDIVIG)  
   hemolytic disease of the fetus and newborn 370, 378  
 heparin-induced thrombocytopenia 414–415  
   intravenous immunoglobulin 417  
   posttransfusion purpura 421  
 histocompatibility testing *see* compatibility testing  
*HIT* *see* heparin-induced thrombocytopenia  
*HLA* *see* human leukocyte antigen; human leukocyte antigens  
*HNA* *see* human neutrophil antigens  
 homology, Rh proteins 105  
 homozygosity, Rh system 105, 106  
 hormones, plasma composition 202–203  
 hospital transfusion services  
   accreditation 73  
   adverse reaction reporting 70–73  
   defective product reporting 72  
   pretransfusion testing 122–130  
   sample collection 123–124  
   transfusion-related death reporting 71  
   United States regulations 70–73  
   World Health Organization programs 74–75  
 hospital-acquired anemia (HAA) 461  
 hospital-based tissue services 679  
*HPA1a* (PIA1) specific antigen, testing 47  
*HPAs* *see* human platelet antigens  
*HPP* *see* hereditary pyropoikilocytosis  
*HSC* *see* hematopoietic stem cells  
*HTLV* *see* human T-cell lymphotropic virus  
 human herpesviruses (HHVs) 519  
 human immunodeficiency virus (HIV)  
   515–517  
   antibody testing 43  
   confirmatory testing 43  
   erythroid growth factors 605  
   identification 10  
   neutropenia 192  
   nucleic acid tests 31, 43  
   P blood group 96  
   type 1 515–516  
   type 2 516–517  
 human leukocyte antigen (HLA)-matched platelet transfusions 172–173, 399–400  
 human leukocyte antigens (HLA) 624–632  
   antibody identification 630–632  
   antigens 625, 628  
   biological functions 625–626, 629  
   cross-reactive groups 172–173  
   disease associations 631–632  
   epitopes 631  
   genotyping 629  
   major histocompatibility complex 624–626  
   medical significance 629  
   next-generation sequencing 628  
   nomenclature 626–627  
   pharmacogenetics 632  
   phenotypes 629  
   platelets 168–169, 399–400  
   polymorphism 626–627  
   sequence-specific oligonucleotide probe hybridization 628  
   testing 47, 628–632  
   TRALI 575–576  
   transplantation 630–632  
 human neutrophil antigens (HNA), TRALI 574–575  
 human platelet antigens (HPAs) 169–170, 399–400  
 human T-cell lymphotropic virus (HTLV)  
   516–517  
   testing 44  
 human tissue allografts 670–683  
   ABO typing 672–673  
   acellular dermal matrix 675  
   adverse event monitoring 682  
   bone 673–674  
   cardiac 676–677  
   connective tissues 674–675  
   donor–recipient matching 672–673  
   general principles 670–672  
   ocular tissue 675–676  
   peripheral nerves 677  
   recalls and lookback 682–683  
   reimbursement 683  
   skin 675  
   surveillance 680–682  
   tissue processing 680  
   vascular tissue 676–677  
*HUS* *see* hemolytic uremic syndrome; hemolytic-uremic syndrome  
*HX* *see* hereditary xerocytosis  
 hydroxyethyl starch (HES), therapeutic plasma exchange 263  
 hyperbilirubinemia 368–369  
 hypergammaglobulinemia, apheresis 270–271  
 hyperhemolysis 343–344  
 hyperimmune globulins 247–248  
 hyperleukocytosis, apheresis 286  
 hypocalcemia 257, 266  
 hypodysfibrinogenemia 441–442  
 hypofibrinogenemia 441  
 hypokalemia 257  
 hypotension  
   apheresis 257  
   transfusion reactions 565  
 hypothermia 563  
 hypoxia, erythropoietin regulation 136–137  
 hypoxia-inducible transcription factors (HIFs) 136  
 I blood group 90–93  
   biochemistry 91  
   biological roles 92–93  
   expression 90–91, 92–93  
   molecular biology 91–92  
   serology 90–91  
   transfusions 92  
 I-null ( $i_{adult}$ ) phenotype 91  
*IAT* *see* indirect antiglobulin test  
*IC* *see* informed consent  
*ICAM-4* 107  
 iceberg model, heparin-induced thrombocytopenia 414  
*ICL670Q* *see* deferasirox  
 idarucizumab 450  
*IDE* *see* iron-deficient erythropoiesis  
*IGF1* *see* insulin-like growth factor-1  
*IGNT* (GCNT2) 91–92  
*IL11* *see* interleukin-11  
 immature platelet fraction (IPF) 167  
 immediate, acute adverse reactions, blood donation 50  
 immune responses 118–122  
   affinity maturation 119–120  
   antigen receptors 118  
   antigen-presenting cells 119  
   clonal selection 120  
   clonality 120  
   immunoglobulins 118–120  
   primary 119  
   secondary 119  
   somatic hypermutation 120  
   T-cell independent 119  
 immune risk factors  
   ABO system 86–87  
   Lewis blood group 90  
   P blood group 93–94, 96–97  
   Sd<sup>a</sup> antigen 99  
 immune thrombocytopenic purpura (ITP)  
   393, 406–411  
   children 410–411  
   clinical features 406–407  
   danazol 410  
   emergency treatment 410  
   fostamatinib 409  
   immunoglobulin therapy 246  
   immunosuppression 409–410  
   intravenous immunoglobulin 407–408  
   monoclonal antibodies 409  
   pathogenesis 406  
   platelet lifespan 165–167  
   pregnancy 373–374, 410

- preoperative management 410  
preplatelet maturation 160–161  
reticulated platelets 166–167  
Rh immune globulin 408  
splenectomy 408–409  
thrombopoietic growth factors 614  
thrombopoietin receptor agonists 409  
treatment 407–411  
vinca alkaloids 410  
*immune tolerance induction (ITI),*  
  hemophilia 433–434  
*immune-mediated thrombocytopenia*  
  402–423  
    alloimmune 417–422  
    drug-induced 411–417  
    heparin-induced 413–417  
    immune thrombocytopenic purpura  
      406–411  
    laboratory tests 402–406  
    passive 421–422  
    posttransfusion purpura 421  
    refractoriness 422  
    vaccine-induced 417  
    *see also* immune thrombocytopenic  
      purpura; *individual disorders...*  
*immunoabsorption, therapeutic plasma*  
  processing 275–276  
*immunocompromised patients,*  
  TA-GVHD 584  
*immunodeficiency syndromes, TA-GVHD*  
  583–584  
*immunoglobulin A (IgA), deficiency*  
  testing 47  
*immunoglobulin G (IgG)*  
  adverse reactions 242–243  
  affinity maturation 119–120  
  antibody structure 120  
  antiglobulin test 122  
  apheresis 260–261  
  depletion, source plasma donation 63  
  in plasma 201  
  primary immune responses 119  
  purification 238–240  
  secondary immune responses 119  
  transfusion 240–248  
*immunoglobulin M (IgM)*  
  antibody structure 120  
  antiglobulin test 122  
  in plasma 201  
  primary immune responses 119  
  T-cell independent response 119  
*immunoglobulins*  
  adverse reactions 242–243  
  affinity maturation 119–120  
  anticomplementary activity 226  
  autoimmune diseases 241, 245–247  
  autoimmune mucocutaneous bullous  
    diseases 247  
  chronic inflammatory demyelinating  
    polyneuropathy 246  
  complement scavenging 244  
conventional-dose therapy 240  
dosing and scheduling 244–247  
drug interactions 241  
European standards 226–227  
Guillain–Barré syndrome 245–246  
hyperimmune globulins 247–248  
immune thrombocytopenic  
  purpura 246  
indirect actions 244  
inflammatory diseases 241, 245–247  
Kawasaki disease 245  
mechanism of action 243–244  
multifocal motor neuropathy 246–247  
oncology patients 486  
pathogen reduction 240, 496  
plasma 201  
primary immune deficiencies 244–245  
products 236–248  
purification 224–227, 238–240  
secondary immune deficiencies 245  
structure 118–119, 236–238, 238  
subcutaneous therapy 240–241  
transfusion-related acute lung  
  injury 576  
transplantation 247  
warm autoimmune hemolytic  
  anemia 350  
  *see also* antibodies  
*immunohematology* 118–130  
  ABO typing 124  
  affinity maturation 119–120  
  agglutination 120–122  
  antibody–antigen interactions  
    120–122  
  antiglobulin tests 122, 124–125  
  automated testing 125–127, 128–129  
  compatibility testing 122–130  
  crossmatching 128–129  
  hemolysis 122  
  Rh typing 124–125  
*immunosuppression*  
  immune thrombocytopenic purpura  
    409–410  
  paroxysmal nocturnal  
    hemoglobinuria 362  
  warm autoimmune hemolytic  
    anemia 350  
*immunotherapy*  
  cancer 633, 636–637  
  chimeric antigen receptor T-cell  
    therapy 634, 635–637  
  dendritic cells 638  
  donor lymphocyte infusions 633–634  
  genetically engineered T cells 634  
  mesenchymal stromal cells 639  
  regulatory T cells 634  
  tumor-infiltrating lymphocytes 634  
  viral infections 637–638  
*implementation, patient blood*  
  management 293–294  
*in vivo recovery, red blood cells* 156–157  
in-date products, retrieval upon positive  
  disease tests 42  
incidence *see* prevalence  
Indian blood group system 115  
indications  
  activated prothrombin complex  
    concentrate 444  
  andexanet alfa 450  
  antifibrinolytics 447–448  
  cell salvage 301  
  cell-based immunotherapies 308  
  chimeric antigen receptor T-cell therapy  
    636–637  
  cryoprecipitate 308  
  desmopressin 448  
  granulocytes 308, 486  
  hematopoietic stem cells 308  
  idarucizumab 450  
  myeloid growth factors 606–609  
  plasma therapy 308  
  platelet therapy 307, 484  
  protamine 449  
  prothrombin complex concentrates 308,  
    443–444  
  recombinant activated factor VII 445  
red blood cells 324  
  sickle cell disease 331–335, 332  
  thalassemia 335–337  
sickle cell disease 331–335, 332  
thalassemia 335–337  
thrombopoietic growth factors 614–615  
vitamin K 446  
indirect actions, immunoglobulins 244  
indirect antiglobulin test (IAT) 122, 124  
*individual donor (IND) testing*  
  HBV 43  
  HIV 43  
infants 381–391  
  anemia 381–382  
  coagulation system development  
    388–389  
  cryoprecipitate transfusions 390–391  
  granulocyte transfusions 198, 199  
  paroxysmal nocturnal  
    hemoglobinuria 363  
  plasma transfusions 388–390  
  platelet transfusions 385–388  
  preterm 382–383  
  red blood cell administration 322  
  red blood cell transfusions 382–383,  
    384–385  
  TRALI 573  
  *see also* children; neonates  
*infections*  
  ABO system 86–87  
  Lewis blood group 90  
  neutropenia-causing 192–193  
  P blood group 93–94, 96–97  
  Sd<sup>a</sup> antigen 99  
  *see also* transfusion-transmitted  
    infections

- inflammatory diseases, immunoglobulin therapy 241, 245–247
- inflammatory disorders, Lewis blood group 89–90
- influenza
- pandemics 521
  - Sd<sup>a</sup> antigen 99
- informed consent (IC)
- donors 31–32
  - recipients 129
- inherited bleeding disorders 424–442
- afibrinogenemia 441
  - combined factor V and factor VIII deficiency 438
  - dysfibrinogenemia 441–442
  - factor V deficiency 438
  - factor VII deficiency 438–439
  - factor X deficiency 439
  - factor XI deficiency 439–440
  - factor XIII deficiency 440–441
  - fibrinogen disorders 441
  - hemophilia 424–434
  - hypodysfibrinogenemia 441–442
  - hypofibrinogenemia 441
  - inherited bleeding disorders 424–442
  - pregnancy 375
  - prothrombin deficiency 437
  - von Willebrand disease 434–437
- inhibition, fibrinolysis 230
- inhibitory antibodies, hemophilia 432–433
- initiation, chelation 594
- iNKTs *see* invariant natural killer cells
- innate immunity, ABO system 87
- inosine 146–147
- INR *see* internationalized normalized ratio
- insertional mutagenesis 644
- inspections, blood collection
- establishments 69
- insulin-like growth factor-1 (IGF1) 136
- integrins, LW system 107
- intensification of chelation 595
- interleukin-11 (IL11), oncology
- patients 486
- International Plasma Fractionation Association (IPFA) 78
- International Quality Plasma Program (IQPP) 34, 65
- International Society of Blood Transfusion (ISBT) 65, 78
- internationalized normalized ratio (INR) 457–458
- intracellular requirements, erythroid differentiation 135
- intravenous immunoglobulin (IVIG)
- adverse reactions 242–243
  - anticomplementary activity 226
  - autoimmune mucocutaneous bullous diseases 247
  - chronic inflammatory demyelinating polyneuropathy 246
  - conventional dose therapy 240
  - dosing 244–247
  - drug interactions 241
  - drug-induced immune hemolytic anemia 357–358
  - European standards 226–227
  - Guillain–Barré syndrome 245–246
  - hemolytic disease of the fetus and newborn 370, 378
  - heparin-induced thrombocytopenia 414–415
  - high-dose therapy 241
  - hyperimmune globulins 247–248
  - immune thrombocytopenic purpura 246, 407–408
  - Kawasaki disease 245
  - mechanism of action 243–244
  - multifocal motor neuropathy 246–247
  - oncology patients 486
  - pathogen reduction 496
  - posttransfusion purpura 421
  - primary immune deficiencies 244–245
  - scheduling 244–247
  - secondary immune deficiencies 245
  - transplantation 247
  - vaccine-induced immune thrombotic thrombocytopenia 417
  - warm autoimmune hemolytic anemia 350
- intravenous (IV) access, venipuncture 307
- intrinsic pathway 204
- invariant natural killer cells (iNKTs) 82, 96
- inverse transfusion-related acute lung injury 576, 579
- IPF *see* immature platelet fraction
- IPFA *see* International Plasma Fractionation Association
- IQPP *see* International Quality Plasma Program
- iron burden
- measurement 590–592
  - transfusional 589–590
- iron deficiency anemia (IDA) 56–64, 140–141
- adverse outcomes 59–61
  - mitigation 61–62
  - neurocognitive effects 60
  - pica 60–61
  - pregnancy-related outcomes 60
  - prevalence in donors 56–58
  - regulation 58–59
  - restless legs syndrome 60
  - risk factors 56–58
  - vulnerable populations 61
- iron depletion
- adverse outcomes 59–61
  - blood donors 56–62
  - mitigation 61–62
  - monitoring 61–62
  - neurocognitive effects 60
  - pica 60–61
  - pregnancy-related outcomes 60
- prevalence 56–58
- regulation 58–59
- restless legs syndrome 60
- risk factors 56–58
- vulnerable populations 61
- source plasma donors 63–64
- iron overload 344–345, 587–597
- chelation 592–596
  - clinical features 590
  - erythroid maturation agents 596
  - fetal hemoglobin inducers 596
  - hepcidin mimetics 596
  - monitoring 345
  - novel approaches 596
  - pathophysiology 587–589
  - prevention 281
  - pyruvate kinase activators 596
  - transfusional iron burden 589–590
  - treatment 345, 592–596
- iron supplementation, donors 61
- iron-deficient erythropoiesis (IDE) 57
- irradiation
- blood components 155, 186, 221, 309–310
  - plasma 221
  - platelets 186, 485
  - red blood cells 155, 483
- ISBT *see* International Society of Blood Transfusion
- issuance
- blood components 307
  - cell-based immunotherapies 308
  - cryoprecipitate 308
  - granulocytes 308
  - hematopoietic stem cells 308
  - plasma 308
  - prothrombin complex concentrates 308
  - recombinant clotting factors 308
- issue dates 129
- ITI *see* immune tolerance induction
- ITP *see* immune thrombocytopenic purpura
- IVIG *see* intravenous immunoglobulin
- Jacobsen syndrome 161
- JR blood group system 116
- Kawasaki disease, immunoglobulin therapy 245
- Kell antigen, testing 47
- Kell blood group system 111–112
- Kidd blood group system 113–114
- kinetics
- platelet production 165–167
  - red blood cell production 138
- kininogen 204–208
- KIVOG, purification 224
- KLF-1 *see* Krüppel-like factor-1
- Knops blood group system 115, 330
- Kostmann syndrome 191
- Krüppel-like factor-1 (KLF-1) 134, 135

- L2 *see* deferiprone  
 LacCer *see* lactosylceramide  
 lactosylceramide (LacCer) 94  
 LAD *see* leukocyte adhesion deficiency  
 Lambert–Eaton myasthenic syndrome (LEMS), apheresis 267  
 LAN blood group system 116  
 Landsteiner, K. 7  
 large volume delayed sampling 537  
*LCMV* *see* lymphatic choriomeningitis  
 LDL apheresis 276  
*leishmaniasis* 527–528  
 LEMS *see* Lambert–Eaton myasthenic syndrome  
 lentiviruses, HIV 10, 31, 43, 96, 192, 515–517  
 leukapheresis 54–55, 195  
 leukemia, chimeric antigen receptor T-cell therapy 636  
 Leukine *see* sargramostim  
 leukocytapheresis 286  
 leukocyte adhesion deficiency (LAD) syndromes 189  
 leukocyte reduction (LR) 33, 309, 557–558 platelets 186, 485 red blood cells 155, 483  
 Lewis blood group 87–90, 330 biochemistry 87–88 biological roles 89–90 biosynthesis 87–88 molecular biology 88–89 nonsecretors 89 serology 87 transfusion considerations 89  
 Lewis gene (*FUT3*) 88–89  
 lifespan neutrophils 189 platelets 165–167  
 ligament allografts 674–675  
 light chains 119  
 liver biopsy plasma administration 467 platelet administration 469  
*LKE* *see* luke antigen on erythrocytes loiasis, apheresis 289 lookback 42, 683 low income countries, obstetrics 378 Lower, R. 4  
 LR *see* leukocyte reduction luke antigen on erythrocytes (LKE) phenotype 93–94, 95, 96  
 lumbar puncture plasma administration 468 platelet administration 470 lung allografts, rejection, extracorporeal photopheresis 288 lung tissue engineering 648–650 lusutrombopag (Mulpleta) 613 Lutheran blood group system 111 LW blood group system 107 lymphatic choriomeningitis (LCMV) 521
- lymphocytes cancer treatment 633, 636–637 chimeric antigen receptor-expressing 634, 635–637 donor infusions 633–634 genetically engineered 634–637 infusions 633–634 mesenchymal stromal cells 639 natural killer cells 638–639 regulatory T cells 634 tumor-infiltrating 634 viral infections 637–638
- lyophilization bone 673–674 development of 9 platelets 186–187
- M blood group system, discovery 8 macrocytic anemia 138–140 macrophages, hemolytic transfusion reactions 547 macrothrombocytopenia syndromes, megakaryopoiesis pathologies 161 major histocompatibility complex 624–626 major obstetric bleeding 375–377 malaria 326 ABO system 86, 330 red cell exchange 281–282 transfusion-transmitted 526 malignancies chimeric antigen receptor T-cell therapy 636–637 thrombocytopenia 394 *see also* cancer; oncology patients management hemolytic transfusion reactions 550 PBM programs 293–305 preoperative anemia 297–299 *see also* treatment manual exchange transfusion, protocol 341 manufacturing activated prothrombin complex concentrate 227–228, 444 albumin 222–224 alpha<sub>1</sub>-proteinase inhibitor 228 andexanet alfa 450 antifibrinolitics 447 antithrombin III 230 C1 inhibitor 229–230 desmopressin 448 factor IX 232 factor VIII 233–234 factor X 227 factor XI 228–229 factor XIII 227 fibrinogen 231 immunoglobulins 224–227, 238–240 protamine 449 prothrombin complex concentrate 231–232
- prothrombin complex concentrates 443 recombinant activated factor VII 445 vitamin K 446 von Willebrand factor 232–233
- mass casualty events administration 478 preparedness 16 massive transfusion protocols (MTP) complications 561–564 obstetrics 376–377 maternal thrombocytopenia 374–375 matrix metalloproteinases (MMP), platelet production 160, 165 maturation, megakaryocytes 159–161 May–Hegglin anomaly 161 MB *see* methylene blue MDDDS *see* Medical Device Data Systems Medical Device Data Systems (MDDDS) 70 medication coadministration 311–312 medicines safety monitoring, Europe 77 megakaryocyte-erythroid precursors (MEPs) 159 megakaryocytes (MKs) differentiation 158–165 maturation 159–161 migration 158–159 platelet formation 161–162 megakaryocytic-erythroid progenitor (MEP) 133–134 megakaryopoiesis 158–165 biophysical niche forces 164–165 cytokines 162–164 differentiation 158–161 genetic regulation 164 pathologies 161–162 platelet formation 161–162 proliferation 158 regulation 162–165 thrombopoietin 158–160, 162–164 melanoma, I blood group 93 membrane complex, Rh system 104–105 membrane elongation, protoplatelets 160 membrane metabolism, red blood cells 149–150 membrane mutations, red blood cells 329, 338–339 membrane topology, Rh system proteins 102 men who have sex with men (MSM), screening 31 meniscus allografts 674 mental stress, plasma composition 202–203 MEP *see* megakaryocytic-erythroid progenitor MEPs *see* megakaryocyte-erythroid precursors MER2 116 mesenchymal stem cells, I blood group 93 mesenchymal stromal cell (MSC) immunotherapy 639

- mesenchymal–epithelial transition (MET) 89, 93, 96
- MET *see* mesenchymal–epithelial transition
- metabolic alkalosis 257, 266
- metabolism
- platelets 181–183
  - red blood cells 143–150
- methoxy polyethylene glycol-epoetin beta (Micera) 604
- N-methyl-D-aspartate receptor (NMDAR) antibody encephalitis 267
- methylene blue (MB) pathogen reduction 493, 496, 500
- MGUS *see* monoclonal gammopathy of unknown significance
- Micera *see* methoxy polyethylene glycol-epoetin beta
- microaggregate debris 563
- microangiopathic hemolytic anemias 393–394
- microcirculatory effects, anemia 316–317
- microcytic anemia 140–141
- microfilariasis 528–529
- microtubules, protoplatelets 160
- migration
- megakaryocytes 158–159
  - neutrophils 189
- milk, as a blood substitute 7
- mini physical exam 32, 34–35
- minipools, HIV testing 43
- minor blood group antigens
- DNA-based typing 127–128, 129
  - see also* protein blood groups
- mitigation
- disaster management 14–15
  - donation-related iron depletion 61–62, 63
  - health emergencies 22
- mixed autoantibody hemolytic anemia 355–356
- MKL1 and MKL2 *see* myocardin-like transcription factors
- MM *see* multiple myeloma
- MMN *see* multifocal motor neuropathy
- MMP *see* matrix metalloproteinases
- MNS blood group system 109–111, 110, 330
- discovery 8
- mobilization
- hematopoietic stem cells 619–620
  - neutrophils 188–189
  - peripheral blood progenitor cells 610–611
- modern transfusion
- beginnings 5–7
  - twentieth century 7–8
- modifications
- aliquoting 310
  - granulocytes 487
  - irradiation 155, 186, 221, 309–310
- leukocyte reduction 33, 155, 186, 309
- platelets 185–186, 485
- red blood cells 483
- washing 155, 186, 310
- molecular biology
- ABO system 83–85
  - human leukocyte antigens 625–626
  - I blood group 91–92
  - Lewis blood group 88–89
  - P blood group 95–96
  - prions 529–530
  - Sd<sup>a</sup> antigen 97–98
- molecular structure
- ABO antigens 81–84
  - antibodies 120
  - Diego antigens 110, 114
  - Duffy protein 112–113
  - I group antigens 91
  - immunoglobulins 118–119, 236–238, 238
  - Kell–XK complex 111–112
  - Lutheran antigens 111
  - MNS antigens 110
  - P group antigens 93–94
  - Rh antibodies 107
  - Rh proteins 100–105
- monitoring
- chelation 595–596
  - hemolytic disease of the fetus and newborn 365–368
  - human tissue allografts 680–683
  - iron overload 345
  - physicians 295–297
  - red blood cell administration 341–342
- monoclonal antibodies 120
- cold agglutinin disease 353
  - immune thrombocytopenic purpura 409
- interference in pretransfusion testing 127
- paroxysmal nocturnal hemoglobinuria 361–362
- warm autoimmune hemolytic anemia 350
- monoclonal gammopathy of unknown significance (MGUS), apheresis 268
- monocytes, transfusion-related acute lung injury 574
- MonoMAC syndrome 192
- MPL gene 161
- MPPs *see* multipotent progenitors
- MS *see* multiple sclerosis
- MSC *see* mesenchymal stromal cell
- MSM *see* men who have sex with men
- MTP *see* massive transfusion protocols
- mucinous A 82
- Mudd, S. 9
- Mulpleta *see* lusutrombopag
- multicomponent collections, apheresis 253, 256
- multifocal motor neuropathy (MMN), immunoglobulin therapy 246–247
- multiple component collection, adverse reactions 54
- multiple myeloma, apheresis 273
- multiple sclerosis (MS), apheresis 268
- multipotent progenitors (MPPs) 133
- Murphy, W.P. 9
- mushroom poisoning, apheresis 273
- myasthenia gravis, apheresis 267
- myelodysplastic syndrome (MDS) 615
- myeloid growth factors 606–611
- adverse effects 608–609
  - indications 606–609
- myeloid transcription factors 133, 135
- Myelokathexis 191
- myeloma cast nephropathy, apheresis 273
- myocardin-like transcription factors (MKL1/MKL2) 164
- N blood group system
- discovery 8
  - see also* MNS blood group system
- NAIT *see* neonatal alloimmune thrombocytopenia
- NAT *see* nucleic acid testing
- NATA *see* Network for Advancement of Transfusion Alternatives
- national authorities, disaster management 18
- National Blood Collection and Utilization Survey (NBCUS) 29
- national competent authorities (NCAs) 77
- National Regulatory Authorities (NRAs) 75
- National Response Framework (NRF) 16
- natural killer (NK) cell immunotherapy 638–639
- NBCUS *see* National Blood Collection and Utilization Survey
- NCAs *see* national competent authorities
- NEC *see* necrotizing enterocolitis
- necrotizing enterocolitis (NEC)
- hemolytic disease of the fetus and newborn 371
  - Lewis blood group 89–90
- neonatal alloimmune neutropenia 192
- neonatal alloimmune thrombocytopenia (NAIT) 29, 385, 395, 419–421
- neonatal treatment 420
  - prenatal management 420–421
- neonatal thrombocytopenia 385
- neonates
- hyperbilirubinemia 368–369
  - I blood group 90–93
  - phototherapy 369–370
  - testing, HDFN 368
  - TRALI 573
- nerve injuries, blood donation 53
- NETosis 189–190

- Network for Advancement of Transfusion Alternatives (NATA) 293
- Neulasta *see* pegfilgrastim
- Neupogen *see* filgrastim
- neurocognitive effects, iron deficiency anemia 60
- neurological disorders, therapeutic plasma exchange 266–269
- neuromyelitis optica spectrum disorder (NMOSD), apheresis 268–269
- neurosurgical procedures
- plasma administration 468
  - platelet administration 470
- neutropenias 190–193
- adult 192–193
  - congenital 191–192
  - medications causing 192
  - myeloid growth factors 609–611
- neutrophilia 190
- neutrophils 188–193
- clearance 189
  - kinetics 188–190
  - migration 189
  - mobilization 188–189
  - NETosis 189–190
  - production 188
  - transfusion-related acute lung injury 573–574
- next-generation sequencing (NGS) 628
- NGS *see* next-generation sequencing
- nineteenth century
- animal-to-human transfusions 7
  - anticoagulants 6–7
  - modern transfusion development 5–7
  - saline solutions 7
- nitric oxide, synthesis, red blood cells 149
- Nivestym *see* filgrastim-aafi
- NK *see* natural killer
- NMDAR *see* N-methyl-D-aspartate receptor
- NMOSD *see* neuromyelitis optica spectrum disorder
- nomogram 35
- nonantibody transfusion-related acute lung injury 576, 579
- nonengineered T-cell
- immunotherapies 633–634
- nonhematopoietic cells, apheresis 289
- nonhemolytic transfusion reactions
- 553–568, **567**
  - allergic responses 558–561
  - anaphylaxis/anaphylactoid reactions 559–561
  - autologous transfusions 566
  - circulatory overload 215, 310, 563–564
  - dimethyl sulfoxide toxicity 565–566
  - electrolyte disorders 562–563
  - febrile 553–558
  - granulocytes 566
  - hypotensive 565
  - hypothermia 563
- microaggregate debris 563
- plasticizer toxicity 565
- nonsecretors, Lewis blood group 89
- nonsteroidal anti-inflammatory drugs (NSAIDs), thrombocytopenia 394
- NOR phenotype 95
- Nplate *see* Romiplostim
- NRAs *see* National Regulatory Authorities
- NRF *see* National Response Framework
- NSAIDs *see* nonsteroidal anti-inflammatory drugs
- nuclear agent attacks 16
- nucleic acid testing (NAT) 39
- babesiosis 45
  - bacterial contamination 538
  - HBV 44
  - HCV 44
  - HIV 43
  - HIV screening 31
  - syphilis 45
  - West Nile virus 45
  - Zika virus 45
- see also* polymerase chain reaction; transcription-mediated amplification
- nutritional requirements, erythropoiesis 138–142
- Nyvepria *see* pegfilgrastim-apgf
- O antigen 81
- OBRR *see* Office of Blood Research and Review
- obstetrics 373–380
- antifibrinolytics 377
  - antiphospholipid antibody syndrome 375
  - chronic anemia 375
  - disseminated intravascular coagulation 375
  - fetal administration 379–380
  - fetal/neonatal alloimmune thrombocytopenia 379–380
  - fibrinogen concentrates 377
  - gestational thrombocytopenia 373–375
  - HELLP syndrome 373–374
  - hemolytic-uremic syndrome 374–375
  - hemorrhage protocols 376–377
  - immune thrombocytopenic purpura 373–374
  - inherited bleeding disorders 375
  - low income countries 378
  - postpartum anemia 377–378
  - thrombotic thrombocytopenic purpura 374–375
  - transfusions 375–380
- see also* pregnancy
- octanoic acid (caprylate) treatment, plasma 221
- Octogam, purification 224
- ocular tissue allografts 675–678
- Office of Blood Research and Review (OBRR) 68
- Office of Tissues and Advanced Therapies (OTAT) 68
- Official Medicines Control Laboratory (OMCL) 77
- OHSt *see* overhydrated hereditary stomatocytosis
- OK blood group system 116
- OMCL *see* Official Medicines Control Laboratory
- Oncley process, immunoglobulins 224–226
- oncology patients 482–488
- adverse reactions 487–488
  - chimeric antigen receptor T-cell therapy 488, 636–637
  - erythroid growth factors 605
  - granulocyte transfusions 486–487
  - hematopoietic stem cell transplants 482–483, 487–488
  - platelet transfusions 484–486
- red blood cell administration 322, 482–484
- risk factors 487
- T-cell immunotherapies 633
- see also* cancer
- operations organization, disaster management 15–16
- ophthalmological complications, sickle cell disease 335
- oral calcium supplementation, apheresis 54
- organization
- emergency operations 15–16
  - patient blood management 293–305
  - United States blood services 12
  - worldwide blood services 12–13
- orthochromatic erythroblasts (OrthoEBs) 134
- orthopedic surgery, red blood cell administration 321
- OTAT *see* Office of Tissues and Advanced Therapies
- OttoEBs *see* orthochromatic erythroblasts
- Ottenberg, R. 8
- overdose, apheresis 273
- overhydrated hereditary stomatocytosis (OHSt) 339
- oversight, human tissue allografts 677–678
- P blood group system 93–97
- biochemistry 93–94
  - biosynthesis 93–94
  - discovery 8
  - epitopes 93–94
  - expression 93–94, 96
  - genetics 95–96
  - molecular biology 95–96
  - serology 94–95
  - transfusions 96

- P.falciparum* see *Plasmodium falciparum*
- p null phenotype 94–95
- P synthase (*B3GALNT1*) 94, 95
- P1PK synthase (*A4GALT1*) 94, 95
- p14 deficiency syndrome 192
- packed red blood cells (PRBCs)
- collection 32
  - separation 32
- PAH see pulmonary arterial hypertension
- PAN see polyarteritis nodosa
- PANDAS see pediatric autoimmune neuropsychiatric disorders associated with streptococcal infections
- pandemic influenza 521
- pandemic management 21–23
- panel-reactive antibody (PRA) tests 630–631
- paracentesis
- plasma administration 468
  - platelet administration 469
- paraproteinemic demyelinating polyneuropathies, apheresis 268
- Paris–Trousseau thrombocytopenia 161
- paroxysmal cold hemoglobinuria (PCH) 96, 354–355
- paroxysmal nocturnal hemoglobinuria (PNH) 358–363
- anticoagulants 360
  - bone marrow disorders 363
  - children 363
  - clinical features 359–360
  - eculizumab 361–362
  - elective surgery 363
  - epidemiology 358
  - hematopoietic stem cell transplants 362
  - immunosuppression 362
  - pathophysiology 358–359
  - pregnancy 363
  - prognosis 363
  - red blood cell administration 362–363
  - risk factors 358
  - treatment 360–363
- partial D phenotype 102, 105–106
- Parvovirus B19 519–520
- aplastic crisis 333–334
  - P blood group system 96
  - testing 47
- passive alloimmune thrombocytopenia (PAT) 421–422
- PAT see passive alloimmune thrombocytopenia
- pathogen reduced plasma 210, 219–221, 495–496
- pathogen reduction 489–500
- albumin 496
  - alloimmunization 498–499
  - amotosalen/UV-A 490, 492, 495, 497, 498, 499
  - amustaline/glutathione 492–493, 496, 499–500
- bacterial contamination 538–539
- efficacy 493–495
- fresh frozen plasma 495–496
- immunoglobulins 240, 496
- immunological effects 498–499
- methylene blue 493, 496, 500
- overview 489–490
- platelets 496–499
- red blood cells 155, 498
- riboflavin/UV 492, 495, 497, 498, 499
- safety and toxicity considerations 499–500
- solvent and detergent 493, 496, 500
- technologies 490–493
- transfusion-associated graft-versus-host disease 499
- ultraviolet C 493, 496, 497, 500
- in vitro platelet function 496–497
- in vivo function tests 497–498
- pathogenesis
- alloimmune thrombocytopenia 417
  - heparin-induced thrombocytopenia 414–415
  - hepatitis B 506–507
  - immune thrombocytopenic purpura 406
  - posttransfusion purpura 421
- pathophysiology
- anemia 137, 381–382
  - cold agglutinin disease 351–352
  - drug-induced immune hemolytic anemia 356–357
  - hemolytic transfusion reactions 545–547
  - iron overload 587–589
  - paroxysmal cold hemoglobinuria 354
  - paroxysmal nocturnal hemoglobinuria 358–359
  - sickle cell disease 330–331
  - transfusion-associated graft-versus-host disease 582
  - transfusion-related acute lung injury 570–571, 573–577
  - warm autoimmune hemolytic anemia 346–348
- patient blood management (PBM) 293–305
- acute nonvolemic hemodilution 301–302
  - antifibrinolitics 303
  - cell salvage 299–301
  - definitions 293
  - desmopressin 303–304
  - evidence-based guidelines 294–295
  - fibrinogen concentrates 304
  - hemostatic agents 302–304
  - limiting phlebotomy blood loss 304
  - physician education and monitoring 295–297
  - point-of-care testing 302
  - preoperative anemia management 297–299
- program implementation 293–294
- prothrombin complex concentrates 303
- recombinant activated factor VII 304
- washed cell salvage 300–301
- patient monitoring 312
- patient safety organizations (PSOs) 129
- PBM see patient blood management
- PBSC see peripheral blood stem cell
- PCC see prothrombin complex concentrates
- PCH see paroxysmal cold hemoglobinuria
- PCR see polymerase chain reaction
- pediatric . . . , see also children; infants; neonates
- pediatric autoimmune neuropsychiatric disorders associated with streptococcal infections (PANDAS), apheresis 269
- pegfilgrastim (Neulasta) 607
- pegfilgrastim-apgf (Nyvepria) 608
- pegfilgrastim-bmez (Ziextenzo) 608
- pegfilgrastim-cbqv (Udenyca) 608
- pegfilgratim-jmdb (Fulphilia) 608
- PEL blood group system 116–117
- pentose shunt (hexose monophosphate shunt) 146, 147
- perioperative practice 453–471
- acute nonvolemic hemodilution 462
  - anemia 453–455
  - anticoagulants 459–460
  - anticoagulation therapies 459–461
  - antiplatelet therapies 459–461
  - blood avoidance techniques 461–462
  - conservative practice 461
  - direct oral anticoagulants 460
  - guidelines 464–466
  - paroxysmal nocturnal hemoglobinuria 363
  - plasma administration 464–470
  - platelet administration 466, 469–470
- point-of-care viscoelastic assays 459
- red blood cell administration 464
- topical hemostatic therapies 462–463
- peripheral blood progenitor cell mobilization 610–611
- peripheral blood stem cell (PBSC) collection 620
- peripheral nerve allografts 677
- peripheral nerve system tissue engineering 651–652
- pH
- albumin 223
  - alpha<sub>1</sub>-proteinase inhibitor 228
  - antithrombin III 230
  - factor IX 232
  - fibrinogen 231
  - immunoglobulins 226
  - platelet storage 182–183
  - prothrombin complex concentrate 232
  - red blood cell storage 153

- virus inactivation 221
- von Willebrand factor 233
- pharmacogenetics, human leukocyte antigens 632
- phenotypes, human leukocyte antigens 629
- PhEur *see* European Pharmacopoeia
- phlebotomy
  - donor collections 32–36
    - acute adverse reactions 52–53
    - limiting blood loss 304
    - pretransfusion testing 123–124
      - see also* blood collection
  - phlebotomy-related nerve injury, donors 53
  - photochemotherapy *see* extracorporeal photopheresis
  - phototherapy, hemolytic disease of the fetus and newborn 369–370
  - PHSA *see* Public Health Service Act
  - physical examination
    - blood donors 32
    - source plasma donors 34–35
  - physical exercise, plasma composition 202
  - physician education 295–297
  - physician monitoring 295–297
  - phytanic acid storage disease, apheresis 269
  - pica, donation-related iron deficiency 60–61
  - P<sup>k</sup> phenotype 94
  - PKD *see* pyruvate kinase deficiency
  - PIA1 *see* HPA1a
  - planning, disaster management 14–15, 16–17
  - plasma 200–235
    - ABO antibody titers 38–39
    - administration 209–215
      - adverse reactions 215
      - angiography 468–469
      - bleeding treatment 212–213
      - bronchoscopy 468
      - burn patients 480
      - central venous catheter insertions 466–467
      - children 388–390
      - clinical use 212
      - combined factor V and factor VIII deficiency 438
      - cryoprecipitate 214–215
      - disseminated intravascular coagulation 213
      - epidural anesthesia 468
      - factor V deficiency 438
      - hemophilia 426–427
      - infants 388–390
      - liver biopsy 467
      - lumbar puncture 468
      - neurosurgical procedures 468
      - oncology patients 486
      - paracentesis 468
    - perioperative guidelines 464–466
    - prophylactic 213–214
    - prothrombin deficiency 437
    - thoracentesis 468
    - transbronchial lung biopsy 468
    - trauma patients 480
    - upper airway procedures 468
    - warfarin effect 214
    - adverse reactions 215
    - albumin 200
    - alpha-1 antitrypsin 201
    - alternate preparations 209–211
    - apheresis collection requirements 254–255
    - bacterial contamination 541
    - C1 inhibitor 201
    - chromatographic separation 218–219, 222
    - clotting factors 201–202, 210–211
    - coagulation factor inhibitors 201–202, 203
    - coagulation screening tests 204–208
    - cold ethanol fractionation 9, 217–218
    - collection
      - chronic effects 63–64
      - donor screening 34–35, 37, 39–47
      - process 34–36
    - composition 200–208
    - convalescent 23, 212
    - cryoprecipitate 210–211, 214–215
    - donor screening 34–35, 37, 39–47
    - dried 209–210
    - European regulation 75–78
    - fibrinolysis 203–208
    - frozen storage 203
    - hemostasis 203
    - immunoglobulins 201
    - infectious disease testing 37, 39–47
      - see also* prerelease testing
    - initial usage 9
    - medicinal product manufacturing processes 222–234
    - octanoic acid treatment 221
    - pathogen reduced 210, 219–221, 495–496
    - pH 221
    - prion safety 222
    - protein purification 216–235
    - separation 33
    - solvent and detergent treatment 33, 220–221
    - storage 203
    - thawing 311
    - ultraviolet treatment 221
    - virus filtration 221
    - virus inactivation 220–221
    - volume reduction 310
    - von Willebrand factor 201–202
    - World Health Organization programs 74–75
      - see also* source plasma; therapeutic plasma exchange
  - plasma composition, acute phase reaction 202
  - plasma exchange *see* therapeutic plasma exchange
  - Plasma Protein Therapeutics Association (PPTA) 65, 78
  - Plasma Protein Therapeutics Association (PPTA)-certified centers 34
  - plasma removal, platelets 186
  - plasma-derived factor FVII (pdFVII) 438–439
  - plasma-derived medicinal products (PDMPs) 12, 216–235
    - activated prothrombin complex concentrate 227–228
    - albumin 222–224
    - alpha<sub>1</sub>-proteinase inhibitor 228
    - antithrombin III 230
    - bacterial contamination 541
    - C1 inhibitor 229–230
    - chromatographic separation 218–219, 222
    - cold ethanol fractionation 217–218
    - European regulation 77
    - factor IX 232
    - factor VIII 233–234
    - factor X 227
    - factor XI 228–229
    - factor XIII 227
    - fibrinogen 231
    - heat treatment 220
    - immunoglobulins 224–227
    - octanoic acid treatment 221
    - oncology patients 486
    - pathogen reduction 219–221
    - pH 221
    - prion safety 222
    - prothrombin complex concentrate 231–232
    - purifications 222–234
    - solvent and detergent treatment 220–221
    - ultraviolet treatment 221
    - validation of virus reduction/removal 219–220
    - virus filtration 221
    - virus inactivation 220–221
    - virus reduction 219–220
    - von Willebrand factor 232–233
    - plasmapheresis
      - anticoagulants 251–252
      - see also* therapeutic plasma exchange
    - Plasmodium falciparum* (*P. falciparum*) 86, 281–282, 330
    - plasticizer toxicity 565
    - platelet-rich plasma (PRP) 179
      - separation 33
    - plateletpheresis 180
      - acute adverse reactions 53–54
      - chronic effects 63
      - requirements 255–256
      - thrombocytosis 285–286

platelets 158–187  
 ABO system antigens 170–171, 484  
 acquired disorders 393  
 additive solutions 184–185  
 administration 395–400  
   bronchoscopy 469  
   cardiac surgery 470  
   central venous catheter  
     placement 469  
   children 385–388  
   dosing 397  
   epidural anesthesia 470  
   fetal 379  
   infants 385–388  
   liver biopsy 469  
   lumbar puncture 470  
   maternal 378  
   neurosurgical procedures 470  
   oncology patients 484–486  
   paracentesis 469  
   perioperative guidelines 464–466  
   preoperative 397–398  
   prophylactic 396–397  
   refractoriness 398–399, 422  
   therapeutic 398–400  
   thoracentesis 469  
   transbronchial lung biopsy 469  
   upper airway procedures 469  
 alloimmunization 171–177, 399–400,  
   485, 498–499  
 alternate sources 181  
 antibody-specific prediction 173–174  
 antigen typing 47, 399  
 antigens 168–171, 418  
 apheresis-derived 180  
 bacterial contamination 46, 533–539  
 buffy-coat method 180  
 centrifugation 186  
 circulatory survival 165  
 congenital disorders 393  
 crossmatching 174, 400, 484  
 differentiation 158–165  
 HLA-matched transfusions 172–173  
 human leukocyte antigen 168–169,  
   399–400  
 human platelet antigens 169–170, 399  
 immunology 168–177  
 irradiation 186, 485  
 issuance 307  
 kinetics 165–167  
 leukocyte reduction 186, 485  
 lifespan 165–167  
 megakaryopoiesis regulation 162–165  
 modifications 185–186  
 novel storage techniques 186–187  
 oncology patients 484  
 pathogen reduction 496–499  
 pathologies of megakaryopoiesis  
   161–162  
 plasma removal 186

platelet-rich plasma 179  
 preoperative transfusions 397–398  
 preparation 179–181  
 prevention of alloimmunization  
   176–177  
 production 158–165  
 prophylactic transfusions 396–397  
 refractoriness 174, 398–399, 422, 485  
 sequestration 167, 392–393  
 storage 181–187, 307  
   additive solutions 184–185  
   containers 183  
   lesions 183–184  
   metabolic needs 181–183  
   pH 182–183  
   respiratory capacity 183  
   temperature 181–182  
 transfusion-related acute lung  
   injury 574  
 volume reduction 186, 310, 485  
 washing 186  
 PNH *see* paroxysmal nocturnal  
   hemoglobinuria  
 point of care immunoassays, bacterial  
   contamination 537–538  
 point-of-care assays, viscoelastic 459  
 point-of-care testing 302  
 polyamines, red blood cells 148–149  
 polyarteritis nodosa (PAN), apheresis 272  
 polychromatophilic erythroblasts  
   (PolyEBs) 134  
 polycythemia vera, apheresis 282–283  
 PolyEBs *see* polychromatophilic  
   erythroblasts  
 polymerase chain reaction (PCR)  
   testing 39  
   human leukocyte antigens 628  
 polymorphism in human leukocyte  
   antigens 626–627  
 pooled plasma, TRALI prevention  
   578–579  
 pooling  
   HIV testing 43  
   nucleic acid testing 39  
 post-transfusion sepsis, prevention  
   strategies 535–540  
 postdonation care 32–33  
 postpartum anemia 377–378  
 posttransfusion purpura (PTP) 395, 421  
 PPTA *see* Plasma Protein Therapeutics  
   Association  
 PRA *see* panel-reactive antibody tests  
 PRBCs *see* packed red blood cells  
 pregnancy 373–380  
   antiphospholipid antibody  
     syndrome 375  
   blood donation 33  
   chronic anemia 375  
   disseminated intravascular  
     coagulation 375  
 donation-related iron deficiency 60  
 fetal administration 379–380  
 fetal/neonatal alloimmune  
   thrombocytopenia 379–380  
 gestational thrombocytopenia 373–375  
 HELLP syndrome 373–374  
 hemolytic disease of the fetus and  
   newborn 364–372  
 hemolytic-uremic syndrome 374–375  
 hemorrhage protocols 376–377  
 immune thrombocytopenic purpura  
   373–374, 410  
 inherited bleeding disorders 375  
 low income countries 378  
 paroxysmal nocturnal  
   hemoglobinuria 363  
 platelet transfusions 378  
 postpartum anemia 377–378  
 red blood cells transfusions 378  
 red cell exchange 281, 334  
 sickle cell disease 334  
 thrombotic thrombocytopenic purpura  
   374–375  
 transfusions 375–380  
 prekallikrein 205–208  
 prenatal genotyping, Rh proteins 105  
 preoperative anemia  
   erythroid growth factors 605  
   management 297–299, 454–455  
 preoperative hemostatic testing 457–459  
 preoperative intravenous immunoglobulin,  
   immune thrombocytopenic  
   purpura 410  
 preoperative platelet transfusions 397–398  
 preoperative red cell exchange 281, 334  
 preparation  
   blood components 309–311  
   irradiation 309–310  
   leukocyte reduction 309  
   platelets 179–181  
   thawing plasma 311  
   washing 310  
 preparedness, disaster management 14–15,  
   16–17  
 preplatelets 160–161  
 prerelease testing 39–47  
   babesiosis 45  
   blood derivatives 46  
   Chagas' disease 46  
   cytomegalovirus 46  
   donated blood 39, 40  
   donor management 41–42, 41  
   donor reentry 41, 42–43  
   extended blood group antigen typing 47  
   false positives 39  
   hemoglobin S 47  
   hepatitis A 47  
   hepatitis B 43–44  
   hepatitis C 44  
   HIV 43

human leukocyte antigen 47  
 human T-cell lymphotropic virus 44  
 IgA deficiency 47  
 lookback 42  
 Parvovirus B19 47  
 platelet antigen typing 47  
 platelets 46  
 pooling 39, 43  
 product management 42  
 recipient notification 42  
 regulations 39–40, 41–42  
 resource-limited settings 40  
 sequence 40  
 syphilis 45–46  
 West Nile virus 45  
 Zika virus 45  
**preservation**  
 cell-based immunotherapies 308  
 cryoprecipitate 308  
 granulocytes 195, 308  
 hematopoietic stem cells 308  
 plasma 308  
 platelets 181–187, 307  
     additive solutions 184–185  
     containers 183  
     cryopreservation 186  
     lesions 183–184  
     lyophilization 186–187  
     metabolic needs 181–183  
     pH 182–183  
     respiratory capacity 183  
     temperature 181–182, 186  
 red blood cells 150–157, 307  
     additive solutions 152–154  
     anticoagulant-nutrient solutions 152  
     effectiveness 155–156  
     frozen storage 156  
     leukocyte reduction 155  
     pathogen reduction 155  
     pH 153  
     principles 150–151  
     rejuvenation 156  
     storage containers 154–155  
     temperature and time lapse 154  
     washing 155  
 presyncopal symptoms, blood donation 50  
 preterm infants, red blood cell transfusions 382–383  
 pretransfusion checks at bedside 311  
 pretransfusion testing 118–130  
     ABO typing 124  
     administration 129  
     antiglobulin tests 129  
     automation 125–127, 128–129  
     biochemistry 118–122  
     blood selection 128  
     compatibility testing 122–130  
     crossmatching 128–129  
     DNA-based blood group typing 127–128, 129

donor testing 122–123  
 donor-recipient 128–129  
 emergency release 129  
 issue dates 129  
 minor blood group antigens 127–128, 129  
 monoclonal antibody interference 127  
 patient testing 123–130  
 prior records checking 128–129  
 red blood cells 339–340  
 sample collection 123–124  
 unit labeling 129  
**prevalence**  
 alloimmune  
     thrombocytopenia 418–419  
 cold autoimmune hemolytic anemia 351  
 donation-related iron depletion 56–58  
 drug-induced immune hemolytic anemia 356  
 hemolytic transfusion reactions 543–544  
 paroxysmal nocturnal hemoglobinuria 358  
 paroxysmal cold hemoglobinuria 354  
 therapeutic plasma exchange complications 264–265  
 transfusion-associated graft-versus-host disease 583  
 transfusion-related acute lung injury 572–573  
 transfusion-related circulatory overload 580  
 warm autoimmune hemolytic anemia 346  
**prevention**  
 allergic responses 559  
 alloimmunization 176–177  
 anaphylaxis 561  
 febrile nonhemolytic reactions 557–558  
 hemolytic disease of the fetus and newborn 367–368  
 hemolytic transfusion reactions 551–552, 557–558  
 post-transfusion sepsis 535–540  
 stroke, sickle cell disease 332–333  
 syncopal reactions, blood donation 51–52  
 transfusion-associated graft-versus-host disease 585–586  
 transfusion-related circulatory overload 580  
**primary immune deficiencies, immunoglobulins**  
 244244–244245  
**primary immune responses** 119  
**primary stroke prevention, red cell exchange** 280–281, 332–333  
**primary thrombocytosis, apheresis** 285–286  
**prions** 11, 31, 222, 529–531  
     molecular biology 529–530  
     regulatory measures 531  
     transmissibility 530–531  
**Privigen, purification** 226  
**procedure requirements, apheresis, collections** 254–256  
**Procrit** *see* epoetin alfa  
**product requirements, apheresis, collections** 254–256  
**ProEBs** *see* proerythroblasts  
**proerythroblasts (ProEBs)** 134  
**prognoses**  
     cold agglutinin disease 354  
     paroxysmal cold hemoglobinuria 355  
     paroxysmal nocturnal hemoglobinuria 363  
     warm autoimmune hemolytic anemia 351  
**programmed cell death, red blood cells** 150  
**proliferation, megakaryocytes** 158  
**Promacta** *see* eltrombopag  
**prophylactic transfusions**  
     granulocytes 198  
     hemophilia 427  
     plasma 213–214  
     platelets 396–397  
**prostate cancer, I blood group** 93  
**protamine** 449  
**protein blood groups**  
     ABCC1 system 117  
     AUG system 117  
     Cartwright system 115  
     Chido/Rogers system 115  
     Colton system 114–115  
     CTL2 system 117  
     Diego system 114  
     Dombrock system 115–116  
     Duffy system 112–113  
     Gerbich system 115  
     GIL system 114–115  
     Indian system 115  
     JR system 116  
     Kell 111–112  
     Kidd system 113–114  
     Knops system 115  
     LAN system 116  
     LW system 107  
     MAM system 117  
     MNS system 109–111  
     OK system 116  
     overview 110  
     PEL system 116–117  
     RAPH system 116  
     Rh system 100–107  
     Scianna system 114  
     VEL system 117  
     Xg system 114

- prothrombin complex concentrates (PCC) 214, 303, 443–444  
adverse reactions 444  
burn patients 480  
dosing 444  
indications 443–444  
issuance 308  
manufacturing 443  
obstetrics 377  
purification 231–232  
storage 443  
surgical protocols 460  
trauma patients 480
- prothrombin deficiency 437  
prothrombin (FII) 201, 437  
prothrombin time (PT) 205–208, 457–458  
protocols  
  obstetric hemorrhage 376–377  
  red blood cell administration 339–341
- protoplatelets 160
- protozoan diseases  
  red cell exchange 281–282  
  *see also* babesiosis; Chagas disease;  
    leishmaniasis; malaria; malaria,  
    babesiosis; microfilariasis;  
    toxoplasmosis
- PRP *see* platelet-rich plasma
- PSOs *see* patient safety organizations
- PTP *see* posttransfusion purpura
- Public Health Service Act (PHSA) 66
- pulmonary arterial hypertension (PAH),  
  sickle cell disease 334–335
- pulmonary endothelial cells, transfusion-related acute lung injury 574
- purification  
  immunoglobulin G 238–240  
  immunoglobulins 224–227, 238–240
- plasma proteins 216–235  
  activated prothrombin complex  
    concentrate 227–228  
  albumin 222–224  
  alpha<sub>1</sub>-proteinase inhibitor 228  
  antithrombin III 230  
  C1 inhibitor 229–230  
  factor IX 232  
  factor VIII 233–234  
  factor X 227  
  factor XI 228–229  
  factor XIII 227  
  fibrinogen 231  
  immunoglobulins 224–227, 238–240  
  pathogen reduction 219–221  
  prion safety 222  
  prothrombin complex concentrate  
    231–232  
  validation of virus reduction/removal  
    219–220  
  virus filtration 221  
  virus inactivation 220–221  
  virus reduction 219–220
- von Willebrand factor 232–233
- red blood cells 151–152  
  *see also* manufacturing
- pVHL *see* von Hippel-Landau protein
- pyruvate kinase activators 596
- pyruvate kinase deficiency (PKD) 338
- QALY *see* quality-adjusted life years
- QC *see* quality control
- quality control (QC)  
  hospital transfusion services 71  
  human tissue allografts 679–680
- quality evaluation, red blood cells 156–157
- quality-adjusted life years (QALY),  
  autologous donations 29
- RA *see* rheumatoid arthritis
- Rac family small GTPases (RAC1 and RAC2) 160
- radiological agent attacks 16
- RAPB blood group system 116
- rapid infusion practices 313–314, 376–377  
  complications 561–564
- Rapoport-Luebering shunt (2,3-DPG shunt) 145–146, 147
- Ras homolog family member A (RhoA) 160
- RBCs *see* red blood cells
- RCE *see* red cell exchange
- real-time polymerase chain reaction  
  (real-time PCR), human leukocyte antigens 628
- rebound thrombocytopenic 614
- recalls, human tissue allografts 682–683
- recipient-specific blood donation 29
- recipients  
  ABO typing 124  
  administration 129  
  medications, apheresis 265–266  
  monitoring 312  
  notification of discovery of a donor disease 42  
  patient blood management 293–305  
  platelet alloimmunization 171–174  
  pretransfusion testing 123–130  
  Rh typing 124–125  
  skin disinfection 535
- recombinant activated factor VII (rFVIIa) 304, 445–446  
  adverse reactions 445–446  
  burn patients 480  
  dosing 445–446  
  indications 445–446  
  manufacturing 445–446  
  oncology patients 486  
  storage 445–446  
  trauma patients 480
- recombinant clotting factors, storage 308
- recombinant factor IX (rFIX) 431–432
- recombinant factor VIII (rFVIII) 427, 436–437
- recombinant factor Xa (andexanet alfa) 449–450
- recombinant products, European regulation 77
- records management, disasters 18
- recovery, disaster management 15
- recruitment  
  blood donation 27–29  
  disasters 28–29  
  recipient-specific 29  
  retention 28  
  theories of behavior 28
- red blood cell antibodies, hemolytic disease of the fetus and newborn 365
- red blood cells (RBCs) 133–157  
  ABO typing 37–38, 38  
  additive solutions 152–154  
  adenosine triphosphate 143, 145, 147–148
- administration  
  acute myocardial infarction 321–322  
  cardiac surgery 319–321  
  children 322, 383–385  
  chronic anemia 324  
  clinical trials 318–322  
  cold agglutinin disease 354  
  complications 342–345  
  critical care 319  
  decision-making 323  
  dosing 324  
  fetal 379  
  guidelines 322–324  
  hemolytic disease of the fetus and newborn 370–371  
  infants 382–383  
  maternal 378  
  microcirculatory effects 316–317  
  monitoring 341–342  
  oncology patients 322, 482–484  
  orthopedic surgery 321  
  paroxysmal cold hemoglobinuria 355  
  paroxysmal nocturnal  
    hemoglobinuria 362–363  
  perioperative guidelines 464–466  
  preterm infants 382–383  
  protocols 339–341  
  sickle cell disease 331–335, 332  
  surgical outcomes 455–457  
  targets 341–342  
  thalassemia 335–337  
  transfusion threshold 324  
  upper gastrointestinal bleeding 321  
  warm autoimmune hemolytic anemia 350–351
- alloimmunization 342–343, 483
- alternate metabolic substrates 146–147
- anemia of chronic inflammation 141
- antibody detection 38
- anticoagulant-nutrient solutions 152
- antigen–antibody interactions 120–121

apheresis requirements 256  
 bacterial contamination 540–541  
 biochemistry 143–150, 144  
 biosynthesis 135, 148–149  
 blood groups *see* blood groups  
 DNA-based group typing 127–128, 129  
 dosing 324  
 2,3-DPG shunt 145–146, 147  
 effectiveness 155–156  
 enzymopathies 328–329, 337–338  
 eryptosis 150  
 erythropoiesis 133–142  
 erythropoietic therapies 141–142  
 expiration time 307  
 frozen storage 156  
 G proteins 148  
 glucose metabolism 145–148  
 guanine nucleotides 148  
 irradiation 155, 483  
 issuance 307  
 leukocyte reduction 155, 483  
 macrocytic anemia 138–140  
 membrane metabolism 149–150  
 membrane mutations 329, 338–339  
 metabolic regulation 147–148  
 metabolism 143–150  
 microcirculatory effects of  
   administration 316–317  
 microcytic anemia 140–141  
 pathogen reduction 155, 498  
 pentose shunt 146, 147  
 preservation 144, 150–157, 307  
   additive solutions 152–154  
   anticoagulant-nutrient solutions 152  
   effectiveness 155–156  
   frozen storage 156  
   leukocyte reduction 155  
   pathogen reduction 155  
   pH 153  
   principles 150–151  
   rejuvenation 156  
   storage containers 154–155  
   temperature and time lapse 154  
   washing 155  
 pretransfusion testing 339–340  
 production kinetics 138  
 quality evaluation 156–157  
 rejuvenation 156  
 Rh proteins function 105  
 Rh typing 37–38, 105–109, 124–128  
 salvage devices 312  
 separation 33, 151–152  
 simple transfusion 340–341  
 storage 307  
 storage containers 154–155  
 structural hemoglobin mutations 47,  
   327–328  
 thalassemia 141, 335–337  
 transfusion threshold 324  
 in vivo recovery 156–157  
 volume reduction 310

washing 155, 300–301  
*see also* alpha thalassemia; beta  
 thalassemia; sickle cell disease;  
 thalassemia  
 red cell exchange (RCE) 278–282  
   acute chest syndrome 279, 333  
   acute multiorgan failure  
     syndrome 279–280  
   acute vaso-occlusive stroke 279  
   aplastic crisis 333–334  
   babesiosis 282  
   chronic transfusion therapy 280–281  
   contraindications 335  
   fraction of the preprocedure cells  
     remaining 278–279  
   iron overload prevention 281  
   malaria 282  
   ophthalmological complications of  
     SCD 335  
   pregnancy 281, 334  
   preoperative 281, 334  
   protocol 341  
   protozoan diseases 281–282  
   pulmonary arterial  
     hypertension 334–335  
   sickle cell disease 279–281  
   sickle hepatopathy 334  
   stroke prevention 280–281, 332–333  
 refractoriness, platelets 174, 398–399,  
   422, 485  
 Refsum's disease, apheresis 269  
 regulation  
   adverse transfusion reaction  
     reporting 70–73  
   blood collection establishments  
     66–70  
   defective products reporting 69, 72  
   devices 70  
   disaster management 17–18  
   erythropoietin production 136–137  
   health emergencies 17–18, 21–23  
   hematopoietic stem cells 623  
   hospital transfusion services 70–73  
   inspections 69  
   megakaryopoiesis 162–165  
   prion safety 531  
   red blood cell metabolism 147–148  
   screening 39–40, 41–42  
   source plasma collection 34  
   United states history 65–66  
   World Health Organization 74–75  
 regulatory T cells 634  
 reimbursement, human tissue  
   allografts 683  
 rejection  
   cardiac allografts, extracorporeal  
     photopheresis 287–288  
   lung allografts, extracorporeal  
     photopheresis 288  
 rejuvenation, red blood cells 156  
 release, blood components 307

relevant transfusion-transmitted infections  
 (RTTIs)  
 autologous donations 29  
 donor screening 29–32, 34–36  
*see also* transfusion-transmitted  
 infections  
 renal tissue engineering 654–656  
 repeat donors 28  
 repeat reactive (RR) samples, HIV 43  
 replacement solutions, therapeutic plasma  
 exchange 261–263, 265  
 reportable deviations, blood collection  
 establishments 69  
 reporting  
   adverse transfusion reactions 70–73,  
   129–130  
   defective products 69, 72  
   transfusion service errors 71–72  
   transfusion-related deaths 71  
 reproductive system tissue engineering  
   650–651  
 resource-limited contexts, infectious  
 disease testing 40–41  
 respiratory capacity, stored platelets 183  
 respiratory infections, ABO system 87  
 responses, disaster management 15  
 restless legs syndrome (RLS), donation-  
   related iron deficiency 60  
 Retacrit *see* epoetin alfa-epbx  
 retention, donors 28  
 reticulated platelets (RPs) 160–161,  
   166–167  
 reticulin fibrosis 614  
 reticulocyte suppression/destruction 547  
 retroviruses 515–517  
*see also* human immunodeficiency virus;  
   human T-cell lymphotropic virus  
 returns, blood components 309  
 Revolade *see* eltrombopag  
 rFIX *see* recombinant factor IX  
 rFVIIa *see* recombinant activated  
   factor VII  
 rFVIII *see* recombinant factor VIII  
 Rh immune globulin 408  
 Rh system 100–107  
   altered CE 103–104  
   antibodies 106, 107  
   biological roles 105  
   C/c & E/e antigens 102–103  
   D antigen 101  
   discovery 8  
   donor testing 37–38  
   expression 101–104  
   fetal typing 105  
   genetics 101–106  
   genotyping 105–106  
   hemolytic disease of the fetus and  
     newborn 106  
   history 100  
   homology 105  
   homozygosity 105, 106

- Rh system (*cont'd*)  
 immune responses 106–107  
 membrane complex 104–105  
 nomenclature 100, 101  
 partial D 102, 105–106  
 RhAG association 101  
 serology 100–104, 106–107  
 silenced CE 103  
 transfusions 106–107  
 weak D 101–102, 103, 105–106
- Rh typing 37–38  
 fetal 105  
 genotyping 105–109  
 hemolytic disease of the fetus and newborn 365–366  
 recipients 124–125
- Rh-associated glycoprotein (RhAG) 101
- RhAG *see* Rh-associated glycoprotein
- RHCE 101
- RHD 101, 105
- rheumatoid arthritis (RA), neutropenia 192
- RhoA *see* Ras homolog family member A
- riboflavin/UV pathogen reduction 492, 495, 497, 498, 499
- RIF 117
- risk, definition 503
- risk factors  
 anemia 317–319  
 donation-related iron depletion 56–58  
 gene therapy 643–644  
 infections, ABO system 86–87  
 oncology patients 487  
 paroxysmal nocturnal hemoglobinuria 358  
 post-donation acute adverse reactions 51, 53  
 Rh antibodies 106
- rituximab  
 cold agglutinin disease 353  
 immune thrombocytopenic purpura 409  
 neutropenia 192  
 warm autoimmune hemolytic anemia 350
- RNA testing, HIV 43
- Robert T. Stafford Disaster Relief and Emergency Assistance Act (1988) 15
- Romiplostim (Nplate) 611–613
- RPs *see* reticulated platelets
- RR *see* repeat reactive
- RTTIs *see* relevant transfusion-transmitted infections
- S/D *see* solvent and detergent
- SABM *see* Society for the Advancement of Blood Management
- safety measures, human tissue allografts 679–680
- SAG-M *see* saline, adenine, and glucose with mannitol
- saline, adenine, and glucose with mannitol (SAG-M) solution 151, 152–153
- saline, adenine, and glucose (SAG) solution 151
- saline solutions  
 innovation 7  
 source plasma donors 35
- salvage devices 312
- sample collection, pretransfusion testing 123–124
- SAO *see* Southeast Asian Ovalocytosis
- sargramostim (Leukine) 608
- SARS-CoV-1, ABO type 87
- SARS-CoV-2 14, 16, 18–23  
 ABO type 87  
 convalescent plasma 23  
 vaccine-induced immune thrombotic thrombocytopenia 417
- SBDS *see* Shwachman–Bodin–Diamond syndrome
- scaffolds  
 gastrointestinal tissue 653–654  
 lung tissue 648–649  
 peripheral nerve system tissue 651  
 renal tissue 654–655  
 reproductive system tissue 650  
 skeletal muscle 652–653
- SCD *see* sickle cell disease
- scheduling, immunoglobulin therapy 244–247
- SCI *see* silent cerebral infarcts
- Scianna blood group system 114
- SCID *see* severe combined immunodeficiency
- SCIG *see* subcutaneous IgG
- SCN1 191
- SCNs *see* severe congenital neutropenias
- screening 29–32, 37–48  
 ABO antibody titers 38–39  
 ABO system 37–39, 38  
 administration 31–32  
 automated systems 40  
 babesiosis 45, 524  
 bacterial infections 535  
 biovigilance 129–130  
 blood derivatives 46  
 Chagas' disease 46  
 Chagas disease 46, 525  
 CJD/vCJD risk 31  
 coagulation tests 204–208, 457–458  
 compatibility testing 122–130  
 cytomegalovirus 46, 519  
 dengue virus 517  
 direct antiglobulin testing 39  
 donated blood 39, 40  
 Donor History Questionnaire 30–31  
 donor management 41–42, 41  
 donor reentry 41, 42–43
- donor–recipient mismatches 128–129
- educational materials 31
- extended blood group antigen typing 47
- false positives 39
- ferritin levels 61–62, 63
- granulocyte donors 194–195
- hemoglobin S 47
- hepatitis A 47, 507–508
- hepatitis B 43–44, 510–511
- hepatitis C 44, 511
- hepatitis D 514
- herpesviruses 519
- HIV 43, 516
- HTLV 517
- human leukocyte antigen 47
- human T-cell lymphotropic virus 44
- IgA deficiency 47
- IgG depletion 63
- infectious diseases 37, 39–47
- informed consent 31–32
- leishmaniasis 528
- limiting blood loss 304
- lookback 42
- malaria 526
- men who have sex with men 31
- pandemic influenza 521
- Parvovirus B19 47, 520
- platelet antigen typing 47
- platelets 46
- pooling 39, 43
- product management 42
- RBC antigens 38
- recipient notification 42
- regulations 39–40, 41–42
- resource-limited settings 40
- Rh groups 37–38
- Rh system 37–38
- sequence 40
- source plasma donors 34–35  
 ABO antibody titers 38–39  
 direct antiglobulin testing 39  
 infectious diseases 40–41  
 RBC antigens 38
- syphilis 45–46
- TT virus 521
- United States regulations 37, 38, 39–40, 41–42
- vital sign measurement 32
- West Nile virus 45, 518
- World Health Organization requirements 39
- Zika virus 45
- see also* pretransfusion testing; testing
- Sd<sup>a</sup> antigen (SID) 97–99
- biochemistry 97
- biological roles 98–99
- expression 97–98
- molecular biology 97–98
- serology 97
- synthesis 97

- transfusion 97  
 xenotransplantation 98–99
- Se** *see* secretor-positive
- secondary culture 536–537
- secondary erythrocytosis, apheresis 283–284
- secondary immune deficiencies (SID), immunoglobulins 245
- secondary immune responses 119
- secondary stroke prevention, red cell exchange 280–281, 333
- secretor gene (*FUT2*) 81, 82, 89
- secretor-positive (Se) individuals, ABO system 81–82
- separation
- blood components 33
  - activated prothrombin complex concentrate 227–228
  - albumin 222–224
  - alpha<sub>1</sub>-proteinase inhibitor 228
  - antithrombin III 230
  - C1 inhibitor 229–230
  - factor IX 232
  - factor VIII 233–234
  - factor X 227
  - factor XI 228–229
  - factor XIII 227
  - fibrinogen 231
  - immunoglobulins 224–227, 238–240
  - pathogen reduction 219–221
  - prion safety 222
  - prothrombin complex concentrate 231–232
  - red blood cells 151–152
  - validation of virus reduction/removal 219–220
  - virus filtration 221
  - virus inactivation 220–221
  - virus reduction 219–220
  - von Willebrand factor 232–233
  - see also* purification
- sepsis, neutropenia 192
- septic phlebitis, blood donation 52
- sequence-specific oligonucleotide probe hybridization (SSOP) 628
- sequence-specific primer polymerase chain reaction (SSP) 628
- sequestration, platelets 167, 392–393
- serological assays, human leukocyte antigens 630
- serology
- ABO system 82
  - I blood group 90–91
  - Lewis blood group 87
  - P blood group system 94–95
  - Rh system 100–104, 106–107
  - Sd<sup>a</sup> antigen 97
- serum albumin in saline, therapeutic plasma exchange 261–262
- seventeenth century
- animal transfusions 4
  - animal-to-human transfusions 4–5
  - discovery of circulation 3
- severe combined immunodeficiency (SCID), gene therapy 644
- severe congenital neutropenias (SCNs) 191–192
- shigatoxins (Stx) 97
- Shigella dysenteriae*, P blood group 97
- Shwachman–Bodian–Diamond syndrome (SBDS) 191
- sickle cell disease (SCD) 330–335
- acute chest syndrome 279, 333
  - acute multiorgan failure syndrome 279–280
  - acute splenic sequestration crisis 334
  - acute stroke 332
  - aplastic crisis 333–334
  - ASPEN 280
  - chronic transfusion therapy 280–281
  - contraindications 335
  - delayed hemolytic transfusion reactions 343–344
  - hemoglobin S testing 47
  - indications for transfusion 331–335, 332
  - LW system 107
  - ophthalmological complications 335
  - pathophysiology 330–331
  - pregnancy 334
  - preoperative red cell exchange 281, 334
  - primary stroke prevention 332–333
  - pulmonary hypertension 334–335
  - red cell exchange 279–281
  - Rh system proteins 106
  - secondary stroke prevention 333
  - sickle hepatopathy 334
  - silent cerebral infarcts 280
  - stroke prevention 280–281, 332–333
  - symptomatic anemia 333–334
- SID *see* Sd<sup>a</sup> antigen; secondary immune deficiencies
- silenced CE phenotype 103
- silent cerebral infarcts (SCI), prevention 280
- simple transfusion
- hemolytic disease of the fetus and newborn 371
  - red blood cells 340–341
- skeletal muscle tissue engineering 652–653
- skin allografts 675
- skin disinfection 535
- skin tissue engineering 658
- SLE *see* systemic lupus erythematosus
- Society for the Advancement of Blood Management (SABM) 293, 294
- software, blood establishments 70
- solid organ transplantation, ABO system 85
- solid-phase adherence assays 125
- solid-phase testing, human leukocyte antigens 630
- soluble transferrin receptor, iron-deficient erythropoiesis 57
- solvent and detergent (S/D)
- pathogen reduction 493, 496, 500
  - plasma treatment 33, 220–221
  - plasma usage 302–303
- somatic hypermutation 120
- sore arms, blood donation 52
- source plasma (SP)
- ABO antibody titers 38–39
  - collection 34–36
  - chronic effects 63–64
  - direct antiglobulin testing 39
  - donor screening 34–35
  - infectious disease testing 34–36, 40–41
  - RBC antigens 38
- Southeast Asian Ovalocytosis (SAO) 339
- SP** *see* source plasma
- Spanish Civil War 9
- specialized therapeutic plasma processing 275–276
- Spectra Optia instrument 254
- splenectomy
- immune thrombocytopenic purpura 408–409
  - warm autoimmune hemolytic anemia 350
- Splenomegaly 192–193
- sponge rinsing 301
- SSOP *see* sequence-specific oligonucleotide probe hybridization
- SSP *see* sequence-specific primer
- polymerase chain reaction
- Stafford Act (1988) 15
- stem cell transplantation
- ABO system 85–86
  - myeloid growth factors 610
- steroid responsive encephalopathy
- associated with autoimmune thyroiditis (Hashimoto's encephalopathy) 269
- stimulation, granulocyte production 195
- storage
- activated prothrombin complex concentrate 444
  - andexanet alfa 450
  - antifibrinolitics 447
  - cell-based immunotherapies 308
  - cryoprecipitate 308
  - desmopressin 448
  - equipment 308–309
  - granulocytes 195, 308, 487
  - hematopoietic stem cells 308, 620–621
  - plasma 308
  - plasma composition effects 203

- storage (*cont'd*)
- platelets 181–187, 307
    - additive solutions 184–185
    - containers 183
    - cryopreservation 186
    - lesions 183–184
    - lyophilization 186–187
    - metabolic needs 181–183
    - pH 182–183
    - respiratory capacity 183
    - temperature 181–182, 186
  - protamine 449
  - prothrombin complex concentrates 443
  - recombinant activated factor VII 445
  - recombinant clotting factors 308
  - red blood cells 150–157, 307
    - additive solutions 152–154
    - anticoagulant-nutrient solutions 152
    - effectiveness 155–156
    - frozen storage 156
    - leukocyte reduction 155
    - pathogen reduction 155
    - pH 153
    - principles 150–151
    - rejuvenation 156
    - storage containers 154–155
    - temperature and time lapse 154
    - washing 155
  - vitamin K 446
  - storage containers
    - platelets 183
    - red blood cells 154–155
  - storage-generated cytokines 555
  - streptococcal infections, PANDAS 269
  - Streptococcus suis*, P blood group 97
  - structural hemoglobin mutations 47, 327–328
  - structure, ABO antigens 81–84
  - Stx *see* shigatoxins
  - subcapsular cataracts, leukapheresis risk 54–55
  - subcutaneous IgG (SCIG) therapy 240–243
  - sulfur metabolism, red blood cells 148–149
  - superinfection, hepatitis D 513
  - surgical outcomes
    - anemia 454
    - red blood cell administration 455–457
  - surgical patients
    - activated clotting time 459
    - acute nonvolemic hemodilution 462
    - anemia 453–455
    - anticoagulants 459–460
    - antiplatelet therapies 460–461
    - blood avoidance techniques 461–462
    - conservative practice 461
    - direct oral anticoagulants 460
    - guidelines 464–466
    - plasma administration 464–470
  - platelet administration 466, 469–470
  - point-of-care viscoelastic assays 459
  - preoperative hemostatic testing 457–459
  - red blood cell administration 455–457, 464
  - topical hemostatic therapies 462–463
    - see also* perioperative practice; preoperative...
  - syncopal reactions, blood donation 51–52
  - syphilis
    - testing 45–46
    - transfusion-transmitted 541
  - systemic lupus erythematosus (SLE)
    - apheresis 273
    - neutropenia 192
    - pregnancy 375
  - T-cell immunotherapies
    - cancer 633, 636–637
    - chimeric antigen receptor-expressing 634, 635–637
    - donor lymphocytes 633–634
    - engineered 634–637
    - nonengineered 633–634
    - regulatory 634
    - tumor-infiltrating lymphocytes 634
    - viral infections 637–638
  - T-cell independent response 119
  - T-cells, chimeric antigen receptor therapy 488
  - TA-GVHD *see* transfusion-associated graft-versus-host disease
  - TACO *see* transfusion-related circulatory overload
  - TAD *see* transfusion-associated dyspnea
  - TAIFI *see* thrombin-activatable fibrinolysis inhibitor
  - TALENs *see* transcription activator-like effector nucleases
  - Tamm-Horsfall protein (uromodulin) 97
  - targets, red blood cell administration 341–342
  - TBO-filgrastim (Granix) 608
  - temperature, red blood cell storage 154
  - tendon allografts 674–675
  - terminal platelet production 160–161
  - test tube methods, Rh typing 124–125
  - testing
    - biovigilance 129–130
    - coagulation 204–208, 457–458
    - compatibility 122–130
    - donated blood 37–48
      - ABO antibody titers 38–39
      - ABO system 37–39, 38
      - automated systems 40
      - babesiosis 45
      - blood derivatives 46
      - Chagas' disease 46
      - cytomegalovirus 46
    - direct antiglobulin testing 39
    - donated blood 39, 40
    - donor communication 41–42, 41
    - donor reentry 41, 42–43
    - extended blood group antigen typing 47
    - false positives 39
    - hemoglobin S 47
    - hepatitis A 47
    - hepatitis B 43–44
    - hepatitis C 44
    - HIV 43
    - human leukocyte antigen 47
    - human T-cell lymphotropic virus 44
    - IgA deficiency 47
    - infectious diseases 37, 39–47
    - lookback 42
    - Parvovirus B19 47
    - platelet antigen typing 47
    - platelets 46
    - pooling 39, 43
    - product management 42
    - RBC antibody detection 38
    - RBC antigens 38
    - recipient notification 42
    - regulations 39–40, 41–42
    - resource-limited contexts 40–41
    - resource-limited settings 40
    - Rh groups 37–38
    - Rh system 37–38
    - sequence 40
    - syphilis 45–46
    - United States regulations 37, 38, 39–40, 41–42
    - West Nile virus 45
    - World Health Organization requirements 39
    - Zika virus 45
  - donor-recipient mismatches 128–129
  - ferritin levels 61–62, 63
  - granulocyte collection 194–195
  - IgG depletion 63
  - limiting blood loss 304
  - neonates, HDFN 368
  - source plasma donors 34–36
    - ABO antibody titers 38–39
    - direct antiglobulin testing 39
    - infectious diseases 40–41
    - RBC antibody detection 38
  - United States regulations 37, 38, 39–40, 41–42
  - World Health Organization requirements 39
    - see also* pretransfusion testing
  - thalassemia 141, 328, 335–337
    - gene editing 645
  - thawing
    - hematopoietic stem cells 621–622
    - plasma 311
  - The Joint Commission (TJC) 17, 72–73
  - theory of planned behavior (TPB) 28

therapeutic phlebotomy 278, 282–285  
hereditary hemochromatosis 284–285  
polycythemia vera 283  
secondary erythrocytosis 283–284  
therapeutic plasma exchange (TPE) 214,  
259–277  
5% serum albumin in saline 261–262  
acute disseminated  
encephalomyelitis 268  
ANCA-associated vasculitis 272  
angiotensin-converting enzyme  
inhibitors 265  
antibody-mediated rejection 274  
anticoagulation 251–252  
autoantibody hemolytic anemia 271  
biochemical changes 263–264  
catastrophic antiphospholipid  
syndrome 271  
children 266  
chronic inflammatory demyelinating  
polyradiculoneuropathy 267  
citrate toxicity 266  
cold agglutinin disease 353–354  
complement-mediated thrombotic  
microangiopathy 270  
complications 264–266  
continuous renal replacement  
therapy 263  
controversial applications 274–275  
cryoglobulinemia 271–272  
cryoprecipitate 262–263  
desensitization in transplant  
recipients 274  
dilated cardiomyopathy 275–276  
exchange volumes 260–261  
extracorporeal membrane  
oxygenation 263  
familial hypercholesterolemia 276  
focal segmental glomerulosclerosis  
272–273  
fresh frozen plasma 262–263  
Goodpasture syndrome 272  
Guillain–Barré syndrome 261, 266–267  
Hashimoto's encephalography 269  
hematologic disorders 269–272  
hemolytic disease of the fetus and  
newborn 370–371  
hydroxyethyl starch 263  
hypergammaglobulinemia 270–271  
immunoabsorption 275–276  
instrumentation 259–260  
Lambert–Eaton myasthenic  
syndrome 268  
LDL removal 276  
N-methyl-D-aspartate receptor antibody  
encephalitis 267  
multiple sclerosis 268  
mushroom poisoning 273  
myasthenia gravis 267  
myeloma cast nephropathy 273  
neurological disorders 266–269

neuromyelitis optica spectrum  
disorder 268–269  
overdose 273  
PANDAS 269  
paraproteinemic demyelinating  
polyneuropathies 268  
phytanic acid storage disease 269  
rejection of transplants 274  
replacement solutions 261–263, 265  
specialized 275–276  
systemic lupus erythematosus 273  
techniques 259–263  
thrombotic microangiopathies 269–271  
thrombotic thrombocytopenic  
purpura 261, 270  
thyroid storm 273  
ticlopidine-associated thrombotic  
microangiopathy 270  
transplantation 273–274  
vascular access 260, 265  
vasculitis 272  
vasculitis with hepatitis B-associated  
polyarteritis nodosa 272  
voltage-gated potassium channel  
antibody diseases 269  
Wilson Disease 273  
*see also* plasmapheresis  
therapeutic platelet transfusions 398–400  
therapeutic thrombocytapheresis 286  
therapeutics, health emergencies 23  
Therasorb 275  
thoracentesis  
plasma administration 468  
platelet administration 469  
thrombin 201, 203  
thrombin time (TT) 205–208  
thrombin-activatable fibrinolysis inhibitor  
(TAFI) 203  
thrombocytapheresis 286  
thrombocytopenia  
alloantigens 418  
alloimmune 417–422  
cancer 394  
chemotherapy-induced 615  
dialysis 394  
dosing 397  
drug-induced immune 411–417, 412  
heparin-induced 394, 413–417  
impaired platelet production 392  
laboratory tests 402–406  
microangiopathic hemolytic anemias  
393–394  
other medications causing 394–395  
passive alloimmune 421–422  
platelet refractoriness 398–399, 422  
platelet substitutes 400  
platelet transfusion 395–400  
platelet transfusions 395–400  
posttransfusion purpura 395, 421  
preoperative transfusions 397–398  
therapeutic transfusions 398–400

thrombopoietic growth factors 611–616  
thrombopoietin receptor agonists 401  
uremia 394  
vaccine-induced 417  
*see also individual thrombocytopenic*  
*disorders...*  
thrombocytosis, apheresis 285–286  
thromboembolic events (TEEs),  
immunoglobulin therapy 242  
thrombolytic agents, paroxysmal nocturnal  
hemoglobinuria 360  
thrombopoietic growth factors 611–616  
adverse effects 613–614  
indications 614–615  
receptor agonists 611–613, 615–616  
thrombopoietin receptor agonists (TPO-  
RAs) 401, 409, 611–613, 615–616  
thrombopoietin (TPO) 158–160,  
162–166, 611  
oncology patients 486  
thrombosis  
erythroid growth factors 605  
thrombopoietic growth factors 613  
thrombosomes, oncology patients 486  
thrombotic microangiopathic anemias  
(TMAAs) 373–374  
thrombotic microangiopathies (TMAs)  
apheresis 269–271  
drug-induced 413  
platelet maturation pathologies 160–162  
thrombotic thrombocytopenic purpura  
( TTP )  
apheresis 261, 263, 270  
pregnancy 374–375  
thyroid storm, apheresis 273  
thyroiditis, apheresis 269  
ticlopidine-associated thrombotic  
microangiopathy 192, 270  
time lapse, red blood cell separation 154  
tissue engineering 648–659  
bone 656  
cardiac 657  
cartilage 657–658  
COVID-19 650  
gastrointestinal 653–654  
lung 648–650  
peripheral nerve system tissue 651–652  
renal 654–656  
reproductive system 650–651  
skeletal muscle 652–653  
skin 658  
urological 658–659  
vascular 656–657  
tissue hypoxia, erythropoietin regulation  
136–137  
tissue processing, human tissue  
allografts 680  
tissue-specific regulation, ABO system 84  
TJC *see* The Joint Commission  
TMA *see* transcription-mediated  
amplification

TMAAs *see* thrombotic microangiopathic anemias  
 TMAs *see* thrombotic microangiopathies  
 toxoplasmosis 528  
 TP *see* *Treponema pallidum*  
 TPB *see* theory of planned behavior  
 TPE *see* therapeutic plasma exchange  
 TPO *see* thrombopoietin  
 TPO-RAs *see* thrombopoietin receptor agonists  
 tranexamic acid (TXA) 377, 447–448  
   adverse reactions 448  
   burn patients 479–480  
   dosing 448  
   indications 447–448  
   manufacturing 447  
   storage 447  
   surgical patients 462  
   trauma patients 479–480  
 transamino, red blood cells 148  
 transbronchial lung biopsy  
   plasma administration 468  
   platelet administration 469  
 transcription activator-like effector nucleases (TALENs) 644  
 transcription factors, erythroid differentiation 133–135  
 transcription-mediated amplification (TMA) testing 39  
 transferrin receptor (CD17) 134  
 transfusion protocols, red blood cells 339–341  
 transfusion safety officers (TSOs) 129  
 transfusion services  
   accreditation 73  
   adverse reaction reporting 70–73  
   defective product reporting 72  
   operational chain 11  
   pretransfusion testing 122–130  
   sample collection 123–124  
   transfusion-related death reporting 71  
   United States regulations 70–73  
   World Health Organization programs 74–75  
 transfusion thresholds, red blood cells 324  
 transfusion-associated dyspnea (TAD) 580  
 transfusion-associated graft-versus-host disease (TA-GVHD) 582–586  
   component irradiation 309–310  
   diagnosis 584–585  
   immunocompromised patients 584  
   immunodeficiency syndromes 583–584  
   incidence 583  
   oncology patients 483  
   pathogen reduction 499  
   pathophysiology 582  
   prevention 252–256  
   treatment 586

transfusion-related acute lung injury (TRALI) 10, 569–579  
 antibodies 571, 574–576, 578  
 biologically active lipids 576  
 blood donation 33  
 CD40 ligand 576  
 children 573  
 clinical features 569–570  
 critically ill patients 577  
 diagnosis 571–572  
 donor investigation and management 577–578  
 human leukocyte antigens 575–576  
 human neutrophil antigens 574–575  
 immunoglobulins 576  
 incidence 572–573  
 inverse 576, 579  
 monocytes 574  
 multiple hit/threshold theory 576–577  
 neonates 573  
 neutrophils 573–574  
 nonantibody 576, 579  
 pathophysiology 570–571, 573–577  
 plasma 215  
 platelets 574  
 pooled plasma 578–579  
 prevention 578–579  
 pulmonary endothelial cells 574  
 therapeutic plasma exchange 265  
 treatment 573  
 transfusion-related circulatory overload (TACO) 563–564, 579–580  
 diagnosis 580  
 incidence 580  
 plasma 215  
 prevention 580  
 treatment 580  
 volume reduction 310  
 transfusion-related deaths, reporting 71  
 transfusion-transmitted infections (TTIs) 507–542  
   1900s 9–11  
   automated testing systems 40  
   babesiosis 45, 523–524  
   bacterial contamination 533–542  
   Chagas disease 46, 524–525  
   chikungunya virus 518  
   cytomegalovirus 519  
   dengue virus 517–518  
   donor screening 34–36  
   ebola virus 520–521  
   Epstein–Barr virus 519  
   hepatitis A 507–508  
   hepatitis B 508–511  
   hepatitis C 511–513  
   hepatitis D 513–514  
   hepatitis E 514  
   hepatitis F 515  
   herpesviruses 519  
   HIV 10, 31, 43, 96, 192, 515–516  
   HTLV 44, 516–517  
   introduction of precautions 10–11  
   leishmaniasis 527–528  
   lymphatic choriomeningitis 521  
   malaria 524–525  
   microfilariasis 528–529  
   pandemic influenza 521  
   parasites 523–529  
   Parvovirus B19 519–520  
   prions 529–531  
   screening 37, 39–47  
     general concepts 39–40  
     resource-limited contexts 40–41  
     *see also individual infectious agents...;*  
       prerelease testing; testing  
   syphilis 541  
   toxoplasmosis 528  
   TT virus 521  
   viral 507–522  
   West Nile virus 518  
 transfusion-transmitted viral infections (TTVs) *see* transfusion-transmitted infections  
 transfusional iron burden 589–592  
 transfusional iron overload *see* iron overload  
 transfusions *see* administration; blood administration  
 Transfusions Medicine Committee 73  
 transmissible spongiform encephalopathies (TSEs) 11, 31, 222, 529–531  
 transmission, hepatitis B 509–510  
 transplantation  
   ABO system 85–86  
   adverse event monitoring 682  
   alloimmune thrombocytopenia 422  
   desensitization 274  
   donation tracking 678–679  
   extraembryonic tissue 677  
   hospital-based tissue services 679  
   human leukocyte antigens 630–632  
   human tissue allografts 670–683  
   immunoglobulin therapy 247  
   Lewis blood group 89  
   oversight 677–678  
   recalls and lookback 682–683  
   reimbursement 683  
   rejection 274  
   Sd<sup>a</sup> antigen 98  
   surveillance 680–682  
   therapeutic plasma exchange 273–274  
   tissue engineering 648–659  
 transplantation-associated alloimmune thrombocytopenia 422  
 transport, blood components 309  
 trastuzumab emtansine 160  
 trauma patients 471–481  
   burns 478–481  
   coagulation factor replacement 480  
   coagulopathies 474–475

- cryoprecipitate 480  
 damage-control resuscitation 475–478  
 freeze-dried plasma 480  
 prothrombin complex concentrates 480  
 recombinant activated factor VII 480  
 tranexamic acid 479–480  
 whole blood 480
- treatment  
 allergic responses 559  
 anaphylaxis 560–561  
 cold agglutinin disease 353–354  
 combined factor V and factor VIII deficiency 438  
 drug-induced immune hemolytic anemia 357–358  
 dysfibrinogenemia 441–442  
 factor V deficiency 438  
 factor VII deficiency 438–439  
 factor X deficiency 439  
 factor XI deficiency 440  
 factor XIII deficiency 440–441  
 febrile nonhemolytic reactions 556–557  
 hemolytic transfusion reactions 550  
 hemophilia 426–434  
 heparin-induced thrombocytopenia 414–417  
 hepatitis B 511  
 hepatitis C 511–513  
 HIV 516  
 hypodysfibrinogenemia 441–442  
 immune thrombocytopenic purpura 407–411  
 iron overload 592–596  
 paroxysmal cold hemoglobinuria 354–355  
 paroxysmal nocturnal hemoglobinuria 360–363  
 posttransfusion purpura 421  
 prothrombin deficiency 437  
 transfusion-associated graft-versus-host disease 586  
 transfusion-related acute lung injury 573  
 transfusion-related circulatory overload 580  
 von Willebrand disease 436–437  
 warm autoimmune hemolytic anemia 349–351  
*see also* administration
- Treponema pallidum* (TP) 45–46, 541
- Trima Accel instrument 254
- Trypanosoma cruzi* (Chagas' disease) 46
- TSEs *see* transmissible spongiform encephalopathies
- TSOs *see* transfusion safety officers
- TT *see* thrombin time
- TT virus (TTV) 521
- TPP *see* thrombotic thrombocytopenic purpura
- TTVs (transfusion-transmitted viral infections) *see* transfusion-transmitted infections
- tumor-infiltrating lymphocytes 634
- twentieth century 7–11  
 anticoagulants development 8–9  
 blood bank development 9  
 blood groups discovery 7–8  
 compatibility testing 8  
 component therapy development 9  
 direct transfusions 7–8  
 discovery of hepatitis viruses 9–10  
 HIV/AIDS epidemic 10  
 transfusion-related acute lung injury 10  
 transfusion-transmitted infections 9–11  
 variant Creutzfeld–Jakob disease 11
- TXA *see* tranexamic acid
- UCB *see* umbilical cord blood
- Udenyca *see* pegfilgrastim-cbqv
- ultraviolet A (UV-A), with amotosalen 490, 492, 495, 497, 498, 499
- ultraviolet C (UV-C) 493, 496, 497, 500
- ultraviolet (UV) treatment  
 with amotosalen 490, 492, 495, 497, 498, 499  
 plasma 221, 495–496, 497  
 with riboflavin 492, 495, 497, 498, 499
- umbilical cord blood (UCB) 621
- unexpected antibodies  
 donor-recipient testing 128  
 Rh typing 124
- unit labeling 129
- United States Food and Drug Administration (FDA)  
 blood collection establishment regulations 66–70  
 defective product reporting 72  
 global regulation 78  
 guidance documents 68–69  
 hospital transfusion services regulation 71–72  
 inspections 69  
 iron depletion regulations 58–59
- Office of Blood Research and Review 68
- reportable deviations 69
- screening regulations 39–40, 41–42
- service error reporting 71–72
- United States (US)  
 albumin regulations 223–224  
 blood collection establishments regulations 66–70  
 blood services organizations 12  
 blood testing requirements 37, 38, 39–40, 41–42  
 hospital transfusion services regulations 70–73
- universal leukocyte reduction 11
- upper airway procedures  
 plasma administration 468  
 platelet administration 469
- upper gastrointestinal bleeding, red blood cell administration 321
- uremia, thrombocytopenia 394
- urological tissue engineering 658–659
- uromodulin (Tamm–Horsfall protein) 97
- US Code of Federal Regulations (CFR) 67–68
- UV-A *see* ultraviolet A
- UV-C *see* ultraviolet C
- vaccinations  
 gene therapy 644  
 health emergencies 23  
 hemophilia 432
- vaccine-induced immune thrombotic thrombocytopenia (VITT) 417
- valganciclovir 192
- validation, virus reduction/removal 219–220
- vapor heat, virus inactivation 220
- variant Creutzfeld–Jakob disease (vCJD) 11, 31, 529–531
- vascular access  
 apheresis 252, 260, 265  
 central venous catheters 260, 466–467, 469
- vascular tissue allografts 676–677
- vascular tissue engineering 656–657
- vasculitis, apheresis 272
- vasculitis with hepatitis B-associated polyarteritis nodosa, apheresis 272
- vasovagal reactions, blood donation 50
- vCJD *see* variant Creutzfeld–Jakob disease
- vectors, gene therapy 643–644
- VEL blood group system 117
- venipuncture 307
- VER 117
- VGCC *see* voltage-gated calcium channel
- VGKC *see* voltage-gated potassium channel
- Vibrio* Spp, ABO type 86
- vinca alkaloids 410
- viral infections  
 chikungunya 518  
 cytomegalovirus 46, 192, 519  
 dengue 517–518  
 ebola 520–521  
 Epstein–Barr virus 519  
 hepatitis A 9–10, 47, 192, 507–508  
 hepatitis B 9–10, 43–44, 192, 272, 507–508  
 hepatitis C 511–513  
 hepatitis D 513–514  
 hepatitis E 514  
 hepatitis F 515  
 hepatitis C 44  
 herpesviruses 519  
 HIV 10, 31, 43, 96, 192

- viral infections (*cont'd*)
- HIV-1 515–516
  - HIV-2 516–517
  - HTLV 44, 516–517
  - human T-cell lymphotropic virus 44
  - lymphatic choriomeningitis 521
  - neutropenia-causing 192
  - pandemic influenza 521
  - Parvovirus B19 47, 96, 333–334, 519–520
  - T-cell therapy 637–638
  - transfusion-transmitted 507–522
  - TT virus 521
  - West Nile virus 45, 518
  - Zika virus 45
- viral testing
- cytomegalovirus 46
  - hepatitis A 47
  - hepatitis B 43–44
  - hepatitis C 44
  - human T-cell lymphotropic virus 44
  - Parvovirus B19 47
  - source plasma donors 34–36
  - West Nile virus 45
  - Zika virus 45
- viral-inactivated purified fibrinogen
- concentrate 211
- virus inactivation, plasma 220–221
- virus reduction, plasma 219–220
- viscoelastic assays, point-of-care 459
- vital sign measurement, donors 32
- vitamin K 446–447
- adverse reactions 447
  - dosing 447
  - indications 447
  - manufacturing 447
  - storage 447
- vitamin K antagonists (VKA), reversal 443–447
- VITT *see* vaccine-induced immune thrombotic thrombocytopenia
- VKA *see* vitamin K antagonists
- voltage-gated calcium channel (VGCC) autoantibodies, apheresis 267
- voltage-gated potassium channel (VGKC)
- antibody diseases, apheresis 269
- volume reduction, platelets 186, 485
- von Hippel-Landau protein (pVHL)
- 136–137
- von Willebrand disease (vWD) 434–437
- acquired 451–452
  - classification 434–436
  - clinical features 434
  - diagnosis 434–436
  - treatment 436–437
- von Willebrand factor (vWF) 436–437
- cryoprecipitate transfusion 210–211
  - in plasma 201–202
  - platelet formation 160–161
  - purification 232–233
- von Willebrand factor-cleaving protease 201
- vWD *see* von Willebrand disease
- WAIHA *see* warm autoimmune hemolytic anemia
- Walter, C. 9
- warfarin effect, plasma transfusion 214
- warm autoimmune hemolytic anemia (WAIHA) 106–107, 346–351
- clinical features 348–349
  - DAT positive without hemolytic anemia 349
  - DAT-negative 349
  - epidemiology 346
  - pathophysiology 346–348
  - prognosis 351
  - treatment 349–351
- warming blood 313
- WAS *see* Wiskott Aldrich syndrome
- washed cell salvage 300–301
- washing
- platelets 186
  - red blood cells 155
- WB *see* whole blood
- WBDPs *see* whole blood derived platelets
- WBIT *see* wrong blood in tube
- weak D phenotype 101–102, 103, 105–106
- weak subtypes, ABO system 82–83
- West Nile virus (WNV) 45, 518
- WFH *see* World Federation of Hemophilia
- white blood cells 188–248
- apheresis 195, 286
  - granulocytes 193–199
  - leukocytapheresis 286
  - neutrophils 188–193
- WHO *see* World Health Organization
- whole blood derived platelets (WBDPs)
- collection 32
  - separation 33
- whole blood (WB)
- administration
    - burn patients 480
    - trauma patients 480
  - collection 32
  - pathogen reduced, function tests 496–498
- Wilson Disease, apheresis 273
- Wiskott Aldrich syndrome (WAS) 161
- gene therapy 644
- WNV *see* West Nile virus
- World Federation of Hemophilia (WFH) 78
- World Health Organization (WHO)
- blood and blood component safety programs 74–75
  - Blood Regulators Network 75
  - blood screening requirements 39
  - blood services recommendations 12–13
  - Expert Committee on Biological Standardization 75
- World War II 9
- wrong blood in tube (WBIT) 306
- xenotransplantation, Sd<sup>a</sup> antigen 98
- Xg blood group system 114
- XK protein 111–112
- Zarxio *see* filgrastim-sndz
- ZFNs *see* zinc finger nucleases
- Zixtenzo *see* pegfilgrastim-bmez
- Zika virus (ZIKV) 45
- ZIKV *see* Zika virus
- zinc finger nucleases (ZFNs) 644