Textbook of
Pediatric Hematology and Hemato-Oncology

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Dedicated to

Zinet Currimbhoy
Teacher of Teachers
in Pediatric Hematology and Oncology
for whom
Children with blood disorders and cancer were the greatest teachers!

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Foreword

It is both a pleasure and a privilege to write a foreword for this first edition of *Textbook of Pediatric Hematology and Hemato-Oncology*. Immense advancement has been made over the past decade in the field of hematology and hemato-oncology providing not only enhanced accuracy in the diagnosis of inherited and acquired malignant and nonmalignant blood disorders but also new therapeutic strategies that have resulted in improved patient outcomes. The book with its illustrations, tables, figures and clinical photographs provides a concise yet thorough comprehension of pediatric hematology and will definitely become a reference book for the students and the practitioners.

I congratulate the Editor-in-Chief, MR Lokeshwar; Editors, Nitin K Shah and Bharat R Agarwal; Co-editors, Mamta Vijay Manglani and Anupam Sachdeva; Publication Editor, Asha Pillai and the 72 reputed and dedicated pediatric hematologists and hemato-oncologists from across the world who after 3 years of exhausting brainstorming sessions, brought out the remarkable book with 50 chapters spread over 7 sections.

I am sure that the information contained herein will be a benchmark in the understanding of the best approaches to the patients that we evaluate and manage.

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Foreword

I am delighted to write the foreword for the first comprehensive book *Textbook of Pediatric Hematology and Hemato-Oncology*. Postgraduate students look up to their teachers for a book by the editorial team of Doynes in the field of Pediatric Hematology and Hemato-oncology with their vast experience in the field and past experience in writing will fill in this void and meet the expectations of the postgraduate students. The Editor-in-Chief, MR Lokeshwar has been well supported by the other editors, Nitin K Shah, Bharat R Agarwal, Mamta Vijay Manglani and Anupam Sachdeva. The descriptive text along with clinical pictures make it an interesting reading material. It covers a vast spectrum of conditions making it very useful to the reader. I congratulate the entire team which includes the contributors, section editors, editorial board and M/s Jaypee Brothers Medical Publishers (P) Ltd, New Delhi, India, who have worked relentlessly to achieve this milestone for the book. Above all, my hearty congratulations to the ever-enterprising respected Dr MR Lokeshwar who thought of this brilliant idea of *Textbook on Pediatric Hematology and Hemato-Oncology*. I wish the best for the success and popularity of the publication among both postgraduate students and practitioners. It will be a landmark publication on Pediatric Hematology Oncology in the history of medical literature.

Vijay N Yewale
President, 2014
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Preface

Pediatric Hematology and Hemato-Oncology as a pediatric specialty has developed rapidly in the Western countries since last few decades and is now catching-up in developing countries. Gone are the days when the 'adult' hematologists and hemato-oncologists used to treat pediatric patients in need of specialized services. To begin with several pediatricians got self-trained in pediatric hematology and hemato-oncology and pioneered training in field of pediatric hematology and hemato-oncology in the form of several informal and formal courses throughout the country. This created interest in budding pediatricians to join this field by undergoing fellowships or even formal training in pediatric hematology and hemato-oncology abroad and now in India. The last step towards the growth of this specialty has been starting of 2 years of Post-Doctorate Fellowship in Pediatric Hematology and Hemato-Oncology by the National Board in India with several pioneering centers now offering this course since last 7 years, as well as 1 year Post-Doctorate Fellowship by the Maharashtra University of Health Sciences (MUHS) and even by the Indian Academy of Pediatrics (IAP). Several formally trained Pediatric Hematologists and Pediatric Hemato-oncologists are now providing the specialized services in private and public hospitals.

With the interest created in the field of pediatric hematology and hemato-oncology, there was also a felt need by the students as well as practitioners alike to have a dedicated textbook on pediatric hematology and hemato-oncology. While there are several reputed textbooks on pediatric hematology and hemato-oncology, many of them are also elaborate and at times bogged down with details of molecular science which may not necessarily fulfill the needs of students and practitioners who are looking at complete yet concise book.

With the single aim in mind of having a complete and yet easy-to-read textbook on pediatric hematology and hemato-oncology, we have attempted to bring out the first edition after 3 years of brain-storming and grueling process. The 50 chapters spread over 7 sections and authored by 72 reputed pediatric hematologists and hemato-oncologists from India and abroad make the book elaborate enough to give enough to all the readers, yet curtailing it to more than 500 pages making it concise enough! Further powered by illustrations, tables, figures and clinical photographs will make reading a unique and memorable event for the readers. Each chapter is further enriched by references at the end and is peer-reviewed making it scientifically as complete as possible. This being the first edition is bound to have some errors which might have escaped our attention in spite of our best efforts. We would request all our readers to send their feedback to us which will help us improve upon in the subsequent editions.

We are sure that the book will become a reference book for the students and a desk companion to the practitioners!

Editors

Acknowledgments

We would like to express our gratitude to many people who saw us sail through the publication of *Textbook of Pediatric Hematology and Hemato-Oncology* by providing support, talking things over, read, write, offering comments and helping us in bringing out the book.

We wish to especially thank the following people for their contributions:

- Shri Jitendar P Vij (Group Chairman), Mr Ankit Vij (Group President), Mr Tarun Duneja (Director-Publishing) without whom the book would have not found its way, Ms Samina Khan (Executive Assistant to Director-Publishing) for guiding us throughout, Mr KK Raman (Production Manager), Mr Sunil Dogra (Production Executive) and other staff of M/s Jaypee Brothers Medical Publishers (P) Ltd, New Delhi, India, for assisting us in the editing, proofreading and designing skills.
- All the authors/contributors mentioned in the list of contributors.
- Special thanks to Mr Harish Raut for assisting us in the editing, proofreading and designing skills.
- All the patients and families for allowing us to continue to learn the subject.
- We would also like to thank our family members for their support.

Last but not least, we beg forgiveness of all those who have been with us over the course of years and whose names, we have failed to mention.

Editorial Board

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SECTION 1

Physiology

CHAPTERS OUTLINE

CHAPTER 1

Ontogeny of Erythropoiesis

Nirav Buch

DEVELOPMENT OF HEMATOPOIESIS

Hematopoiesis occurs in three different waves in humans: yolk sac liver and the bone marrow named so based upon the main sites of hematopoiesis. Their characteristics reflect the oxygen needs and the characteristics of the developing embryo (Fig. 1).[1]

Yolk Sac Phase

After 19 days of fertilization islands of hematopoietic tissue appear in the yolk sac and develop within the vasculature.[1] Initially, clusters of mesodermal cells called hemangioblasts develops in the extra-embryonic region. They are initially solid but later inner cells disappear and peripheral cells acquire morphology of vascular endothelium, opening up vessel lumens. Cells adhering to these endothelium form hematopoietic cells and are called blood islands (Figs 2A to C). These cells (endothelium and hematopoietic precursors) show CD 34 expression (Figs 3A and B).[2]

They are macrocytic and contain embryonal hemoglobins: Gower I (ζ2ε2), Gower II (α2ε2) and Portland

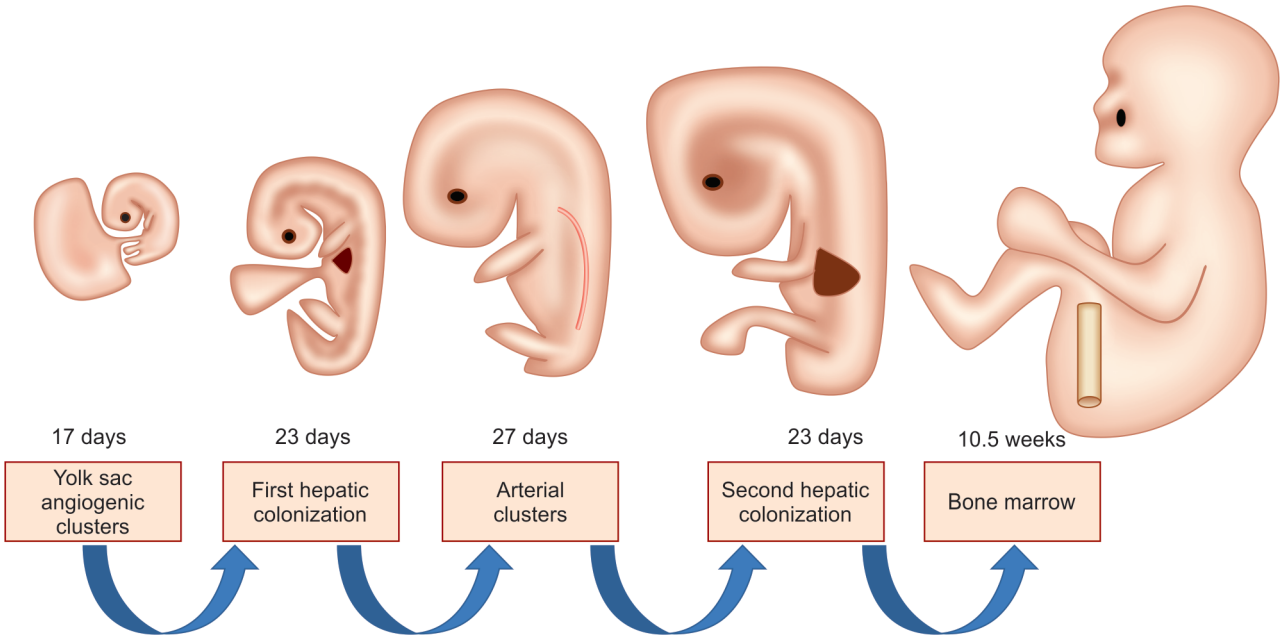
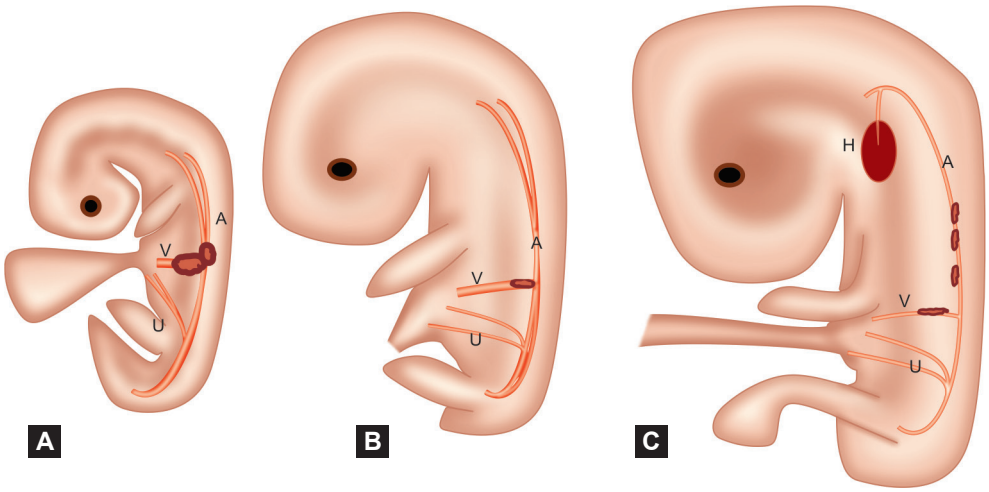
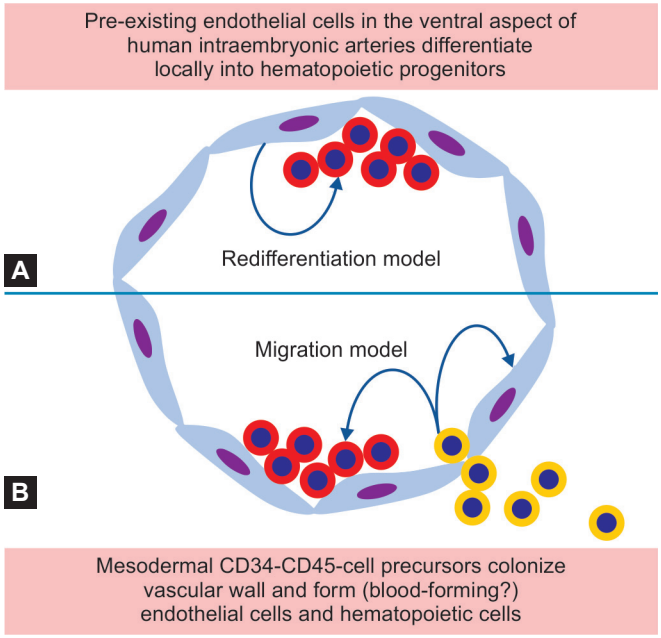


Fig. 1 Chronology of appearance of hematopoietic stem cells in the developing human embryo[2]



Figs 2A to C Sequence of emergence of hematopoietic stem cell cluster within the human embryo. (A) Beginning from 27 days of development, scattered groups of a few hematopoietic stem cells appear, adhering to the aortic endothelium in the preumbilical region. Groups of 2 to 3 cells are also often detected in a more rostral region, where the aorta is still bifurcated; (B) From day 30, the hematopoietic cell clusters increase in size and groups of cells are also encountered at the bifurcation of the vitelline artery, always associated with the ventral aspect of the vascular endothelium; (C) The size of hematopoietic progenitor clusters attains several hundreds of cells at 36 days of development. At subsequent stages, stem cell clusters undergo gradual decrease till 40th day

Abbreviations: A: Aorta; V: Vitelline duct; U: Umbilical Arteries; H: Heart.



Figs 3A and B Possible origin hematopoietic stem cell emergence within the human embryo.[2] (A) Redifferentiation hypothesis at 27 days of development, pre-existing endothelial cells in the ventral aspect of human intraembryonic arteries differentiate locally into blood cell progenitors; (B) Migration hypothesis scattered mesodermal CD34-CD45–cell precursors colonize the ventral vascular wall and give rise to (blood-forming?) endothelial cells and hematopoietic cell clusters

I ($\zeta 2\gamma 2$)—and later in the development hemoglobin F ($\alpha 2\gamma 2$). Cells are large and are nucleated. These consist mainly of erythroid precursors but during 6th and 7th week few megakaryocytes also develop.[1]

This first wave of erythrocyte production in the yolk sac is known as primitive, and erythrocyte production that takes place in is known as definitive erythropoiesis.[2]

Onset of Blood Circulation

Primitive erythrocytes are detected in embryo at day 21 (3 somite stage) suggesting vascular connections between the yolk sac and the embryo.[2]

Transition from Yolk Sac to Hepatic Erythropoiesis

Primitive nucleated erythroblasts are seen in early hepatic rudiment from 4.5 to 5 weeks onwards. They rapidly decrease in number and are replaced by definitive macrocytes. At day 23, rare CD 34 negative cells of erythromyeloid lineage are detected followed by CD 34 +ve cells at day 30 suggesting two distinct waves of hepatic colonization.[2]

Hepatic Phase

Hepatic colonization of hematopoietic progenitors start by 6 weeks and the liver becomes a major hematopoietic organ in the 2nd trimester with about half of nucleated

cells of liver being erythroid precursors. These are smaller than their yolk sac predecessors and result in forming anucleate red cells that are megaloblastic. Chief hemoglobin is HbF. The maturation of cells is extravascular in association with macrophages of the erythroid islands. The hematopoiesis now is multilineage and has erythroid myeloid megakaryocytic and lymphoid precursors.[1]

Bone Marrow Phase (Table 1)

With hepatocyte proliferation hematopoiesis becomes restricted in the liver and bone marrow hematopoiesis begins in the fetal bone marrow by 16th to 18th weeks. Fetal marrow becomes major site for hematopoiesis by 6th month of gestation. Hematopoiesis is multilineage with normoblastic maturation. Chief hemoglobin contents are HbF and HbA. Fetal marrow has dominant erythropoiesis with M : E ratio at about 1:4.[1]

CYTOKINE REGULATION OF ONTOGENY AND HEMATOPOIESIS

Several cytokines play an important role in hematopoiesis. They are granulocyte colony-stimulating factor, interleukin (IL)-6, IL-1, IL-4, IL-9, insulin growth factor-1 and EPO. EPO plays an important role in erythropoiesis. Loss of EPO/EPO receptor leads to failure of fetal erythropoiesis causing fetal death. In adults, EPO provides antiapoptotic and proliferative signals to erythroid precursors (Fig. 4).[1]

Hematopoietic Cytokines, Transcription Factors and Lineage Commitment

Close Relation with Endothelium

The endothelium and hematopoietic progenitor cells share several antigens supporting theory of hemogenic endothelium or hemangioblasts. They are SCL, GATA-2, C-kit, AA-4.1, CD34, Flit-3 ligand, Sca-1, VEGFR-1 and -2, only with the exception of CD45 (Fig. 5).[4]

Table 1 Comparison of embryonic, fetal and adult erythropoiesis[3]

	Yolk sac	*Liver*	*Bone marrow*
Lineages	Erythroid	All	All
Stem cell	Cycling	G0	G0
Erythroid site	Yolk sac	Liver	Bone marrow
Nucleated RBC	Yes	No	No
α-globulin	ζα1, α2	α1, α2	α1, α2
β-globulin	ε	γA, γD	βδ

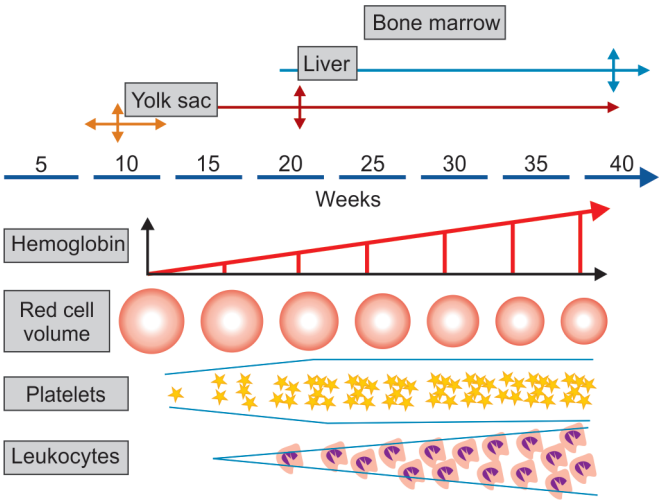


Fig. 4 The phases of embryonal and fetal hematopoiesis. There is a considerable overlap and gradual transition from stage to stage[1]

Transcriptional Regulation of Early Hematopoietic Development (Fig. 5)

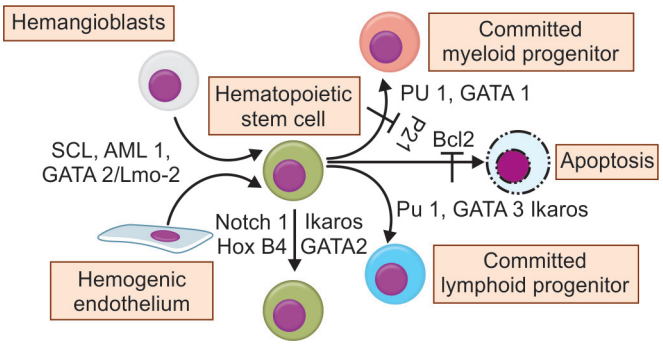


Fig. 5 Important transcription factors for primitive and definitive hematopoiesis are SCL (stem cell leukemia hematopoietic transcription factor), GATA-2 and Lmo-2. AML-1 is required for definitive hematopoiesis[4]

Regulation of Self-renewal and Differentiation of HSCs

Transcription factors HoxB4 and Ikaros, activated nuclear form of Notch1, cell, cycle inhibitor P21, and TGF/BMP-4 family members, TNF-α receptor P55 signaling may be important in the maintenance or promotion of the hematopoietic stem cell renewal. Adjacent cells stromal cells, endothelial cells and local cytokines are thought to play an important role in regulation of HSC cell into renewal or differentiation. TGF-β_1, p21, p27, IL-3, GM-CSF, BMP-4 and TNF-α are some of the important cytokines involved.[4]

Commitment to Lymphoid and Myeloid Lineage (Fig. 6)

After 10 to 15 divisions, the descendent cells daughter cells become fixed towards a single lineage. First cells are believed to be either common lymphoid precursor or common myeloid precursor. These have been differentiated using specific expression patterns.[4] It is believed to be brought about by differential expression of transcription factors. Several candidate transcriptional factors differentially expressed in committed cells have been identified using cDNA library and RT-PCR technologies.[4]

ONTOGENY OF HEMOGLOBIN

Hemoglobin production "switches" from embryonic to fetal hemoglobin at 6 to 7 weeks of gestation and finally to adult hemoglobin at birth (Fig. 8). This is brought about of sequential activation of ζ and ε-genes on chromosomes 16 and 11 respectively. This is unrelated to site of erythropoiesis (Fig. 7).[5] The pattern of expression of genes occurs in 5' to 3' region as development proceeds from embryonic, fetal and then adult life. In fetus, ζ and ε genes are expressed forming Gower 1, Gower 2, and Portland hemoglobins and are seen in yolk sac, para-aortic region and then the liver. Later 2α genes and the 2γ genes are expressed forming hemoglobin F. Later they are downregulated and adult hemoglobins predominate (Table 2 and Fig. 10).[6]

Reduction of MCV, erythroblast count and CD 71 count is believed to be due to switch of erythropoiesis from liver to bone marrow and maturation of hematopoietic tissues.[5]

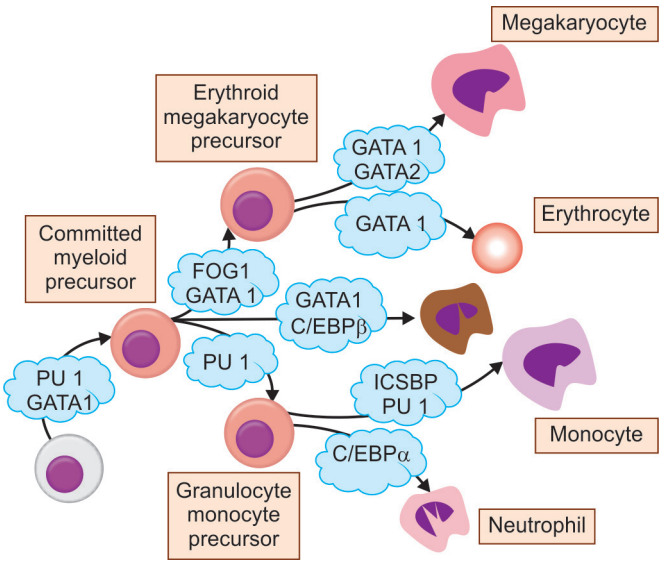


Fig. 6 Transcriptional regulation of common myeloid precursor (CMP) commitment. CMPs differentiate into either common precursors for granulocytic and monocytic lineages (GMPs) or common precursors for both erythroid and megakaryocytic lineages (EMPs). A separate pathway leading to eosinophils is shown may be possible

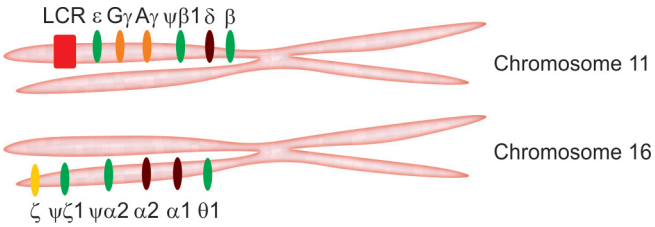


Fig. 7 Chromosome map of human globin chains[9]

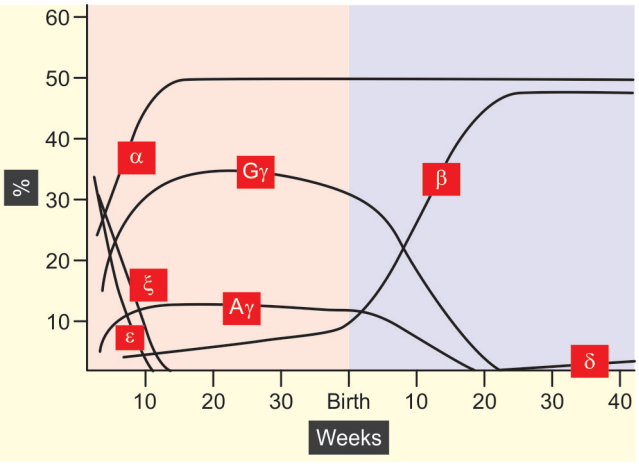


Fig. 8 Production of globin chains during the fetal and neonatal period[9]

Hemoglobin and Hematocrit Rise in Fetal Period (Fig. 9)[7]

It has been determined that hemoglobin concentration increases by 0.21 g/dL and hematocrit by 0.64 percent each week from 22 weeks to 40 weeks. Thus

reference values of hemoglobin and hematocrit can be predicted using the formula : hematocrit = 28.59 + (GA × 0.6359) and hemoglobin concentration = 9.92 + (GA × 0.2087) where GA is the gestation age in weeks. Hematologic values of normal fetuses are described in Table 3.

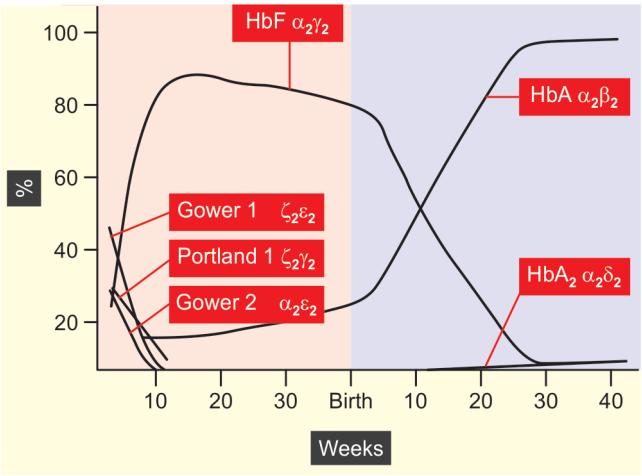


Fig. 9 Hemoglobin production during the fetal and neonatal period[9]

Table 2 Hemoglobins in embryo fetus and adult life[8]

Developmental phase	*Hemoglobin name*	*Chain composition*
Embryo	Portland	ζ2Gγ2 ζ2Aγ2
	Gower I	ζ2ε2
	Gower II	α2ε2
Fetus	F	α2Gγ2
		α2Aγ2
	A	α2β2
Adult	A	α2β2
	A2	α2δ2
	F	α2Gγ2 α2Aγ2

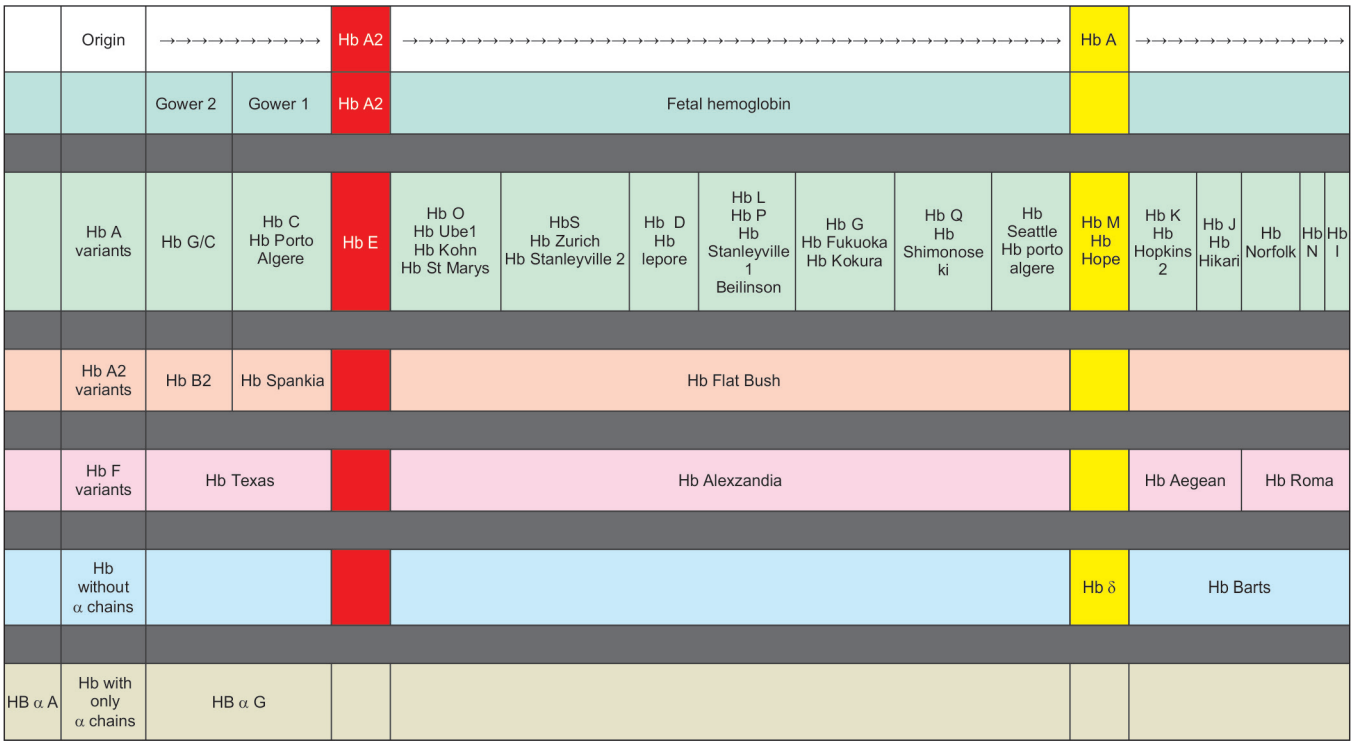


Fig. 10 Relative electrophoretic mobilities on starch gel electrophoresis at pH 8–6 of human hemoglobin variants[10] positions of HbA2 and HbA are marked as red and yellow respectively

Table 3 Hematologic values of normal fetuses

Weeks of gestation	*RBC Count (× 10^{12}/L)*			*Hemoglobin (g/dL)*			*Hematocrit (%)*			*MCV (fL)*			*WBC count corrected (× 10^9/L)*			*Platelet count (× 10^9/L)*		
	Mean	*+ 2 SD*	*–2 SD*	*Mean*	*+ 2 SD*	*–2 S D*	*Mean*	*+ 2 SD*	*–2 SD*	*Mean*	*+ 2 SD*	*–2 SD*	*Mean*	*+ 2 SD*	*–2 SD*	*Mean*	*+ 2 SD*	*–2 SD*
Reference	Millar et al																	
15	2.43	2.17	2.69	10.9	11.6	10.2	34.6	38.2	31	143	151	135	1.6	2.3	0.9	190	221	159
16	2.68	2.89	2.47	12.5	13.2	11.7	38.1	40.2	36	143	155	131	2.4	4.1	0.7	208	265	151
17	2.74	2.97	2.51	12.4	13.3	11.5	37.4	40.2	34.6	137	145	129	2.0	2.8	1.2	202	227	177
18	2.77	3.1	2.44	12.4	13.6	11.2	37.3	41.5	33.1	135	146	126	2.4	3.3	1.5	192	237	147
19	2.92	3.19	2.65	12.3	13.5	11.1	37.5	40.6	34.4	129	135	123	2.5	3.3	1.7	211	259	163
20	3.12	3.48	2.76	13.0	14.1	11.9	39.3	43.4	35.2	126	132	120	2.6	3.8	1.4	170	230	110
21	3.07	3.49	2.65	12.30	13.1	11.5	37.3	40.8	33.8	123	131	115	2.7	3.4	2	223	284	162
Reference	Forestier et al																	
18–21	2.8	3.22	2.38	11.69	12.96	10.42	37.3	41.62	32.98	131.1	142.07	120.13	2.57	2.99	2.15	234	291	177
22–25	3.09	3.43	2.75	12.20	13.8	10.6	38.59	42.53	34.65	125.1	132.94	117.26	3.73	5.9	1.56	247	306	188
26–29	3.46	3.87	3.05	12.91	14.29	11.54	40.88	45.28	36.48	118.5	126.46	110.54	4.08	4.92	3.24	242	311	173
> 30	3.82	4.46	3.18	13.64	15.85	11.43	43.55	50.75	36.35	114.38	123.72	105.04	6.40	9.39	3.41	232	319	145

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CHAPTER 2

Physiology of Blood Coagulation

Shrimati Shetty

The three major components of blood coagulation are platelet, plasma and the endothelium. The platelets adhere to damaged endothelium with the help of von Willebrand factor (VWF) and when activated, they aggregate and make a platform for coagulation factors which then initiate a series of reactions on the damaged blood vessels. The reaction begins with the activation of contact factors which then results in the sequential activation of these clotting factors, resulting in thrombin generation, which converts fibrinogen to fibrin clot. A series of inhibitors to these coagulation factors keep them under check to maintain the thrombohaemorrhagic balance. The most important of these inhibitors are protein C, protein S, antithrombin, tissue factor pathway inhibitor (TFPI) and heparin cofactor II . The fibrinolytic system has an important role in the removal of clot formed, thus maintaining the hemostatic balance. Thrombin has both pro- and anticoagulant role in the coagulation cascade and it is thrombomodulin which converts thrombin into an anticoagulant enzyme by a negative-feedback regulation of its prothrombotic activity through its association with activated protein C (APC). It also has an important role of linking coagulation with fibrinolysis through thrombin activable fibrinolytic inhibitor (TAFI). The deficiency of factors in the Kallekrein-kinin system does not result in a bleeding phenotype; have a role in various noncoagulant functions including apoptosis, proinflammatory and prothrombotic manifestations.

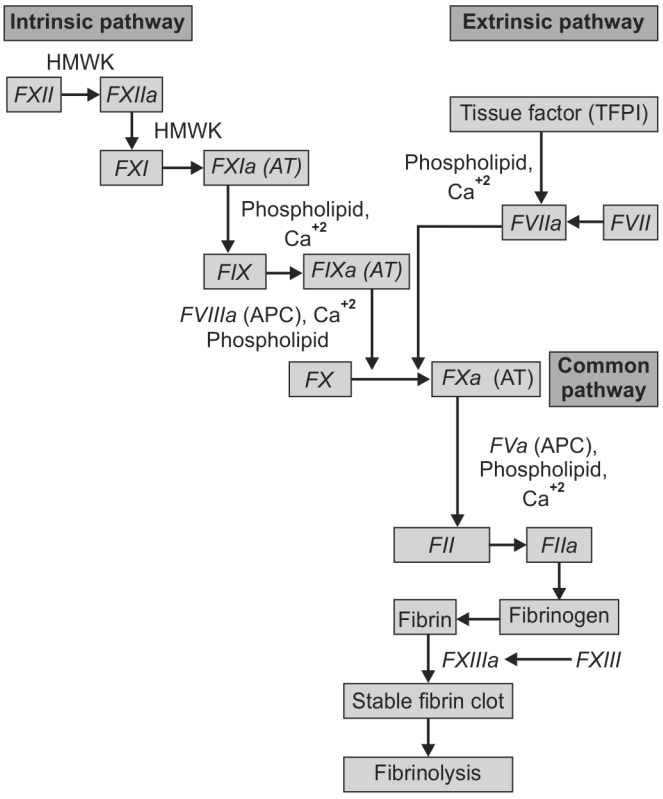
PLATELET ACTIVATION

Upon breach of vasculature, platelets get exposed to collagen and VWF which facilitate their adhesion to the subendothelium through GP-1b-V-IX receptors. The adhesion of the platelets to the subendothelium results in platelet activation which results in an "inside out" signal, causing the exposure of phosphatidyl serine to the outer surface, which forms catalytic surface for its procoagulant activities. The activation also results in secretion of platelet contents along with the exposure of fibrinogen receptors resulting in platelet aggregation at the site of injury. Platelets when activated also trigger the coagulation reaction by providing a catalytic surface which results in the formation of thrombin. More than 250 active substances are released into circulation from the granules present within platelets, when platelet gets activated. Thrombin further stimulates platelet activation through G-protein-coupled protease activated receptors (PAR-1 and PAR-4), resulting in the release of adenosine diphosphate (ADP) and thromboxane A2 (TXA2).[1,2] Thus both platelet activation and coagulation are interdependent. Platelets have a wide array of receptors on their surface which help both in adhesion and aggregation and it is these receptors which have become the target for anti-platelet therapies. Platelet aggregation requires the platelet membrane glycoprotein receptor i.e. GP IIb-IIIa, which helps in platelet aggregation by binding to fibrinogen and fibronectin.[3] Once the platelets are activated, they release different types of granules into the circulation which include ADP, platelet-activating factor (PAF), VWF, serotonin, TXA2 and platelet factor 4.[4] These stimulate and activate more and more platelets resulting in the primary hemostatic platelet plug at the site of wound. It is the same nature of the platelets of showing a quick response to the vascular breach, which is responsible for myocardial infarction or stroke under pathological conditions.

THEORIES OF BLOOD COAGULATION

Morawitz in 1905 put forward the first theory of blood coagulation which is also referred as "four clotting factor" theory.[5] According to this theory, blood clotting is possible due to the presence of four clotting factors, namely, thromboplastin, prothrombin, thrombin and calcium. The thromboplastin gets released on tissue injury which converts prothrombin to thrombin in the presence of calcium. Subsequent to the identification of the remaining coagulation factors, two groups independently put forward a revised blood coagulation model referred to as the "waterfall cascade" model.[6,7] According to this model, all the blood coagulation factors remain in an inactive state and they get activated by the upstream clotting factor. The coagulation cascade shows two distinct pathways i.e. extrinsic pathway initiated by the interaction of tissue factor (TF) with the circulating serine protease factor VIIa. This increases the catalytic activity of FVIIa several fold which then activates factor X to Xa. Similarly in the intrinsic pathway, there is a sequential activation of factors XII, XI, IX the activated form of which then activates factor X to Xa . Thus the two pathways converge into the common pathway by the activation of FX to FXa which then binds to FVa to form prothrombinase, which rapidly converts prothrombin to thrombin. Calcium and phospholipids are required for these sequential activation of coagulation factors. Both extrinsic and intrinsic pathways cannot work independent of each other as deficiencies of any of these factors in either of the two pathways (excluding contact factors and factor XII) can lead to lifelong bleeding tendency (Flow chart 1).

Flow chart 1 The coagulation cascade



Cell Based Model of Blood Coagulation

Though the waterfall hypothesis of blood coagulation has expanded our understanding of blood coagulation, more recent observations demonstrate that the cascade/ waterfall hypothesis does not fully simulate the *in vivo* hemostasis. The cascade model of blood coagulation highlights the importance of coagulation factors *in the* generation of thrombin and overall hemostasis and the role of cells is only to provide phospholipids surface for the coagulation factors to generate thrombin. The cell based model of coagulation shows the significance of cells in coagulation. While the extrinsic pathway is initiated on the TF bearing cells the intrinsic pathway takes place on the platelets. The contact factors and factor XII are not included in the cell based model of coagulation as the deficiency of these factors does not result in bleeding.[8-11] According to this model coagulation takes place in 3 phases i.e. initiation phase, amplification phase and propagation phase.

Initiation Phase

This phase begins with TF bearing cells and is referred to as the 'extrinsic' pathway. TF binds to activated FVII (FVIIa) which in turn activates factor IX to Fix factor X to Xa. Subsequently FXa activates FV to FVa forming the "prothrombinase complex" on TF bearing cells. The dissociation of FXa from these TF bearing cells is prevented by inhibitors like antithrombin and tissue factor inhibitor (TFPI). The FV can either come from activated platelets at the sites of injury or from plasma, both of which can be activated by factor Xa.[12] When present on the TF bearing cells FXa is resistant to its inhibitor i.e. antithrombin. It has also been reported that some amount of FVIIa remains bound to TF even in the absence of injury thus facilitating mild activation of FX and FV all the time.[13]

Amplification Phase

The amplification phase involves the activation of platelets by the initial thrombin generated on the TF bearing cells. Besides thrombin also activates coagulation factors V, VIII and XI on the platelet surface resulting in small amounts of thrombin on the platelet surface.[14]

Propagation Phase

This phase takes place on the surface of activated platelets. FIXa produced during the first step binds to FVIIIa to activate FX to FXa (Tenase complex). FXa then activates FV to FVa which then activates prothrombin to result in thrombin burst (prothrombinase complex). As more and more platelets are recruited to the site of injury, the thrombin generation gets amplified several fold resulting in a thrombin burst which then acts upon fibrinogen to form fibrin. The fibrin clot also consists of erythrocytes, leukocytes and platelets which are held together by fibrin chains. The thrombin activated FXIII then cross links these fibrin chains to form a firm fibrin clot.

The Kallekrein-Kinin System

The Kallekrein-kinin system consists of 3 factors: prekallekrein (PK), high molecular weight kininogen (HMWK) and factor XII (FXII). Their role in the initiation of the intrinsic pathway of coagulation is still not clear as FXII can get autoactivated even in the absence of prekallekrein.[15] The deficiency of any of these factors does not result in bleeding phenotype. Though the absence of these factors prolongs the *in vitro* investigations like activated partial thromboplastin time (APTT), their physiological significance in hemostasis is unclear. Recently there are several reports to support their role in thrombosis.[16] A polymorphism in FXII i.e. 46C/T has shown strong association with arterial thrombosis.[17] The homozygous carriers of this polymorphism were found to have reduced FXII levels which in turn will result in reduced fibrinolytic activity, thus being implicated in thrombosis.

FXIIa also activates FXI to FXIa which then activates FIX to Fix. FXIa along with PK also releases bradykinin, the proinflammatory factor from HMWK.[18]

COAGULATION PROTEASES AND COFACTORS

Biochemically, coagulation factors can be classified into two groups i.e. serine proteases–FVII, FIX, FX, FXI and cofactors-FVIII and FV. The two main cofactors required in most of the steps in blood coagulation including the formation of tenase and prothrombinase complexes are phospholipids and calcium. Calcium also has an important role of binding the coagulation factors to the platelet membrane.[19]

Vitamin K is an important cofactor for coagulation factors which require post translational modification i.e. gamma carboxylation of their glutamic residues which is important for their efficient functioning (factors II, VII, IX, X and their inhibitors i.e. protein C, protein S and protein Z). Two enzymes take part in these processes–vitamin K epoxide reductase (VKORC1) and gamma carboxylase (GGCX). The gamma carboxylase enzyme adds the gamma carboxyl group to the glutamate residues during which Vitamin K epoxide gets reduced. The VKORC1 enzyme converts it into its active form. The gamma carboxylation is required for an effective binding of these serine proteases to the phospholipid surfaces.

Blood Coagulation Inhibitors

Blood coagulation inhibitors as well as fibrinolytic inhibitors neutralize the coagulant or fibrinolytic proteins to prevent excessive thrombosis or hemorrhage . The major anticoagulant pathway is the protein C anticoagulant pathway in which both FVIIIa and FVa required for tenase and prothrombinase complex formation get neutralized. Protein C shows a very high homology to all the remaining vitamin K dependant clotting factors. Protein C shows a very high homology to all the remaining vitamin K dependant clotting factors. Protein S is an important cofactor for activated protein C (APC) in its anticoagulant action on FVa and FVIIIa. A mutation at Arg 506 in factor V gene factor V Leiden) makes it resistant to APC cleavage. Homozygotes for this mutation show 20-30 fold increased risk of thrombosis.[20] As the concentration of thrombin increases it binds to another protein i.e. thrombomodulin which then activates the inactive protein C to its activated form. The APC inactivation by thrombin-thrombomodulin complex takes place on the surface of endothelial cells , where the APC is bound by a receptor i.e. endothelial protein C receptor (EPCR).

Though protein S has initially been reported as a cofactor for APC in neutralizing FVIIIa and FVa, studies have shown that PS can independently neutralize these factors.[21] This is possible because PS generally remains combined to C4BP protein. Besides, PS also has inhibitory action against FXa by facilitating the interaction of FXa with TFPI.[22] It has also been reported that FV acts as a cofactor in the inactivation of FVa by APC and PS.[23]

Antithrombin is another strong inhibitor which inactivates both FXa and thrombin, besides its inhibitory action against IXa, XIa and XIIa along with some of the factors in the fibrinolytic pathway. The activity of AT is increased several fold in the presence of heparin. Though rare, AT deficiency leads to one of the most severe form of thrombophilia. Heparin cofactor II is another inhibitor of thrombin. Protein Z is another inhibitor which is an inhibitor of FXa.

The major inhibitor of tissue factor is tissue factor pathway inhibitor (TFPI) which is expressed on the endothelial cells. The extrinsic pathway involving the activation of X to Xa gets inhibited and there is a shift in the

balance towards the intrinsic pathway of coagulation. TFPI inhibits both TF-VIIa complex and FXa. Thus the initial phase of extrinsic pathway is inhibited. However once Xa takes part in the prothrombinase complex, it becomes resistant to the action of TFPI. Various strategies have been used to use inhibitors of TFPI as a novel therapeutic protocol for haemophilia.[24]

FIBRINOLYTIC PATHWAY

Fibrinolytic pathway plays an important role in maintaining the thrombohaemorrhagic balance. A bleeding tendency is seen in patients with hyperfibrinolysis, and hypofibrinolysis is often accompanied with thrombosis. The major fibrinolytic proteases are plasmin which dissolves fibrin and the two plasminogen activators i.e. tissue plasminogen activator (tPA) and urokinase (uK). One of the important regulators of fibrinolysis is thrombomodulin as it converts thrombin not only to an anticoagulant enzyme but also to a inhibitor of fibrinolysis by activating TAFI·. Fibrinolytic activity is also controlled by inhibitors of fibrinolysis like plasminogen activator inhibitor 1, alpha 2 antiplasmin, thrombin activatable fibrinolysis inhibitor, plasminogen activator inhibitor 2, alpha 2 macroglobulin. Thrombin-activable fibrinolysis inhibitor (TAFI) is a fibrinolytic inhibitor with carboxypeptidase activity and it neutralizes the lysis of fibrin clots by removal of the carboxyl-terminal lysine residues from fibrin.[25]

Platelet Interaction with Coagulant and Anticoagulant Factors

It is now well known that platelets provide the phosphatidyl serine surface for the tenase and prothrombinase reactions to take place due the high affinity of PS membranes to these coagulant proteins. The only vitamin K protein which binds through receptors is thrombin i.e. through PAR1 and PAR4 receptors on the surface of the platelets. But besides this, platelets also have other important functions in coagulation. Platelets have the most important receptor for fibrinogen i.e a2bb3, the congenital absence of which results in a platelet aggregation defect termed as "Glanzmann thrombasthenia". Though TF has been found only in small concentrations on the platelets, its inhibitor, TFPI has been found in high concentrations.[26] Similarly, about 20% of FV in the plasma comes from platelets which when activated comes out of the platelets to take part in the prothrombinase complex.[27] Whether there exists any difference between the plasma FV and platelet FV is not clear. Similarly FVIIa is known to bind platelets via GP 1b-V-IX receptor. Besides FVIIIa and FIXa also bind the phosphatidyl serine membrane facilitating the formation of tenase complex. The binding of FXIa and FXIIa to the platelet surface however is not clear. Among the anticoagulant proteins both PC, PS and TFPI can bind to the platelet surface to neutralize the corresponding proteins. Thus platelets have a important role in blood coagulation.

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CHAPTER 3

Structure, Function and Physiology of Platelets

K Ghosh, Bipin P Kulkarni

Platelets are one of the formed elements of blood. These are anucleate, disc-shaped cells 2 to 4 μ in diameter and are present in blood at a conc. 1,50,000 to 4,50,000/μL. The function of platelets is to bring about primary hemostasis and localize the active coagulant enzymes which develop by a cascade of reactions from inert procoagulant precursors at the site of tissue injury. Platelets admirably performs this job and when somebody gets severe thrombocytopenia (<10,000/μL) or, inherited or acquired platelet defects, their silent but admirable function becomes apparent in the form of microcapillary or more serious cerebral bleeding and on the other extreme, when their function is over—done various types of thrombosis may be the end result.

Platelets are produced from megakaryocytes in bone marrow. Megakaryocytes are naturally occurring giant cells where ploidy value can reach up to 128N, but modal ploidy value for megakaryocytes are 16N to 32N. Megakaryocytes are produced from their precursor progenitor cells by extensive proliferation and endoreduplication of the nuclei. A specific growth factor, thrombopoietin, acts on committed megakaryocyte precursors by acting on c-MPL (CD110) receptor and can produce large number of megakaryocytes and platelets.[1] Presently nonpeptide analogs of thrombopoietin are available (eltrombopag®) and are being used for various causes of thrombocytopenia.

Human hemopoiesis being extravascular, the megakaryocytes once matured, produce very long pseudopod like structures through depolymerization of cellular microtubules and microfilaments. These structures are called proplatelets. Eventually, these proplatelets throw off platelets through the endothelial gaps in the marrow sinusoids.

STRUCTURE AND ULTRASTRUCTURE OF PLATELETS

In a well-stained blood film made from EDTA anticoagulated blood and stained with Romanowsky's stain, platelets appear as single 2 to 4 μ anucleate cells which are purple in color. These cells have granules in the center (Chromomere) and denser peripheral nongranular hyalomere (Fig. 1). If the blood smear is made directly from fingerprick then the platelets are found in small and large aggregates. Occasionally, the platelets can form satellites around neutrophils (Satellitism) due to EDTA dependent cold antibody present in some of the blood samples. Under scanning electron microscope, platelets appear as a small ball with small surface projections. These projections become irregular and longer when platelets are activated (Fig. 2A).[2]

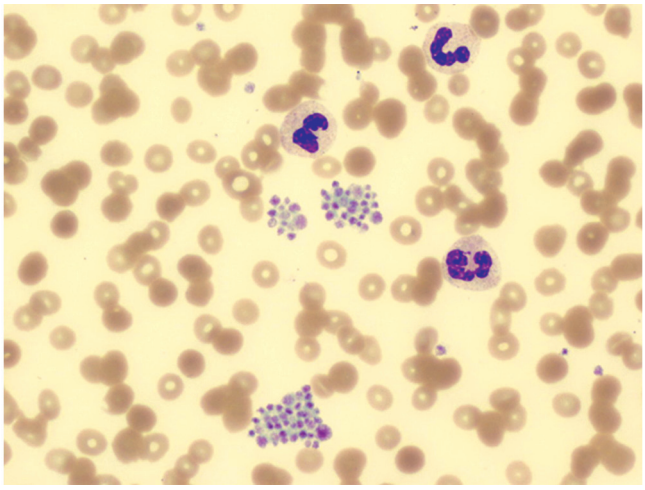
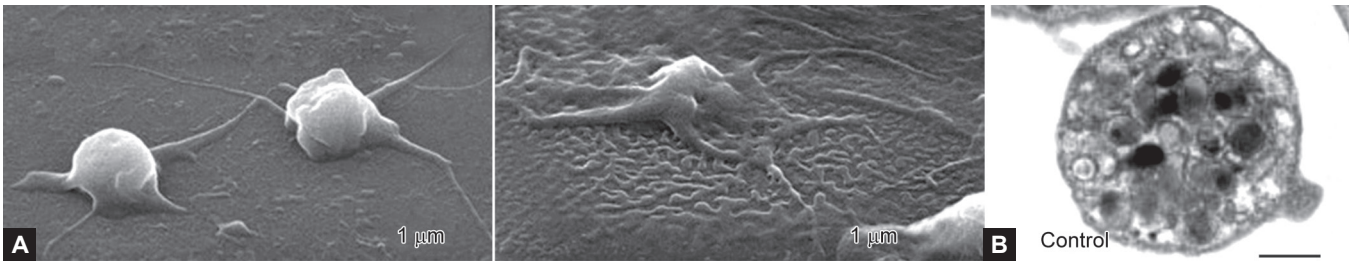


Fig. 1 Peripheral blood showing platelet clumping



Figs 2A and B SEM images of platelets adhered onto topographically structured polymethylmethacrylate (PMMA) surfaces

Transmission electron microscope shows detailed cyto- architecture of platelets (Fig. 2B). It has a trilaminar cell membrane, of which outermost layer is carbohydrate rich and it invaginates the whole cell as surface canalicular system, thus enormously increasing the surface area of the cell membrane on which various glycoprotein receptors are located. Platelets can also secrete a large number of peptides and active chemicals and ions into this canalicular system. Under the cell membrane of platelets the myosin heavy chain, actin and other tubular proteins are arranged in parallel array.

In the cytoplasm of platelet, (1) Dense Granule (called delta granule), (2) Alpha granule, and (3) Lysosomes and mitochondria are seen. Alpha and Delta granule contains large number of peptides and other chemicals (Table 1). These chemicals are responsible for various platelet functions. Some of the chemicals such as 5-hydroxytryptamine (5HT) and peptides are absorbed by the platelets from plasma. While some of the proteins and chemicals are synthesized internally.[3]

Table 1 Platelet granules and their contents[4]

α granules	*Dense granules*	*Lysosomes*
PDGF	ATP	Acid hydrolases
TGF-β	ADP	
CTAPIII	GTP	
PF4	GDP	
TSP	Serotonin	
Fibronectin	Calcium	
Fibrinogen	Magnesium	
Vitronectin		
vWF		
Albumin		
FV, FVIII		
Protein S		
PAI-1		
HMWK		
C1 inhibitor		

Function of Platelets

Platelets main function is its involvement in primary hemostasis, and in addition through release of growth factors such as platelet derived growth factor, platelets also help in wound healing. Hemostatic function of platelet takes place in the following stages. Each of the stage is possible through enactment of a series of biochemical reactions involving ligand-receptor interaction, signal transduction, release of cations like Ca^{2+} and active chemicals and peptides, reverse signal transduction leading to changes in receptor conformation and further reinforcement of interaction and finally activation of coagulation cascade on the surface of the platelet membrane, largely localizing the clot at the site of tissue injury.[5]

Platelet function can be envisioned to take place *in vivo* as:

- Adhesion to injured capillary endothelium and sub-endothelium
- Shape change, aggregation and secretion
- Further aggregation and activation of coagulation.

Adhesion to Injured Capillary Endothelium and Subendothelium

With this reaction, platelets are initially activated to adhere to the collagen of capillary/vascular endothelium at the site of the injury. Adhesion reaction is initiated by interaction of Glycoprotein Ib/IX on the platelet membrane with activated von Willebrand factor (Activated by shear stress at the site of injury) and collagen in the injured vessel. The interaction is strengthened by interactions of platelet receptor Glycoprotein IIb/IIIa with fibrinogen.[6,7]

Shape Change, Aggregation and Secretion

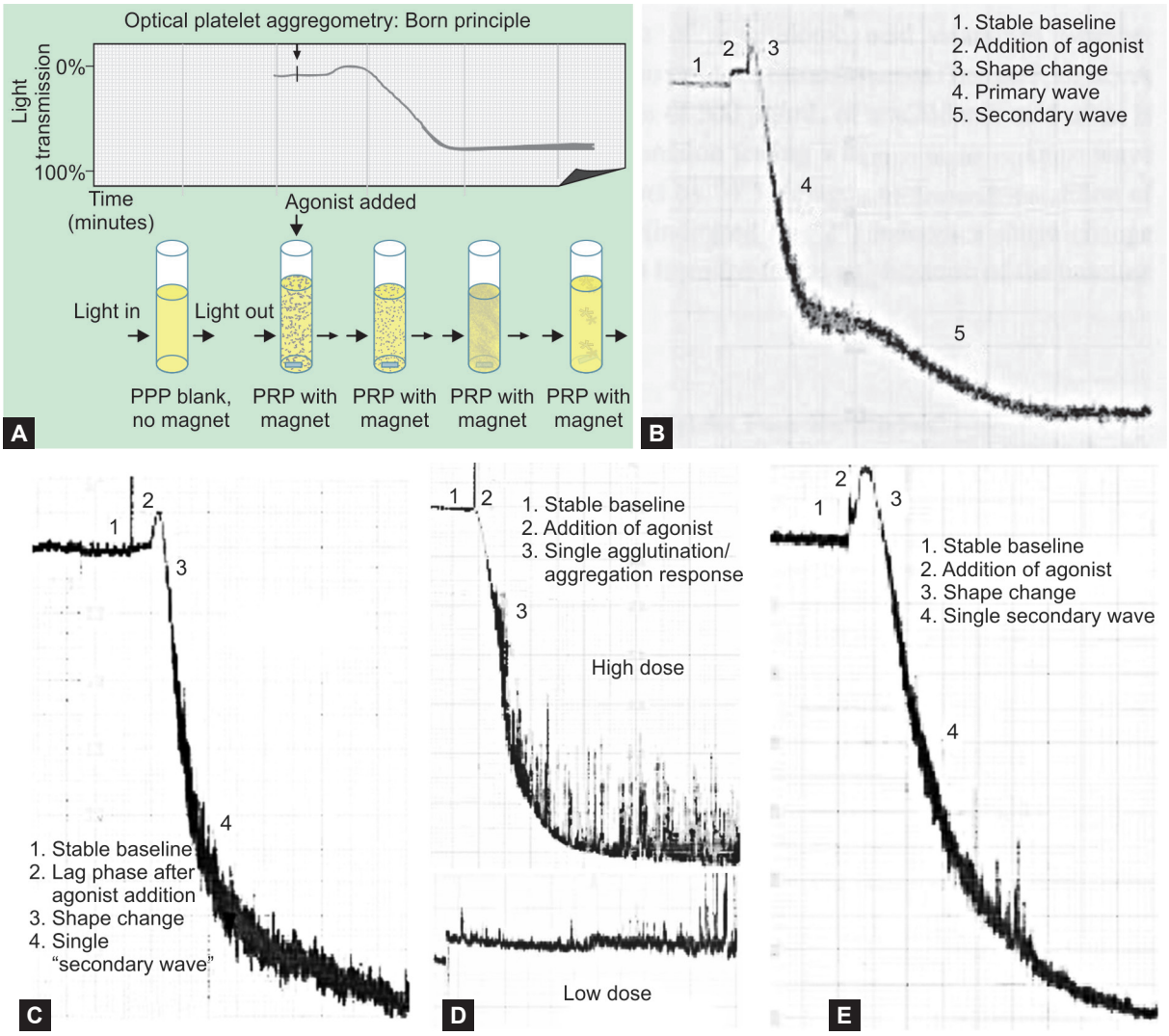
At the site of tissue injury several platelet agonists are released in small amounts, i.e. Thrombin, ADP, ATPase, etc. Injury to the endothelium also exposes subendothelial collagen which is an aggregation promoting ligand for platelets. Small amounts of ADP from surrounding tissue

(red cells) cause platelets to change its shape through contraction of microtubules and microfilaments. This leads to appearance of pseudopodia like structures, centralization of granules. As the process continues and platelets are activated through activated von Willebrand factor and fibrinogen via its GP Ib/IX and GP IIb/IIIa receptors, a graded amount of secretion takes place leading to release of ATP, ADP, 5HT from dense granules, and various peptides involved in coagulation, fibrinolysis, wound healing from alpha granules. This causes initial weak aggregation reaction between platelets into a strong irreversible aggregation of adhered platelets at the site of tissue injury. Platelets also activates production of Thromboxane A_2 during its initial activation and this is another pathway through which platelets are activated and this pathway can be inhibited by drugs like aspirin, which inhibits the enzyme cyclo-oxygenase irreversibly preventing the entry of arachidonic acid into prostaglandin synthesis pathway. Normally, healthy endothelium produces many antiaggregatory and anticoagulant substances. One such antiaggregatory substance is called prostacyclin. When endothelial cells are damaged, postacyclin production is reduced or stopped and this leads proaggregatory thromboxane A_2 to take the upper hand. Healthy endothelium also produces anti-aggregatory nitric oxide and anti-coagulant heparin.[3-7]

With mild stimulus, dense granule products are released and with stronger stimulus alpha granules and finally acid hydrolases from lysosome are released.

Further Aggregation and Activation of Coagulation

As the reinforcement of adhesion and aggregation continues at the site to tissue injury, the liquid phase of blood



Figs 3A to E Platelet aggregometry (A) Born principle, (B) ADP-induced platelet aggregation, (C) Collagen-induced platelet aggregation, (D) Ristocetin-induced platelet aggregation, and (E) Arachidonic aggregation acid-induced platelet aggregation

coagulation is also activated. Initial liberation of tissue thromboplastin produces a small amount of thrombin. This thrombin and some ADP from tissue (mainly red cells) initiate platelet activation. As part of activation of platelets, the platelets change the nature of exposed phospholipids on its surface membrane which in resting state is electroneutral or slightly electropositive. Activation causes the inner membrane phospholipids of platelet to express outside. This phospholipid which is largely phosphotidyl serine is electronegative and supports on it

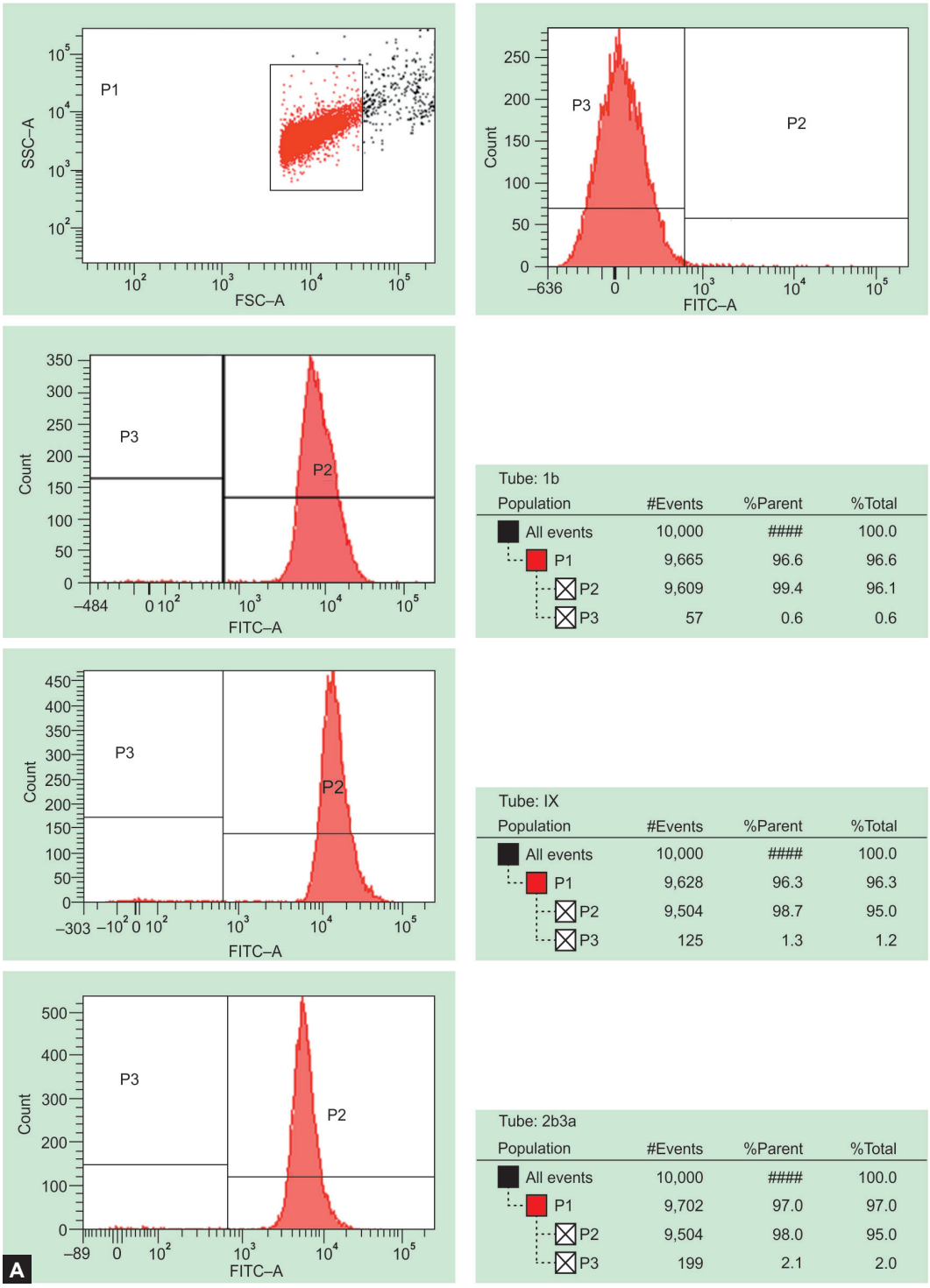


Fig. 4A Platelet flow cytometry: Normal platelet rich plasma (PRP) sample

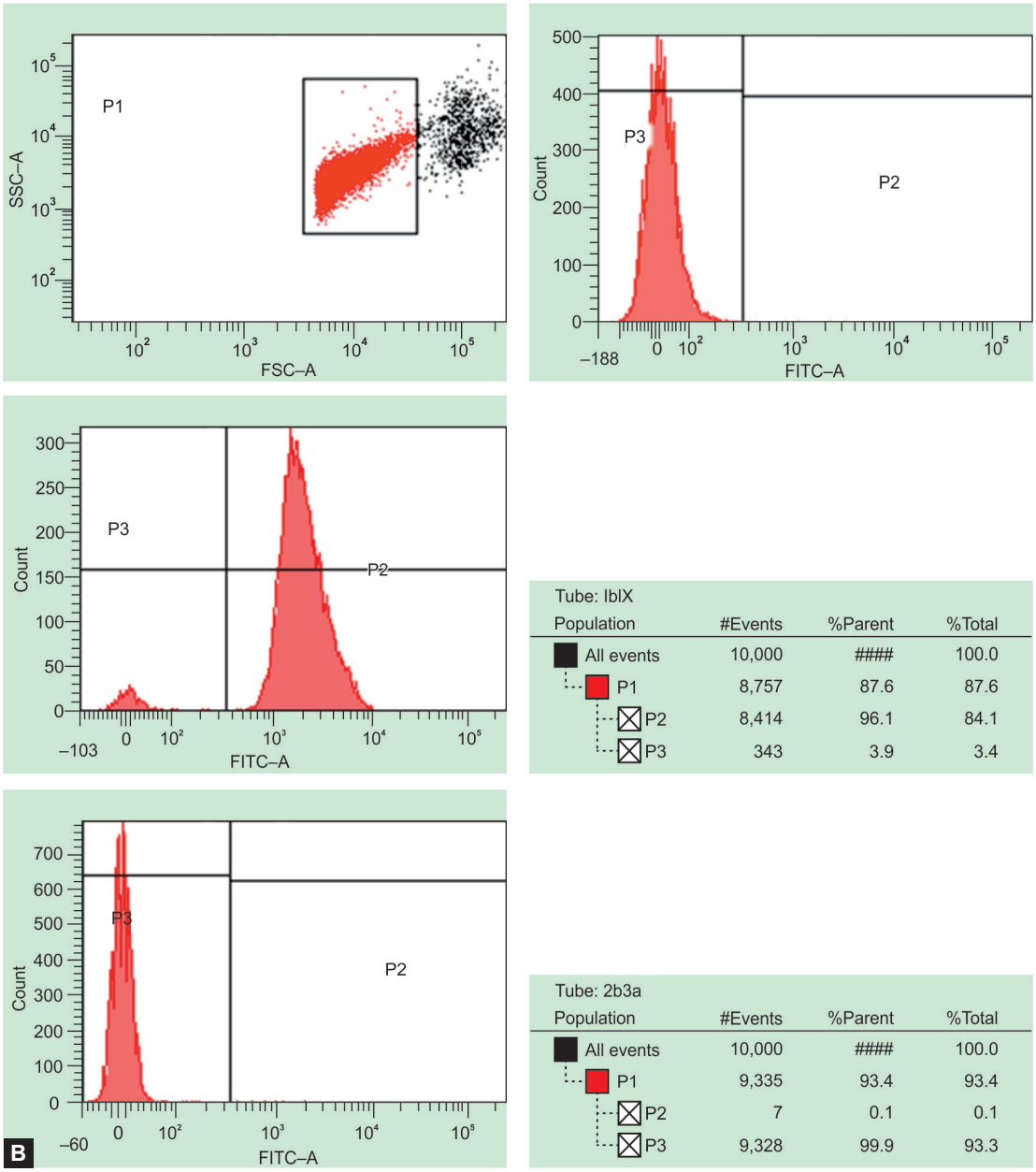


Fig. 4B Platelet rich plasma (PRP) of Glanzmann's thrombasthenia sample

the assembly of Tenase complex (Factor IXa, factor VIIIa, calcium and phospholipids) and prothrombinase complex (Factor Va, Xa and prothrombin). These complexes ultimately produce explosive amount of thrombin locally and strengthens locally adhered and aggregated platelets by a blood clot.[8]

With passage of time, this aggregated platelet along with clot, contracts through platelets actin-myosin machinery and the clot is consolidated.

In the Born aggregometer, PRP is stirred in a cuvette at 37°C and the cuvette sits between a light course and a photocell. When an agonist is added the platelets aggregate and absorb less light and so the transmission increases and this is detected by the photocell.

All the facets of platelet function can be investigated in the laboratory by using platelet aggregometry (Figs 3A to E). Glycoprotein antigen expression and activation on platelet surface can be quantitatied and visualized by platelet flow cytometry (Figs 4A and B). Retraction of platelets can be tested simply in the coagulation laboratory by testing the changes in the volume of blood clot after it has been incubated for 2 hours at 37°C. Ultra-structural

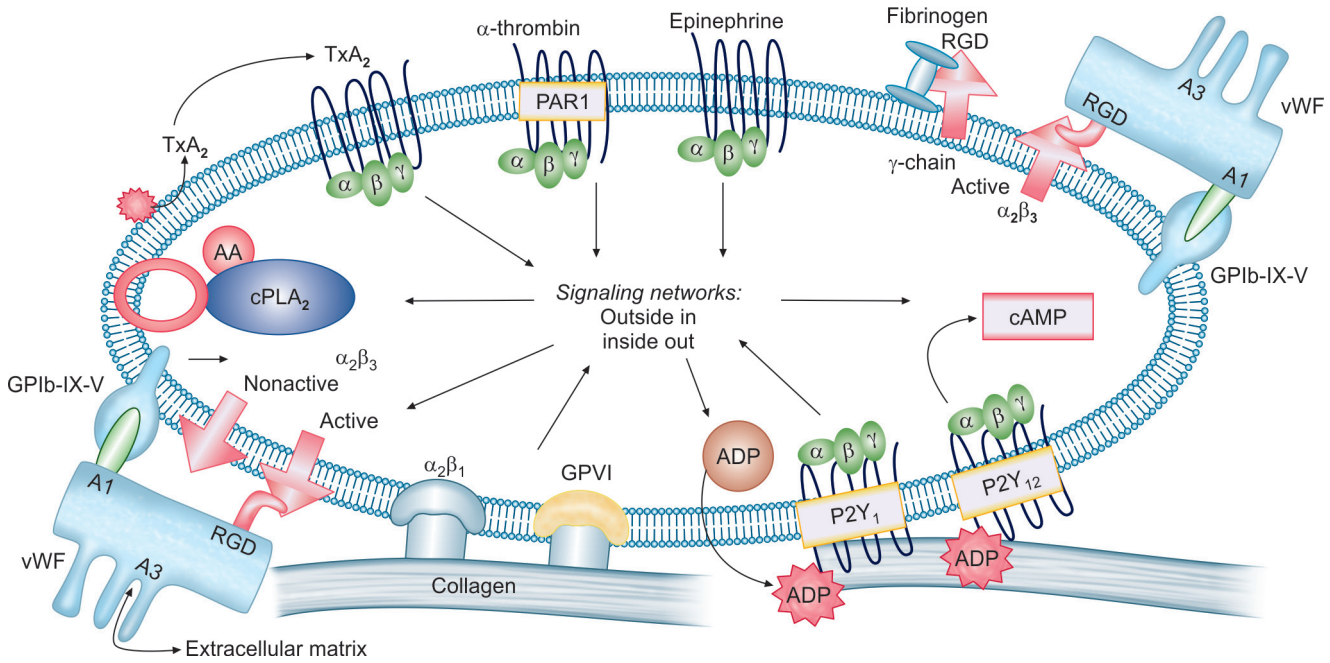


Fig. 5 Schematic diagram of the platelet activation showing the major receptors and effectors.[6-8] Biochemistry of platelet activation (expert review of cardiovascular therapy)

study of platelets can show presence or absence of various granules or granular contents. Secretory functions of platelets can be tested by either seeing ^{3}H labeled 5 HT release or by quantitation of ADP and ATP release by Chemiluminescence assays.

Platelets are biochemical dynamos. Activation of platelets leading to its final aggregation and adhesive state relates to complex chemical interaction of ligands and agonists *in vivo*. A large part of such reactions are still unknown and is being continuously elucidated.

But what we already know is substantial (Fig. 5) and several reactions of this pathway are already exploited in therapy and are useful in understanding hereditary and acquired platelet dysfunction.

To summarize, platelets are of utmost importance in the hemostatic system for the formation of primary hemostatic plug. The granule secretions help recruit more platelets to the primary plug, which eventually provides a surface on which the secondary hemostatic system forms a sturdy clot. The ruptured endothelium gradually repairs itself and the vessel wall structure is restored. Dysfunction of these processes and systems may lead to inherited or acquired platelet function distorders.

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SECTION 2

Neonatal Hematology

CHAPTERS OUTLINE

CHAPTER 4

Variation in the RBC Parameters in the Newborn

MR Lokeshwar, Ambreen Pandrowala, Jayashree Mondkar

The fetal and neonatal period is a most dynamic phase, as during this period there occur profound alterations and adjustments, especially during transit of fetus from dependent hypoxic, intrauterine life—to totally independent extrauterine existence. Erythrocytic system undergoes serial adaptation to meet progressively changing demands of oxygen in embryo, fetus and neonate.

Hematology of newborn, remains a concern even today, not only because of unique blood picture during this period and normal variation in hematological parameters but also in no other period of life is anemia known to occur due to such varied causes.

Although the fetus is nourished and protected by the mother during this period, fetus may suffer adverse effects related to maternal malnutrition, illnesses, infections, drug ingestions, etc.

Moreover, the proximity of two circulatory systems (mother and the baby) also permit the free passage of formed blood elements between mother and fetus as seen in fetomaternal hemorrhage leading to anemia and sensitization to RBC antigens. Also maternofetal hemorrhage may occur, leading to hyperviscosity syndrome.

NORMAL HEMATOLOGICAL VALUES IN THE NEWBORN

Hemoglobin

Various authors have reported values for the normal mean hemoglobin concentration of the cord blood ranging from 15.7 to 17.9 gm% (Table 1). Approximately, 95 percent of all values fall between 13.7 and 20.1 g/dL[1,3,5-11]

Table 1 Hemoglobin concentration of cord blood[3]

	Mean g/dL	*Hb g/dL range*
Mollison (1951)[5]	16.6	–
Dochain et al. (1952)[6]	17.9	14.4 – 21.6
Walker et al. (1953)[7]	16.5	
Marks et al. (1955)[8]	16.9	12.3 – 22
Guest et al. (1957)[9]	17.1	13.6 – 25
Sturgeon (1956)[10]	15.7	–
Dalal and Lokeshwar[3]	16.2	13.2 – 22
Rama Rao[11]	15.13	14.16 – ± 3.26

Shortly after birth the Hb concentration increases by as much as 2.5 to 6 gm%/dL depending on the amount of placental transfusion. The redistribution of body fluids with decrease in plasma volume after birth also accounts for this rise. Failure of hemoglobin to rise during this period is a marker of blood loss.

- Hemoglobin levels return to cord blood values by the end of the first week.
- A significant Hb decrease during this time even if absolute values of Hb are within the normal range is also suggestive of hemorrhage or hemolysis.

During first several hours after birth, there is increase in Hb concentration. Hb increases by 17 to 20 percent of the initial level in the first 24 hours of life but then falls slightly during the next 24 hours.[3,19,25]

At the end of the first week of life, the Hb concentration is as high as it was in the cord blood.

Anemia

Anemia during the first week of life is defined as a hemoglobin value less than 14 g/dL.

Beyond the first week of life many factors influence what is considered as normal hematological parameters in newborn period. Naiman and Oskin,[1] Mollison et al,[5,31] Lokeshwar et al.[3] and others[10,12,13,15] have suggested that 13.5 g/dL be considered as lowest normal value for cord blood Hb. Most authorities suggest that an Hb concentration of 13.5 g/dL in cord blood be considered as the lower limit of normal. Hb value for umbilical artery blood tend to be about 0.5 g/dL higher than sample obtained from umbilical vein.[8]

In a study (Lokeshwar et al.) of 100 newborn babies, only 2 percent of neonates had Hb level less than 13 gm% in cord blood.[2,3]

Hb concentration decreases in both term and preterm infants to reach minimal levels of 9.4 to 14.5 g/dL in term infants by 7 to 9 weeks of age. This "physiological" anemia occurs because of a decline in erythrocyte mass due to the following reasons:

- *In utero* the fetal oxygen saturation is low at around 45 percent, erythropoietin levels are high and RBC production is rapid. Reticulocyte counts are 3 to 7 percent reflecting erythropoiesis.
- With improved oxygen saturation to 95 percent after birth, the erythropoietin levels become undetectable hence RBC production stops, reticulocyte counts are low and the hemoglobin level falls.
- This factor coupled with a reduced life span of fetal RBCs results in anemia that is not a functional one as oxygen delivery to the tissue is adequate as the levels of Hb A and 2,3DPG increased.
- At 8 to 12 weeks, hemoglobin levels reach their nadir (Tables 2 and 3), oxygen delivery to the tissues is impaired, erythropoietin production is stimulated and hemoglobin starts increasing. The hemoglobin and RBC count fall earlier and to a greater extent in preterm infants leading to "anemia of prematurity".

Table 2 Hematological parameters in the full term normal infant studied at LTMG Hospital, Mumbai[3]

	Hb (g%)	*Hematocrit (%)*	*RBC (million/mm³)*
Cord blood	16.2 ± 3.6	46.66 ± 5.1	4.9 ± 1.2
12–18 hours	18.79 ± 2.8	49 ± 4.8	5.3 ± 0.8
72 hours	17.38 ± 3.0	46.9 ± 5.3	5.2 ± 0.6
15 days	16.36 ± 2.2	43.4 ± 4.1	5.01 ± 0.9
28 days	14.17 ± 2.4	42.1 ± 3.8	4.7 ± 1.0

Table 3 MCV, MCH, MCHC and normoblast count in the full term normal infant studied at LTMG Hospital, Mumbai[3]

	MCV (fL)	*MCH (Pg)*	*MCHC (%)*	*Normoblasts (cells/mm³)*
Cord blood	113.04 ± 5.3	34.33 ± 1.4	33.9 ± 0.8	600 ± 186
12–18 hours	108.96 ± 5	35.1 ± 1.9	34.4 ± 0.6	283 ± 122
72 hours	98.54 ± 2.9	35.82 ± 0.8	34.9 ± 0.5	36 ± 48
7 days	96.0 ± 3.4	34.0 ± 1.0	34.6 ± 0.8	–
15 days	95.5 ± 4.0	33.2 ± 9.0	34.54 ± 0.5	–
28 days	96.1 ± 3.2	31.6 ± 0.93	34.2 ± 0.7	–

Table 4 Normal hematological values during the first-two weeks of life in the term infants[1]

Value	*Cord blood*	*Day 1*	*Day 3*	*Day 7*	*Day 14*
Hb g/dL	16.8	18.4	17.8	17.0	16.8
Hematocrit (%)	53.0	58.0	55.0	54.0	52.0
Red cells (mm³)	5.25	5.8	5.6	5.2	5.1
MCV (fL)	107	108	99.0	98.0	96.0
MCH (Pg)	34	35	33	32.5	31.5
MCHC (g/dL)	31.7	32.5	33	33	33
Reticulocytes	3–7	3–7	1–3	0–1	0–1
Nucleated RBCs	500	200	0–5	0	0

Blood Volume

Immediately after birth, the blood volume of term infants may range from 50 to 100 mL/kg, with mean of 85 mL/kg.[4,5,7] If cord clamping is done early for instance at 30 minutes of age, blood volume is 78 mL/kg as compared to 98.6 mL/kg in case of delayed cord clamping. By 72 hours, this difference in blood volume decreases.

The blood volume of premature infants ranges from 89 to 105 mL/kg during first few days of life (Table 4).[6,7] This is mainly because of increase in plasma volume, with the total RBC volume per kg of body weight being the same to that of term infants.

By 1 month of age, this value remains at 73 to 77 mL/kg. Newborns with tight cord around neck and with hyaline membrane disease have low blood volume and those born after late intrauterine asphyxia have higher blood volume.

Hematocrit

Normal values of hematocrit ranges from mean of 51.3 to 56 percent.[1,3,13,20] Just as Hb value, hematocrit value also

shows increase during first few hours of life and reaches original value of cord blood by one week and mean capillary hemotocrit value is two percentage point higher than the mean venous hematocrit value at one week age.

Gatti et al.[16] reported capillary hematocrit on first day of life to be 62.9 ± 3.2 percent reaching 56.6 ± 2.6 by day 7 and 53.7 ± 2.5 percent by day 10, whereas Guest and Brown[17] recorded mean cord blood hematocrit of 52.3 percent and 58.2 percent on first day, 54.31 percent on 3rd day and 54.9 percent on 7th day. Mean capillary hematocrit value is two percentage points higher than the mean venous hematocrit values[1] at 1 week of age.

RED CELL COUNT AND RED CELL INDICES[7,8]

Red Blood Cell Count

Red blood cell count also shows a great variability at the time of birth and ranges from 4.6 to 5.2 million/cumm.[1,3,8,9,18]

Our study of 100 newborn babies[2,3] showed red blood cell count in cord blood 4.9 ± 1.2 million/cmm reaching 5.3 ± 0.8 million/cmm after 12 to 18 hours and 5 ± 1.12 million/cmm at 7th day and stabilizing at same level thereafter throughout the neonatal period. Other studies also have reported similar changes in red blood cell count.[1,8,9,18]

Mean Corpuscular Volume[7,21-24]

Newborn red cells are generally much larger than adults and average diameter of RBC is 8.5 to 9.3 cumm at birth reaching the adult value of 7.5 Cu around 6 months of age.[19-21] Relative macrocytosis is observed in the newborn period. Mean corpuscular volume (MCV) at birth ranges from 104 to 118 fL. Compared to normal adult value of 82 to 92 fL. Mean corpuscular volume rapidly decreases in first week of life and at the age of 2 months, cell size is comparable to those in adult cells.[7,21-24]

MCV values less than 92 fL should strongly suggest alpha thalassemia trait or iron deficiency. MCV is higher in preterm infants 115 ± 5 fL[1,30] and decline to mean value of 95 ± 5 by 7th week of life.[31]

MCH is also increased in newborn period, values ranging from 33.5 to 41.4 Pg as compared to adult values of 27 to 31 Pg.[1,3] However, MCHC in the newborn period is quite similar to that in adult ranging from 30 to 35 percent in newborn and 30 to 36 percent in adults.[1,3]

Reticulocyte Count and Nucleated RBC[1,3,14,19,30]

Average reticulocyte count at birth ranges from 1.6 to 6.2 percent, infants born prematurely have higher retic counts, with values ranging between 6 and 16 percent in infants born between 30th and 34th week (Fig. 2).

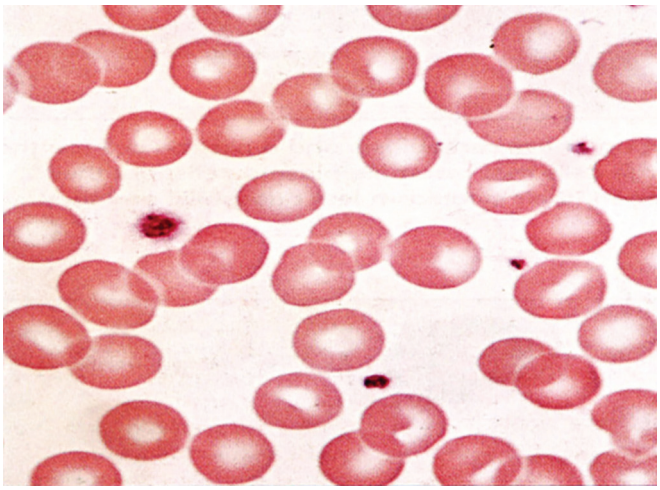

Fig. 1 Normocytic normochromic RBC in newborn

Term infants have an average 7.3 nucleated RBC/100 WBC with normal range of 0 to 24 at birth.

Infants born prematurely have higher retic count with values ranging between 6 and 16 percent in infants born between 30 and 36 weeks of gestation.

This increased retic count in first 2 to 3 days of life reflects very active erythropoiesis during newborn period and value drops to about 1 percent by 7th day of life.[1,17,26,27,29]

Persistent reticulocytosis in cord blood suggests

- Hemolytic process[1]
- Hypoxia
- Blood loss.

The term infant has approximately 500 nucleated RBCs/cμm at birth (0.1% of red cell population) which drops to 50 percent by 12 hours and 20 to 30 nucleated RBC/cμm by 48 hours (Fig. 1). It is unusual to see nucleated RBCs in the peripheral smear of term infant after the age of 4 days.[1,18] In other words the term infants have 7.3 nucleated red cells per 100 leukocytes at birth (ranging from 0 to 24). In premature infants the average figures are 21 NRBC/100 WBC.[28]

In premature babies nucleated red cells may vary 1000 to 1500/mm^3 at birth[1] and decreases rapidly during first week of life. However occasional nucleated RBCs may be seen in the peripheral blood smear of 7-days-old infant.

Increased number of nucleated RBCs are seen in following:[1,28]

- After hemorrhage
- Hypoxia
- Hemolytic disease of newborn
- Down's syndrome and congenital anomalies.

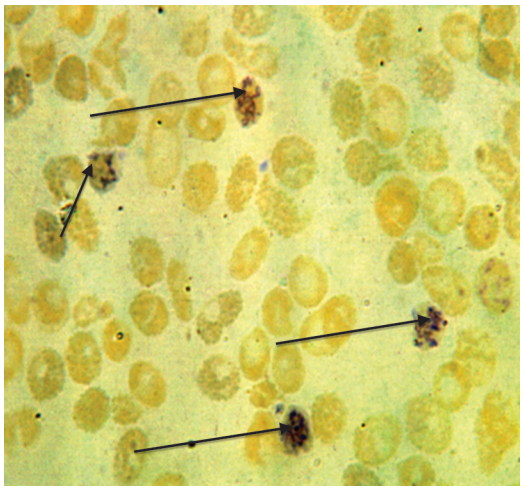

Fig. 2 Reticulocytes count

Factors affecting normal hematological values in newborn[1,3]

- Site of sampling
- Time of sampling
- Treatment of umbilical vessels at the time of delivery
- Position of neonate after delivery
- Gestational age of the infant
- Fetomaternal transfusion
- Maternofetal transfusion.

Various variables influence the interpretation of normal values of Hb, HCT, RBC indices, reticulocyte count at the time of birth and during early weeks of life.

Site of sampling

Capillary sampling collected by skin prick from heal or the toe has a 5 to 10 percent higher hemoglobin concentration than simultaneously collected venous sample.[1]

Oettinger and Mills[29] reported this difference around during 1st hour of life 3.6 to 8 gm%. However, in some instances the capillary hemoglobin–venous Hb difference may exceed 5 to 10 gm%.[29-33]

Stasis of the blood in the peripheral vessels–because of sluggish circulation and resultant transudation of plasma is believed to be the cause of higher capillary hemoglobin as compared to venous hemoglobin.[1]

Capillary/venous hematocrit ratio is greater than 1 in virtually all infants. A ratio higher than 1.2 is observed in premature infants (before 30 weeks of gestation), infants with acidosis (pH less than 7.2) and hypotension.[32]

Thus capillary Hb and hematocrit are falsely elevated in sick infants with altered microcirculation. However, an accurate determination of Hb concentration is most important in the clinical management. Capillary venous HCT ratio gradually decreases with increasing gestational age. Diagnosis of anemia can be missed by evaluating capillary blood Hb.

Moe et al.[33] (1967) in their study of 54 infants with erythroblastosis fetalis 25 out of 41 infants found to anemic whereas only 14 could be considered as anemic according to the value obtained from capillary sample.[34]

Capillary values should not be compared to previously obtained cord venous blood values when one is looking for changes in Hb concentration during first week of life and venous blood should be obtained for this purpose. The selection of the vein is unimportant as blood from different sites of vein gives similar results.[15,31]

Time of sampling

During the first few hours after birth, an increase in hemoglobin concentration takes place (as great as 2.5–6 g/dL)[1,31,33] which is due to placental transfusion that occurs during the time of delivery. Readjustment of the blood volume after birth resulting in increased red cell count, hematocrit and Hb concentration. Magnitude of increase depends on the amount of placental transfusion.[31]

Treatment of umbilical vessels

The amount of blood received by the neonate depends upon time of clamping umbilical cord at birth.[36]

At birth by allowing complete emptying of placental vessel before cord is clamped, the blood volume of infant may be increased by as much as 61 percent.[43] Placental vessels contain 75 to 125 cc of blood, i.e. 1/3rd to 1/4th fetal blood volume.[37]

Within 15 seconds of birth about a quarter and at the end of 1 minute, about half of placental transfusion takes place. Placental transfusion occurs more rapidly in women who receive ergometrin derivatives at the onset of 3rd stage of labor.[1,3,35,37-40]

During first hour of birth, plasma leaves the circulation. Greater the placental transfusion the greater plasma loss. On the third day of life, there are only small differences in total blood volumes regardless of method of cord clamping. In the group with late cord clamping, at 24 hours of age the red cell mass was approximately 32 percent greater and hematocrit was 15 percent higher.[41,42]

Hb percent difference can range from 2 to 4 gm% between early clamping and delayed clamping with hematocrit difference of 2 to 16 percent by various authors[37,40,43,44] during first week of life. By 6 weeks the difference are no longer apparent.[37,43,44]

Position of the neonate after delivery

As umbilical arteries generally constrict shortly after birth, no blood flows from the infant to mother. However, as the umbilical vein remains dilated it permits the blood flow in the direction of gravity.

Infants held below the level of placenta, as it happens during normal delivery, continue to gain blood from placenta, whereas infants held above the placenta which is often seen during cesarean delivery, infant may bleed into mother, thus leading to anemia in the neonate.[35,37-41]

In infants delivered at term with cesarean section, maximal placental transfusion is achieved in seconds after birth.[37] Delay of 3 minutes in cord clamping after cesarean section has been associated with signs of respiratory and metabolic acidosis indicating that earlier clamping may be preferable.[37] Infants with delayed cord clamping had an average red cells mass of 49 mL/kg at 72 hours as compared to 31 mL/kg in infants with immediate cord clamping.[38-42]

However, in infants in whom cord ligation was delayed, decreased incidence of RDS has been reported and hence delayed cord clamping is indicated for premature infants. Premature infants with increased red cell mass, as consequence of delayed cord clamping, have been found to have higher serum bilirubin level.[43,45] There have been reports of circulatory overload and congnitive cardiac failure in the setting of delayed clamping (symptomatic neonatal plethora).

However, when there is obvious evidence of feto-placental or maternal bleeding before or during the birth and infant appears pale and in shock, cord clamping should be delayed, if resuscitation can be given simultaneously.

Thus numerous physiological changes occur in succession and rapidity in fetus and neonates to adapt to the changing pattern of life. This leads to rapid change in normal hematological parameters from fetal period to immediately after birth and throughout neonatal period even hours, days and weeks after birth.

Interpretation of laboratory findings and institution of appropriate therapy requires understanding of the maturational process and normal physiological variations that takes place during this period.[1-3]

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CHAPTER 5

Physiological Anemia of Newborn, Anemia of Prematurity and Role of Erythropoietin in the Management

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Anemia is the most common hematological abnormality in the newborn. Anemia is defined as a hemoglobin or hematocrit value that is more than two standard deviations below the average for a particular gestational as well as chronological age.[1] As a "rule of thumb" a hemoglobin value less than 13 g/dL in first 2 weeks of life is labelled as anemia and warrants evaluation.

Anemia is a sign and not a diagnosis. There are two categories of anemia:

1. Pathologic
2. Physiologic

Pathologic anemia in newborns results from accelerated blood loss, destruction of red blood cells, or a defect at some stage of red blood cell production.

Physiologic anemia is common and a normal physiologic process in term infants. It is typically asymptomatic requiring no intervention.

Anemia of prematurity (AOP) is an exaggerated and pathologic response of the preterm infant (<32 weeks gestation) to the transition from fetal to postnatal life. The etiology, symptomatology, diagnosis, treatment and prevention of anemia of prematurity are addressed.

PHYSIOLOGIC ANEMIA OF INFANCY

Immediately after birth with successful transition from fetal to neonatal circulation, there is increase in blood oxygen content and tissue oxygen delivery. This downregulates erythropoietin production so that erythropoiesis is suppressed. As a result of this, the hemoglobin concentration in healthy full-term and premature infants undergoes typical changes during the first weeks of life. All infants experience a decrease in hemoglobin and hematocrit concentrations after birth. This physiological decrease in Hb level is principally due to a reduced response capacity for erythropoietin (EPO) production in the face of anemia, even in the presence of symptoms and in spite of the fact that erythroid precursors present in both the bone marrow and the blood are highly sensitive to EPO. It is not known why the bone marrow does not respond adequately to this hypoxic stimulus. The maximum decline in Hb is reached by 4 to 12 weeks and is earlier and of greater severity in preterms compared to terms[2] (Table 1).

Table 1 Postnatal changes in Hb

Maturity	*Hb at nadir (g%)*	*Time of nadir (weeks)*
Term	9.5–11	6–12
1200–1500 gm	8–10	5–10
< 1200 gm	6.5–9	4–8

The Hb concentration continues to decrease until tissue oxygen needs are greater than oxygen delivery. Term infants remain asymptomatic and tolerate the physiologic process well, but very preterm infants, <32 weeks gestation, may become symptomatic or the Hb drops to a critical level warranting a blood transfusion. Further in preterms several other factors contribute in exacerbating the physiologic fall in Hb leading to AOP which are discussed later in the article.

ANEMIA OF PREMATURITY

Background

First described by Shulman in 1959, anemia of prematurity (AOP) is a common hematological problem of premature infants. AOP is an exaggerated and pathologic response of the preterm infant to the transition from fetal to postnatal

life. It is often, unlike the physiological anemia in term babies, a true anemia since oxygen delivery is diminished. AOP is a normocytic, normochromic, hyporegenerative anemia that is characterized by the existence of a low serum EPO level in an infant who has what may be a remarkably reduced hemoglobin concentration.

Incidence[3]

AOP typically is not a significant issue for infants born beyond 32 weeks' gestation. Frequency of AOP is related inversely to the gestational age and/or birth weight. Approximately 80 percent of very LBW (VLBW) infants (<1500 g) and 95 percent of extremely LBW (ELBW) infants (<1000 g) receive at least one red blood cell (RBC) transfusion during their stay in the neonatal intensive care unit (NICU). AOP spontaneously resolves by the time most patients are aged 3 to 6 months.

Pathophysiology

The three basic mechanisms which occur singly or in combination for the development of AOP include:

- **Inadequate red blood cell (RBC) production** for a growing premature infant. Fetal and maternal erythropoiesis occurs independently throughout gestation. Erythropoietin does not cross the placenta. Erythropoietin is the main growth factor responsible for erythropoiesis. The liver is the principal site of EPO production during fetal life. By 32 weeks' gestation, RBCs are produced approximately evenly from both the liver and bone marrow. Production of erythropoietin shifts to the peritubular cells of the kidney after term gestation.[4] Interrupting a pregnancy prematurely does not alter these ontological processes. Erythropoietin (EPO) production is thought to be controlled by an oxygen-sensing mechanism in the liver and kidney and both anemia and hypoxia stimulate mRNA transcription and EPO protein production. The liver is less sensitive than the kidney in response to these stimuli.[5] Because it remains the major source of EPO in the preterm infant, RBC production may be blunted.

  Premature infants also have relatively poor iron stores. Erythroid progenitors of premature infants are quite responsive to EPO when that growth factor finally is produced or administered, but the response may be blunted if iron stores are insufficient.[6] Iron stores are accrued during the last trimester of pregnancy; premature infants therefore miss out on this opportunity.
- **Shortened RBC life span or hemolysis:** The average life span of neonatal RBCs is approximately a half to two thirds that of the adult RBC.[7] This shorter life span is due to the lower levels of intracellular ATP, enzyme activity, and carnitine levels as well as the increased susceptibility to lipid peroxidation and susceptibility of the cell membrane to fragmentation. This shorter life span means that the bone marrow must provide new red blood cells at a very high rate just to maintain adequate hemoglobin and hematocrit levels.[8]
- **Increased blood loss:** Preterm infants loose blood in different situations, some more avoidable than others. The most common form of blood depletion in the preterm population is iatrogenic losses secondary to blood sampling. Phlebotomy losses for blood tests are directly related to the length of an infant's stay in the NICU and the severity of illness. Hidden blood loss (e.g. blood adherent to sampling syringes, gauze pads, bedding, tubing) is inevitable and under recognized. Accurate assessment of the volume of blood removed for laboratory testing is imprecise leading to underestimation of laboratory losses. **One milliliter of blood represents 1 percent of total blood volume, especially in preterm babies.** As a result very preterms can lose close to their entire blood volume within a few days of life, necessitating frequent blood transfusions.[9]

  Nutritional deficiencies of iron, vitamin E, vitamin B_{12} and folate may exaggerate the degree of anemia.

  Several other factors predispose to anemia of prematurity.[6,8,10] Some of these factors are common to both the term and the preterm infant, but may be more profound in the premature infant.

Lists factors that can contribute to the exaggerated physiologic anemia of prematurity (Table 2).

Clinical Manifestations[11]

Infants with anemia of prematurity may be completely asymptomatic. The signs and symptoms listed in Table 3 are commonly seen but nonspecific and are not diagnostic

Table 2 Factors contributing to exaggerated physiologic anemia of prematurity

• Decreased RBC mass at birth
• Rapid body growth resulting in hemodilution
• Poor iron stores along with limited ability to build iron stores
• Increased iatrogenic losses from laboratory sampling
• Shorter RBC life span
• Inadequate erythropoietin production
• Initial reliance on the liver as the primary site of erythropoietin production
• Abrupt decline in reticulocyte counts after the first few days of life

Table 3 Signs and symptoms of anemia of prematurity

• Tachycardia	• Apnea and bradycardia
• Tachypnea	• Poor feeding
• Lethargy	• Poor growth
• Pallor	• Metabolic acidosis

by themselves of AOP.[5,7,11,12,17] Historically these clinical symptoms have been attributed to anemia by clinicians despite the fact that there is no relationship established between low levels of hemoglobin and onset of symptoms. Asymptomatic premature infants may tolerate hemoglobin levels as low as 6.5 g/dL without showing any compromise of their physiologic functions. Although some studies conclude that the use of red blood cell transfusions in anemic premature newborns revert these symptoms, others affirm that this association is not so clear. It is now recognized that resolution of these symptoms may be a maturational process, not a hematologic issue.

Diagnosis[12]

In preterm infants, the hemoglobin and hematocrit levels may be followed periodically, although no specific criteria exist as to how often to monitor these levels. A rule of thumb is to monitor the hemoglobin and the hematocrit if symptoms of anemia of prematurity are present. The CBC count demonstrates normal white blood cell and platelet series. The hemoglobin is less than 10 g/dL but may descend to a nadir of 6 to 7 g/dL; the lowest levels generally are observed in the very premature. RBC indices are normal (e.g. normochromic, normocytic) for age. The reticulocyte count is low for the severity of anemia. The finding of an elevated reticulocyte count is not consistent with the diagnosis of AOP. Peripheral blood smear shows no abnormal cells. *Essentially, AOP is a diagnosis of exclusion.*

MANAGEMENT OF ANEMIA OF PREMATURITY

Options available to the clinician treating an infant with anemia of prematurity (AOP) are prevention, blood transfusion, and recombinant EPO treatment.

Goal of Anemia of Prematurity Therapy

- Maintaining the premature newborn's erythrocyte mass as "intact" as possible.
- Provide the growing neonate with adequate oxygen carrying capacity to prevent symptomatic manifestations of anemia while minimizing morbidity from treatment.

Prevention Strategies

- Delay in clamping the premature infant's umbilical cord during birth contributes to reducing the number of transfusions to which they will later be subjected.[13] It may be beneficial to maintain the fetus 20 cm below the level of the placenta during 30 seconds after birth. Although a tailored approach is required in the case of cord clamping, the balance of available data suggest that delayed cord clamping should be the method of choice.
- A direct relationship exists between the volume of blood extracted and the number of transfusions performed. It is therefore necessary to conscientiously limit the number of laboratory tests performed.
- To avoid excess iatrogenic blood loss, the amount of blood collected should be recorded.
- Administration of iron may begin at 1 month of age (2 mg/kg/d) and continued until 6 to 12 months of age. However, iron supplementation should not be started in growing premature infants until adequate vitamin E is supplied in the diet or supplemented; otherwise, iron may increase blood cell hemolysis.[14,15]
- Vitamin E is an antioxidant that is vital to the integrity of erythrocytes. In its absence, these cells are susceptible to lipid peroxidation and membrane injury. The logical conclusion is that vitamin E deficiency might contribute to the anemia of prematurity.[16]
- The use of noninvasive monitoring devices, such as transcutaneous hemoglobin oxygen saturation, partial pressure of oxygen and partial pressure of carbon dioxide, may allow clinicians to decrease blood drawing; however, no data currently support such an impact of these devices.[3]

Role of Blood Transfusion

Despite disagreement regarding timing and efficacy, packed red blood cell transfusions continue to be the mainstay of therapy for AOP. The frequency of blood transfusions varies with gestational age, degree of illness, and hospital practices. The goal of transfusion in infants with anemia of prematurity is to 'restore or maintain oxygen delivery' without increasing oxygen consumption.

Rationale for not Transfusing Based on Hb Value Alone

There is no trustworthy marker for tissue hypoxia, hence it should be considered that the appearance of symptoms attributable to reduced tissue oxygen delivery may not be solely due to low Hb levels, but also to other non-hematological factors such as cardiac output and oxygen partial pressure, the percentage of fetal Hb and the activity

of 2,3 diphosphoglycerate. Therefore considering the Hb level alone when making a transfusion decision, would appear to be inadequate. There is no consensus as to whether transfusions alleviate the signs and symptoms of anemia of prematurity.[17]

Indications for Blood Transfusion

Transfusion practices vary markedly across units and there is a lack of evidence- based studies to guide practice.[18] None of the clinical signs has been consistently useful either alone or as a group in determining when to transfuse an infant with low hemoglobin of (physiologic) anemia of prematurity and iatrogenic losses. The most common problem associated with anemia of prematurity is identifying the threshold for transfusion. The guidelines use specific hematocrit levels, clinical status, and sometimes infant's age to determine when to transfuse[19,20] (Table 4). In many cases, the hematocrit levels are lower than those used previously. In the Premature Infants in Need of Transfusion (PINT) study, Kirplani et al.[19] demonstrated that transfusion threshold in ELBW infants can be moved downwards by at least 1g/dL without incurring a clinically important increase in the risk of death or major neonatal morbidity.

Issues in Blood Transfusion[11,12]

- **Donor exposure:** A concern for infants who might need multiple transfusions is exposure to multiple donors. The use of multiple donors increases the risk of infection and transfusion reactions. Donor exposure can be reduced for infants who need small volume transfusions (<15–20 mL/kg) by using stored packed red blood cells (PRBCs) from a single unit. This unit is divided into multiple aliquots that are reserved for a specific infant. This procedure has reduced donor exposure to one or two donors for most infants.
- **Preservatives:** Use PRBCs stored in preservatives (e.g. citrate-phosphate-dextrose adenine [CPDA-1]) and additive systems (e.g. Adsol). Preservatives and additive systems allow blood to be stored safely for up to 35 to 42 days. The additives having mannitol should not be used for neonates.
- **Screening:** The risk of cytomegalovirus (CMV) transmission can be reduced dramatically (but not entirely) through the use of CMV-safe blood. This can be accomplished by using either CMV serology-negative cells or blood processed through leukocyte-reduction filters. This latter method also reduces other WBC-associated infectious agents (e.g. Epstein-Barr virus, retroviruses, *Yersinia enterocolitica*).

Table 4 Transfusion guidelines

Hemoglobin (g/dL)	*Mechanical ventilation or symptoms of anemia*	*PRBC volume*
11 or less	Moderate or significant mechanical ventilation requirement (MAP >8 cm H_2O and FiO_2 > 0.4)	15 mL/kg PRBCs over 2–4 hours
10 or less	Minimal mechanical ventilation requirement (any mechanical ventilation or CPAP >6 cm H_2O and FiO_2 >0.4)	15 mL/kg PRBCs over 2–4 hours
8 or less	No mechanical ventilation requirement and one or more of the following present: • 24 or more hours of tachycardia (HR >180) or tachypnea (RR >80) • An increased oxygen requirement from the previous 48 hours • An elevated lactate concentration (2.5 mEq/L or more) • Weight gain <10 g/kg over previous 4 days while receiving 100 kcal/kg per day or more • An increase in episodes of apnea and bradycardia (10 or more episodes in a 24 hours period or 2 or more episodes in 24 hours requiring bag-mask ventilation) while receiving therapeutic doses of methylxanthines • Undergoing some surgery	20 mL/kg PRBCs over 2–4 hours (divide into two 10 mL/kg volumes if fluid sensitive)
Less than 7	No symptoms and an absolute reticulocyte count < 100,000 cells/mL (RBC x % reticulocyte count)	15 mL/kg PRBCs over 2–4 hours
• Do not transfuse to replace blood removed for laboratory tests or low hematocrit alone unless above criteria are met		

Abbreviations: CPAP: Constant positive airway pressure; HR: Heart rate; MAP: Mean airway pressure; PRBC: Packed red blood cell; RBC: Red blood cell; RR: Respiratory rate.

Why Minimize Transfusions in Neonates? (Table 5)

Although transfusion may be lifesaving, like all medical interventions, it is not without risk. Following are hazards which need to be taken into account prior to transfusing blood:

- The number of infectious agents that may potentially be transmitted by this route continues to grow and is the most feared complication.
- Risk of fatal transfusions contaminated with bacterial agents, hyperkalemia and graft versus host disease.
- Alterations of the immune system.
- Inferior quality of practices in developing countries – collection, storage, screening policy and irradiation.

The safest blood transfusion is the one not administered.

There is lack of evidence that early EPO versus late EPO confers any substantial benefits (Role of erythropoietin) As a relative deficiency of EPO is present in the anemia of prematurity it appears logical to supplement EPO in these infants. Recombinant erythropoietin (rEPO) has been studied extensively since it became available for human use in 1980. Despite several large EPO trials, there remains no clear consensus for the efficacy of EPO in neonates, and currently its use remains inconsistent between centers.[3,11,12,20]

Early versus late erythropoietin: A Cochrane analysis showed the use of early EPO (<7days of life) did not significantly reduce the primary outcome of "use of one or more red blood cell transfusions", or "number of transfusions per infant" compared to late EPO (>7 postnatal days) administration.[20,21]

Side effects[20]: Neutropenia and sepsis, rash, hypertension, convulsions, poor weight gain and SIDS have been reported in some but not all studies. Apparently the problem of aplastic anemia reported in adults, was related to a specific type of EPO, and even to a single shipment of this drug sent out by a single laboratory. Animal data and observational studies in humans support a possible association between treatment with EPO and the development of ROP.

Table 5 Approaches to reducing transfusion needs

- Development of, and adherence to, strict guidelines for transfusion
- Reducing iatrogenic blood loss
- Autologous cord transfusion
- Targeted use of erythropoietin
- Iron supplementation

Current status of erythropoietin:[3,11,12,20] Clearly, rEPO has efficacy in stimulating erythropoiesis in preterm infants, but success in the elimination or marked reduction in the need for RBC transfusions has not been definitively demonstrated.

- rEPO therapy must be carefully considered and used only when there is strong evidence of its need and effectiveness.
- There is no agreement regarding timing, dosing, route, or duration of therapy exists. Meta-analysis of controlled clinical trials show some benefit to EPO, but they cannot give firm guidelines on its use or recommend its routine use to prevent AOP. In short, the cost-benefit ratio for EPO has yet to be clearly established, and this medication is not accepted universally as a standard therapy for the individual with AOP.
- When the family has religious objections to transfusions, the use of EPO is advisable.

CLINICAL GUIDELINES FOR TARGETED USE OF ERYTHROPOIETIN[22-24]

Indications for Erythropoietin

The greatest hope of success in reducing need for RBC transfusions seems likely in preterm infants who are:

- < 28 weeks.
- Infants 28 to 32 weeks who are <3rd centile for weight. **With** phlebotomy loses expected to be >30 mL/kg.

Dose and Route of Administration

In 750 Units/kg/week given as 3 doses on alternate days by subcutaneous or intravenous injection beginning at the end of the first week of life and for 6 weeks. The subcutaneous route is preferred.

Iron supplementation: 0.4 to 1 mg/kg/day of iron dextran is given via the parenteral nutrition solution. Once enteral nutrition is initiated, 3 mg/kg/day of elemental iron should be provided by supplement. When full enteral nutrition is reached, elemental iron supplementation is increased to 6 mg/kg/day.

Vitamin E (Table 6) supplementation of 25 units/day should be given orally when EPO is administered.

Practice pointers in management of anemia of prematurity (Table 7):

- It seems clear that the major clinical goal for neonates with anemia is to reduce RBC transfusions, so as to minimize multiple donor exposures, infection, and immune risks. EPO alone cannot achieve this goal reliably.

Table 6 Medications used in the treatment of anemia of prematurity[10,15,23]

Generic name	*Dosage/Route*	*Common adverse reactions*	*Comments*
Epoetin alfa	400–1400 U/kg/wk subcutaneously given every day or every other day	Neutropenia, rash, hypertension convulsions, SIDS	Supplement with iron and vitamin E
Ferrous sulfate	6 mg/kg/day PO based on elemental iron	GI upset	Interferes with vitamin E absorption
Folic acid	50 µg/day PO	Caution if used concurrently with phenytoin	May contain benzyl alcohol as preservative
Vitamin E	25 IU/day PO		May induce vitamin K deficiency
Vitamin B_{12}	0.4 µg PO IM		

Abbreviations: GI: Gastrointestinal; IM: Intramuscular; PO: By mouth.

Table 7 Anemia of prematurity

- Anemia of prematurity is a diagnosis of exclusion and of concern in preterms <32 weeks gestation
- The critical threshold of hematocrit in which transfusion is necessary in preterm infants remains to be determined
- Reduction of the need for blood transfusions is associated with rigorous transfusion policies and restrictive blood testing
- Exogenous replacement of erythropoietin has minimally altered transfusion practice in preterm infants

- Rather, a combination of strategies is best, including reduction of phlebotomy losses, strict adherence to a conservative transfusion protocol, selected use of EPO with sufficient dosing, and optimizing nutrition to promote growth and hematopoiesis.
- We need to be prepared to accept lower Hb/Hct levels in asymptomatic neonates, recognizing that overall growth and stability are more important than any specific Hb number for an individual infant.
- Should rEPO therapy, as it is currently prescribed, be discarded as a relatively useless and expensive transfusion alternative? The answer may be yes for those nurseries willing to apply restrictive transfusion criteria and a single-donor system of blood banking. For nurseries employing liberal transfusion practices, rEPO therapy using multidose vials may be a safe, effective alternative to transfusion.[25]

Key Points: Physiologic anemia of infancy

- Newborns do not mount adequate erythropoietin response to hypoxia and anemia.
- The postnatal drop in Hb is universal in newborns. Term newborns reach a nadir of Hb between 6 and 12 weeks and tolerate the physiologic process well. Few, if any, clinical signs and symptoms are seen.
- The exaggerated and pathologic response of the preterm infant to the transition from fetal to postnatal life is called anemia of prematurity (AOP). It is seen by 4 to 10 weeks and is more profound and occurs earlier than anemia of infancy.
- The clinical importance of knowing the postnatal drop in Hb is in curbing the tendency of subjecting these infants to un-necessary blood transfusions.

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CHAPTER 6

Effect of Maternal Iron Status on Placenta, Fetus and Newborn

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Maternal anemia (hypoferremia) results in increased preterm and low birth weight deliveries and higher rate of stillbirths. There are irreversible structural alterations in placenta. The transfer of iron to fetus is reduced in spite of gradient in relation to severity of maternal hypoferremia. The fetal hepatic and brain iron contents were reduced. The brain iron reduction was irreversible on rehabilitation and was associated with irreversible neurotransmitter and their receptor alterations.

The outcome of severe pregnancy anemia has been associated with increased incidence of premature births, fetal distress, increased perinatal mortality and a higher frequency of maternal deaths.[1] In the case of moderate to severe anemia—breathlessness, edema, congestive heart failure and even cerebral anoxia have been observed. 200 anemic pregnant women observed in the University Hospital, Institute of Medical Sciences, Varanasi, showed: reduced gestation; higher incidence of premature labor, preterm, low birth weight and stillbirth deliveries. These newborns had low Apgar score and there were increased number of neonatal deaths. Maternal mortality was 13 out of 200 anemic as compared to 1 in 50 controls. Similar findings were reported in other Indian studies. Anemic mothers do not tolerate blood loss during childbirth; as little as 150 mL can be fatal. Normally a healthy mother during childbirth may tolerate a blood loss of up to 1000 mL.[2]

CURRENT KNOWLEDGE IN THE DEVELOPMENT OF IRON DEFICIENCY

Iron deficiency is an end result of a long period of negative iron balance mainly due to poor dietary availability, rapid growth and blood loss. The pathological stages are;

- *Prelatent deficiency:* Hepatic (Hepatocytes and macrophages), spleen and bone marrow show reduced iron stores (reduced bone marrow iron and serum ferritin).
- *Latent deficiency:* As the bone marrow iron stores become absent, plasma iron decreases and bone marrow receives little iron for hemoglobin regeneration (bone marrow iron absent, serum ferritin <12 ug/L, transferrin saturation <16 percent and free erythrocyte protoporphyrin is increased), however, hemoglobin concentration remains normal.
- *Iron deficiency anemia:* This is a very late stage of iron deficiency with progressive fall in hemoglobin and mean corpuscular volume.

PREVALENCE OF NUTRITIONAL ANEMIA IN PREGNANT WOMEN (INDIA)

National studies by the Indian Council of Medical Research (ICMR)[3]—covering 11 states; reported in 1989, prevalence of anemia by estimating hemoglobin using cyanmethemoglobin method in pregnant rural women as 87.6 percent, hemoglobin being <10.9 g/dL. These anemic women were given different doses of iron 60, 120 and 180 mg with 500 ug folic acid daily for 90 days in 6 states; 62 percent in spite of iron-folate therapy for 3 months, continued to remain anemic.[4] Thus indicating that short-term treatment as recommended in the National Anemia Control Program may not be sufficient to control anemia in pregnancy. However, it was observed that birth weight improved and low birth weight deliveries were significantly reduced.[5] The administration of higher dose 335 mg of ferrous sulfate and

500 ug of folic acid for 14 weeks as weekly or daily dose, both doses were found to be effective in control of pregnancy anemia. This suggested that, even once a week iron—folate administration will be of help.[6]

National Family Health Survey 1998–99 (NFHS-2) using hemocue method reported prevalence of anemia as 49.7 percent in pregnant women; 56.4 percent in breastfeeding nonpregnant women and 50.4 percent among nonpregnant nonbreastfeeding women. Hemocue method estimates higher levels of hemoglobin thus difficult to compare with the other national studies. In 2005, NFHS-3 demonstrated increase in prevalence of anemia, suggesting marginal rise in anemia nation wide.[7]

Nutrition Foundation of India in 2002–2003—studied prevalence of anemia in pregnancy and lactation in 7 states (Assam, Himachal Pradesh, Haryana, Kerala, Madhya Pradesh, Orissa, Tamil Nadu). The prevalence of pregnancy anemia was—86.1 percent (Hb <7.0 g/dL—in 9.5%) and in lactation up to 3 months was 81.7 percent (Hb <7.0 g/dL in 7.3%). The interstate differences responsible for differences in prevalence of anemia were particularly related to fertility, women education, nutrition status and occupation, availability of antenatal services and iron folate tablets as possible factors.[8,9]

The ICMR, 1999–2000—conducted District Nutrition Survey in 11 states covering 19 districts pregnancy anemia prevalence was 84.6 percent (Hb <7.0 g/dL—in 9.9%). The study also found 90 percent adolescent girls with anemia in these districts.[10]

The prevalence as well as severity of anemia during pregnancy and lactation is grave. This is the period when brain cells grow and neurotransmitters develop, iron is essential for it.

IRON STATUS IN PREGNANCY

- Fetal growth depends, to a large extent, on the availability of iron from the mother.
- Normal nonpregnant woman needs iron 1.3 mg/day.
- Total pregnancy need of iron is 1000 mg or more. Absorption—6 mg/day in the last 2 trimesters.
- 350 mg of iron is transferred to the fetus and placenta.
- 250 mg is lost in blood at delivery.
- 450 mg is needed to increase the RBC mass.
- Lastly around 240 mg is lost as basal losses.
- In cesarean delivery blood loss is almost twice (500 mL). In moderate and severe anemia mother will die, if blood loss is >150 mL.
- During lactation iron loss is 0.3 mg/day.

PLACENTA IN IRON DEFICIENCY

Iron transport: Normally 'placental iron transfer' to fetus becomes 3 to 4 times during 20 to 37 weeks of gestation. The placenta traps maternal transferrin, removes iron and actively transports it across to the fetus where it becomes bound to fetal transferring and is distributed to the liver, spleen and other fetal hemopoietic tissues, maintaining higher levels of fetal iron as compared to the mother. Placenta plays an important role in maintaining iron transport to fetus. This process of iron transport is purely a placental function over which mother and fetus has no control, as placenta continues to trap iron even when fetus is removed in animals.[11] The placental trophoblastic membrane appears to act as an effective barrier against the further transport of iron to the fetus. In spite of this efficient protective mechanism the placental iron content reduces significantly in maternal hypoferremia.[2,12-14] This was a very important finding as earlier studies on Swedish and American women had shown that cord iron does not change in iron deficient pregnant women.[15,16] However, recent studies have confirmed that the maternal anemia affects the placentofetal unit.[17-20]

Morphometry and biochemical alterations: Beischer et al.[21] analyzed data (from Australia, India, New Guinea, Singapore and Thailand) and demonstrated that in all the studies placental weight in maternal anemia was higher than the control. This increase in placental weight was higher with increasing parity. The placental hypertrophy did not correspond to fetal size and had no correlation with maternal serum protein. Ratten and Beischer[22] confirmed that the placental weight exceeds the 90th centile in 20 percent of patients with hemoglobin <8.2 g/dL and in 13.2 percent of those with hemoglobin 8.2 to 9.1 g/dL. The placental hypertrophy is postulated to be due to hypoxia, which is supported by evidence of similar phenomenon at higher altitudes. In our studies maternal anemia was associated with low maternal serum albumin. Both deficiencies were associated with reduced weight and volume of placenta. Placentae in maternal anemia showed reduced number of cotyledons and increase incidence of ill-defined cotyledons and eccentric attachment of cord. There was increased shrinkage in formalin in pregnancy anemia.[23-26] This reduction in placental weight was due to reduced DNA (cell number), however cell size was increased (weight/DNA). In maternal hemoglobin RNA content per cell remained constant.[27] Placental succinic dehydrogenase activity was decreased, total NADP-dependent ICDH was more than NAD+dependant ICDH in severe maternal hypoferremia; suggesting impaired citric acid cycle.[2]

Histology

There was decreased villous vascularity leading to fibrosis with increased endarteritis obliterans reflecting response to hypoxia. There was progressive decrease of surface area and volume of villi per unit volume of blood vessel

in relation to degree of anemia; suggesting maturational arrest.[2,26,28,29] On treatment with iron there was increase in hemoglobin, cord iron and placental (nonheme iron) and placental shrinkage in formalin reduced. However, the reduced villus vascularity, increased villus fibrosis and endarteritis obliterans in placenta of anemic mother did not reverse. It was postulated that moderate-severe anemia present from the early days of pregnancy induces irreversible structural alteration, as iron is needed in 2nd week of pregnancy for placenta formation.[2]

FETUS—NEWBORN

Cord serum iron and hemoglobin were reduced in preterm as well as full term infants of hypoferremic mothers. There is an increased gradient in presence of maternal iron deficiency for transport of iron from mother to fetus but the transport remains proportionate to the degree of maternal hypoferremia. The weight of full term singleton babies born of anemic mothers was reduced in direct relation to hemoglobin level. Similarly these babies showed a progressive decrease in Apgar scores also.[2] Fetal liver iron stores are reduced significantly in maternal hypoferremia. Normally bigger the infant and more advanced the gestational age higher was the amount of iron in fetal liver, spleen and kidney. The tissue iron content increases steeply in last 8 weeks of gestation. Infant born before 36 weeks of gestation, had half the iron content in hepatic reserve.[30] Breast milk—iron content is increased in hypoferremic mothers, a phenomenon of "Physiological Trapping".[31,32]

To understand more a rat model was created with latent iron deficiency (low hepatic iron with out change in hematocrit) in pregnancy.[33-38] Fetal brain iron content and neurotransmitters in maternal (Rat) latent iron deficiency. Iron as a micronutrient is required for regulation of brain neurotransmitters by altering the pathway enzymatic system. To study iron deficiency a rat model was developed to create iron deficiency (low hepatic iron) without change in hematocrit.[33]

In postweaning rats iron decreased irreversibly in all brain parts except medulla oblongata and pons. Susceptibility to iron deficiency showed variable reduction in different parts of the brain: corpus striatum-32 percent, midbrain 21 percent, hypothalamus 19 percent, cerebellum 18 percent, cerebral cortex 17 percent and Hippocampus 15 percent.

Alterations in brain iron content also induced—significant alterations in Cu, Zn, Ca, Mn, Pb and Cd.[34]

Fetal Latent Iron Deficiency (Rat) and Brain Neurotransmitters

In latent iron deficiency there was irreversible reduction in neurotransmitters:

Brain 'Glutamate metabolism'—(GAD, GDH, GABA-T) [35,37]

- Marked reduction in levels of brain GABA, L-glutamic acid and enzymes for biosynthesis of GABA and L-glutamate like glutamate decarboxylase and glutamate transaminase.
- Binding of H3 Muscimol at pH 7.5 and 1 mg protein/assay (GABA receptor) increased by 143 percent, but glutamate receptor binding decreased in the vesicular membranes of latent iron deficient rats by 63 percent[33,38]
  - Brain 'TCA-cycle' enzymes-mitochondrial NAD+ linked dehydrogenase significantly reduced.
  - Brain '5-HT metabolism'—tryptophan, 5-HT, 5-HIAA significantly reduced.
  - The whole brain and corpus striatum showed reduction in catecholamine, dopamine nor-epinephrine, tyrosine and monoamine oxidase, while tyrosine aminotransferase increased in corpus striatum, in spite of reduction in whole brain suggesting that latent iron deficiency induced irreversible neurotransmitter alterations.[39]
  - Brain 'catecholamine metabolism'—whole brain-dopamine, neonephrine, tyrosine and TAT significantly reduced; in 'corpus striatum'—same as in whole brain, except TAT was increased.[40]

These changes were specific to iron deficiency as neurotransmitter alterations in fetal brain due to malnutrition get normalized partially or completely on rehabilitation.[41,42]

The significant effects on neurotransmitter receptors (glutamate mediators) during early stages of iron deficiency clearly indicate the deficits in both excitatory and inhibitory pathways of the central nervous system, showing an important role of iron in brain.[33]

To test the above findings in humans, babies born of moderate to severely anemic mothers were examined for "impact of iron deficiency on mental functions". The intrauterine growth retarded offspring's of anemic as well as undernourished mothers showed hypotonia in 72 percent and hypoexcitability in 56 percent.[43-45]

There was modification of responses in several neonatal reflexes, e.g. limp posture, poor recoil of limbs, incomplete Moro's and crossed extensor responses.

Their EEG had shortening of sleep cycle (REM AND NERM), the reduction was more marked for REM sleep. There was some inter and intrahemispheric asymmetry and abnormal paroxysmal discharges; suggesting dysmaturity of brain.[43,44]

The above findings were not specific to affects of anemia on mental functions. Therefore effects of anemia (nutrition controlled) on mental functions were then studied in rural children during a period of three years. Mental functions in nutrition controlled 388 rural

primary school (6–8 years of age), matched for social and educational statuses were studied by WISC and arithmetic test to assess "intelligence, attention and concentration". Anemia does not affect intelligence, except subtest-digit span. In arithmetic test, attention and concentration was poor in anemic children.[46]

Effects of iron deficiency and/or anemia—on brain: iron deficiency anemia in infancy has been consistently shown to negatively influence performance in psychomotor development. Short-term iron therapy did not improve the lower scores, despite complete hematological replenishment. Neurological maturation was studied in infants 6 months old, including auditory brainstem responses and naptime 18 lead sleep studies. The central conduction time of the auditory brainstem responses was slower at 6, 12 and 18 months and at 4 years, despite iron therapy beginning at 6 months. During sleep-wakefulness cycle, heart rate variability—a developmental expression of the autonomic nervous system—was less mature in anemic infants. This is possibly due to altered myelination of auditory nerves.[47] It has been observed that these changes are resistant to iron therapy in children <2 years of age with iron deficiency with anemia, but not in older children.[48]

These studies supported our earlier findings that brain functions are significantly affected in latent iron deficiency in the brain growth period and such changes are irreversible. These have serious consequences, e.g. poor cognition and learning disabilities.

SUMMARY

The above researches by our group are mainly on effects of maternal hypoferremia on iron status of placenta, cord blood (hemoglobin and ferritin), and fetus (brain and hepatic iron content). The rat model of "latent iron deficiency" showed irreversible brain iron reduction and irreversible neurotransmitter alterations in 'brain growth period'. Once anemia sets in, the additional effects are due to anoxia. Our nation is faced with the problem of iron deficiency that leads to anemia—a clinical condition due to deficiency of many nutrients—mainly iron, folic acid and vitamin B_{12}. Folic acid is essential from prenatal period its deficiency causes neural tube defects.

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CHAPTER 7

Developmental Aspects of Hemostasis in the Fetus and Newborn

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Development of the hemostatic system in the human fetus has long been an area of interest and research. Hemostatic system starts developing in the embryonic period and undergoes evolution in the postnatal period as well till adult levels are reached. As the process spans over a long period of time, multiple age appropriate (gestational and postnatal age) reference ranges are necessary. An understanding of development of hemostasis helps in diagnosis and management of hemostatic problems during childhood.

INTRODUCTION

The hemostatic system is functionally intact in healthy full-term newborns and clinical presentation of bleeding or clotting is seen only in sick newborns especially the preterm infants. All the screening tests of coagulation are prolonged in the plasma of healthy infants compared with adult values, in the absence of bleeding. Stable preterm infants have more prolonged values than the healthy full term infants.

Plasma concentrations of various coagulation proteins mature at different rates in the fetus and newborn. The normal adult range may be achieved as early as mid-gestation for some proteins and as late as several months after birth for others. Thus, the fetus demonstrates a unique balance in levels of specific coagulation proteins in the maintenance of hemostasis.

The vitamin K status of a newborn is precarious at birth and may cause significant bleeding in the absence of other problems. Complications of birth process may result in birth asphyxia, which is an important cause of a consumptive coagulopathy with significant bleeding.

EARLIEST EVIDENCE OF THE FETAL HEMOSTATIC SYSTEM

Fibrinogen has been found in the fetal liver as early as 5th week of gestation. von Willebrand factor (vWF) can be demonstrated in the endothelial cells of the placenta from 4th week onwards, with successive detection in the fetal bone marrow and tissues over the next four weeks. Synthesis of coagulation proteins is among the most active in the fetus after production of plasma proteins such as albumin.

COAGULATION SYSTEM

Coagulation Proteins

It has been demonstrated that the blood of fetuses does not clot before 10 to 11 weeks of gestation. Thereafter, the clotting time rapidly becomes equal to the adult values or even less. Fibrinolytic activity can be detected by 10 to 11 weeks gestation and thereafter is similar to adult values or even greater (indicating shorter lysis times). The coagulation proteins do not cross the placenta or do so in negligible quantities and need to be independently synthesized by the fetus. Results obtained on healthy full-term infants are significantly higher than are those documented on healthy full-term fetuses *in utero*. Similarly, prematurely born infants have higher levels of coagulation proteins than their age matched counterparts *in utero* indicating fast maturation of the coagulation system soon after birth.

Levels of factor V approximate the adult range by 12 to 15 weeks. Plasma concentration of fibrinogen reaches 100 mg/dL by 12 to 15 weeks of gestation. Plasma concentration of most coagulation proteins are maintained at a constant

level throughout gestation until some time after 33 weeks, when a maturational burst happens. From 19 weeks onwards, all the coagulation proteins except factor V, VII, VIII, XII, and antithrombins circulate at 50 to 100 percent of the level achieved by full term. Factor V, VII, VIII, XII, and antithrombins increase steadily throughout the second and third trimesters.

The levels of vitamin K dependent factors and the contact factors (XI, XII, prekallikrein and high molecular weight kininogen) gradually increase to those approaching adult levels by six months of life. The low levels of contact factors are partly responsible for the prolonged APTT during the first months of life. Plasma levels of fibrinogen, factor V, VIII, XIII and von Willebrand factor are similar to adult values at birth. Fibrinogen levels continue to increase after birth. Plasma levels of factor VIII at birth are towards the higher range of normal and levels of both vWF and high molecular weight multimers are increased at birth and remain so till three months of life.

Reduced production of coagulation factors and increased clearance of plasma proteins leads to the differences in the level of various plasma proteins. Premature infants have accelerated clearance of fibrinogen which can be attributed in some part to their increased basal metabolic rate. Fetal fibrinogen has an increased content of sialic acid which accounts for the differences in its structure and function as compared to adult fibrinogen.

Regulation of Thrombin

Newborns have delayed and decreased regulation of thrombin compared with adults. The amount of thrombin generated is directly proportional to the concentration of prothrombin and the rate of thrombin generation depends on the concentration of other procoagulants.

Thrombin is directly inhibited by antithrombin (AT), heparin cofactor II (HC II), and alpha 2 macroglobulin. Alpha 2 macroglobulin is a more important inhibitor of thrombin in plasmas from newborns as compared to plasma from adults. The rate of inhibition of thrombin is slower in newborn infants than it is in adults plasma concentrations of protein C are very low at birth, and remain decreased during the first six months of life. Neonates have a two fold increase in the single chain form of protein C, compared with the double chain form that is predominant in adults and no difference has been found in the functioning of the two forms. Newborns have lower levels of total protein S at birth but functional activity is similar to that in the adult because in newborns, protein S is completely present in the free, active form due to the absence of C4 binding protein. The interaction of protein S with activated protein C in the newborn plasma is regulated with by the increased levels of alpha 2 macroglobulin. Plasma levels of thrombomodulin are increased in early childhood, and decrease to adult values by the late teenage years. Total tissue factor pathway inhibitor (TPFI) levels in newborn infants are same as in older children or adults, but free TPFI is significantly lower. Thrombi in newborn infants do not propagate with the same propensity as in adults, due to significantly low levels of fibrinopeptide A in cord plasma.

PLATELETS

Mega-karyocytes appear in the liver at 8 weeks gestation and platelets can be found in fetuses from 11 weeks gestation. Initially the platelets are large and beyond 12 weeks the mean platelet volume is essentially normal. Cord blood of preterm babies has increased number of all megakaryocyte precursors as compared with term babies and term infants have higher circulating megakaryocyte progenitor numbers at birth compared with adults. After 20 weeks of gestation, the platelet counts and the mean platelet volume are similar to those in adults with values between 1.5 and 5.5 lakh per cu.mm and 7 to 9 fL respectively. The platelet function in term and preterm babies has been found to be impaired *in vitro*.

The most consistent abnormalities are reduced aggregation in response to adrenaline, ADP and thrombin. Electron microscopic studies on cord platelets have shown normal number of granules, however, the concentration of serotonin and adenosine diphosphate (ADP), which are stored in dense granules, is less than 50% of adult values. GP Ib is present on fetal platelet membrane in adult quantities, however GP IIb/IIIa is significantly reduced. Despite these differences, the bleeding time is normal in term and preterm infants.

Newborns have higher levels of thrombopoietin in the cord blood as compared to adult values. The relatively higher hematocrit of neonatal blood contributes to increased blood clotting by increasing the number of platelets directed to the vessel wall by virtue of laminar flow and by offering a larger surface for the formation of fibrin clots. vWF facilitates adhesion of platelets to collagen, and it reaches normal adult values by 20 weeks gestation. At full term, it has a higher total concentration and increased number of larger (stickler) UlvWF.

Platelet receptors in the fetus mature around 12 to 16 weeks' gestation except for decreased coupling of epinephrine receptors. In response to activators, the granular release is decreased in the fetus due to diminished calcium channel transport and impaired signal transduction. Platelets get activated during the birth process by interplay of various factors which include thermal changes, hypoxia, acidosis, adrenergic stimulation and the thrombogenic effects of amniotic fluid.

Cord plasma levels of thromboxane B2, thromboglobulin and platelet factor-4 are increased, the granular content of cord platelets is decreased and epinephrine receptor availability is reduced.

Bleeding Time

Infants during the first week of life have significantly shorter bleeding times as compared to those in adults. Enhanced platelet and vessel wall interaction, higher plasma concentrations of vWF, enhanced function of vWF due to a disproportional increase in the high molecular weight multimeric forms, active multimers, large red cells and high hematocrits all contribute to the shorter bleeding time in infants.

VESSEL WALL

The endothelial cells and extracellular matrix components of the vessel wall have procoagulant and anticoagulant properties which are significantly influenced by age and have a significant bearing on the hemostasis. One of these anticoagulant properties is mediated by lipoxygenase and cyclo-oxygenase metabolites of unsaturated fatty acids. Prostaglandin I2 (PGI2) production from cord vessels is higher than that of adult vessels. Levels of soluble endothelial cell adhesion molecules and selectins are also age dependent, due to differences in development of endothelial cell expression and secretion of these molecules. Nitric oxide (NO) modulates vascular tone in fetal and postnatal lungs and is responsible for the normal decline in pulmonary vascular resistance at birth. NO is a potent inhibitor of platelet adhesion, aggregation and stimulates disaggregation of platelet aggregates. NO interacts with PGI2 and other metabolites of the lipoxygenase pathway to modulate platelet function.

ANGIOGENESIS

Angiogenesis plays an important role in development of the alveoli in the fetal and neonatal lung. Angiogenic factors-angiogenin, basic fibroblast growth factor (bFGF) and vascular endothelial growth factors (VEGF) in the serum increase soon after birth.

FIBRINOLYTIC SYSTEM

Plasminogen levels in the neonates are approximately 50 percent and antiplasmin (AP) levels are approximately 80 percent of the normal adult values. Plasma concentrations of plasminogen activator inhibitor-1 (PAI-1) and tissue plasminogen activator (TPA) are significantly higher as compared to adults due to enhanced release of these two factors from the endothelium shortly after birth. The enzymatic activity of fetal plasmin and its binding to cellular receptors for fetal plasminogen is decreased but still clot lysis is more rapid in fetal plasma due to decreased inactivation of fetal plasmin by alpha-2 antiplasmin. The proof of activation of the fibrinolytic system at birth lies in the short whole blood clotting times, short euglobulin lysis times (ELTs) and increased plasma concentrations of fibrin related peptides.

CONCLUSION

Decreased concentration and activities of coagulation proteins is responsible for the mild prolongations of all the screening tests detected in healthy full-term infants. Except for vWF, factors V, VIII and fibrinogen. All other plasma coagulation proteins are generally low in the newborn. These four coagulation proteins which are all within or above the normal adult range at full-term gestation, together with hematocrit, platelet number and platelet adhesiveness make a major contribution to hemostatic potential in the neonate and are to a certain extent responsible for the hypercoagulability observed in sick infants.

Full-term newborn infants have a balanced and intact hemostatic system, despite apparent deficiencies of procoagulant, regulatory and fibrinolytic activities. The neonatal hemostatic system lacks adequate reserve capacity to cope with massive stresses of low blood flow, acidosis and sepsis, which becomes a clinical challenge in the sick preterm infant.

Fetal hemostasis is a dynamic system that gradually evolves by stages towards the adult state, but always maintains equilibrium between activators and inhibitors throughout intrauterine life until birth. The vascular and hemostatic systems of the fetus and neonate are continually evolving and this must be taken into account when evaluating these systems for dysfunction.

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CHAPTER 8

Anemia in the Newborn

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INTRODUCTION

Pediatricians caring for sick/full term/premature newborn infants are often confronted with a variety of routine as well as life-threatening hematological problems. Anemia in neonatal period remains a cause for concern due to likelihood of rapid decompensation in this vulnerable group. Numerous physiological changes occur in succession and rapidity in fetus and neonate, as the erythrocyte system *in utero* undergoes serial adaptation to meet progressively changing demand for oxygen from embryo stage to term. Thus, there is rapid change in normal hematological parameters from fetal period to immediately after birth and throughout neonatal period even hours, days and weeks after birth.[1-5]

An understanding about the basic physiology of hematopoiesis and appreciation of normal hematologic and laboratory values at birth is important because they form the basis for the diagnosis, treatment and prevention, of many diseases that afflict these neonates. Interpretation of laboratory findings and institution of appropriate therapy requires understanding of maturational process and normal physiological variations that takes place during this period.[1-5]

ANEMIA

Anemia in the term infants is defined as a hemoglobin value of less than 13.5 g/dL during the first week of life. Values for umbilical artery blood tend to be about 0.5 g/dL higher than sample obtained from umbilical vein. Preterm infants have lower baseline values. Capillary specimens obtained by heel stick have higher hemoglobin and hematocrit values than samples obtained from the umbilical vein or peripheral blood.[4-9]

Beyond the first weeks of life many factors influence what is considered as normal hematological parameters in newborn period. Hb concentrations decrease in both normal term and preterm infants after birth to reach minimal levels of 9.4 to 14.5 g/dL in term infants by 7 to 9 weeks of age.[1,4,5]

Physiological Anemia

This "physiological" anemia[1,3-6] occurs because of a decline in erythrocyte mass due to the following reasons:

- During intrauterine period the fetal oxygen saturation is low at around 45 percent, erythropoietin levels are high and RBC production is rapid. Reticulocyte counts are 3 to 7 percent reflecting ongoing erythropoiesis. With improved oxygen saturation to 95 percent after birth, the erythropoietin levels become undetectable hence RBC production stops, reticulocyte counts are low and the hemoglobin level falls.
- This factor coupled with a reduced lifespan of fetal RBCs, results in anemia that is not a functional one as oxygen delivery to the tissue is adequate.

At 8 to 12 weeks, hemoglobin levels reach their nadir, oxygen delivery to the tissues is impaired, erythropoietin production is stimulated and hemoglobin starts increasing. Hemoglobin values rise from 8 to 10 g/dL at 12 weeks of gestation to 13.7 to 20.1 g/dL (mean of 16.8) at term.[1,5]

The hemoglobin and RBC count fall earlier and to a greater extent in preterm infants leading to "Anemia of Prematurity".

Anemia of Prematurity

Anemia of prematurity (AOP)[6,8-12] is an exaggeration of the physiologic anemia of infancy. The hemoglobin and

RBC count fall earlier and to a greater extent as low as 7.8 to 9.6 g/dL in preterm infants leading to "Anemia of Prematurity". Shortened survival of RBCs to an average of 60 days (120 days life span in adult RBCs) and rapid body growth with relative hemodilution are the contributory factors. Besides, iatrogenic blood losses may be higher. Premature infants may require additional folate and B12 to reduce severity of anemia of prematurity.[9,10] Vitamin E deficiencies is more common in small preterm infants.[11]

Nonphysiologic Anemia in Neonate

Pathological anemia in neonate may be caused by wide spectrum of diseases and could be due to

- Hemorrhage[12-25]
- Hemolysis, increased RBC destruction[26-36]
- Failure of red cell production.[37-42]

Nutritional anemia is not a cause of anemia during neonatal period (unlike in older children) even when the mother is severely iron deficient, as fetus is an effective parasite of the mother. Anemia in the newborn often accompanies and is complicated by conditions like asphyxia, shock, jaundice which make the situation even worse.

ETIOLOGY

Hemorrhage[12-25]

Profound anemia appearing at birth or during first 24 hours of life is most often due to hemorrhage or isoimmune hemolytic disorder. Bleeding in the newborn though is often visible and evident, but if it occurs inside the body—gastrointestinal tract and in the body cavity, may not be recognized and may go unnoticed initially.

High index of suspicion in an anemic neonate without jaundice and with negative direct Coomb's test will help in suspecting diagnosis of acute hemorrhage.

Degree of anemia depends upon whether acute or chronic.

Incidence of Hemorrhage in Newborn[12-25]

- Twenty-five percent of all infants admitted to neonatal intensive care.
- Five to ten percent of all severe neonatal anemias are due to hemorrhage.
- One percent of all newborn nursery admissions.

Anemia due to hemorrhage may occur *in utero*, during labor and delivery or after birth.

Obstetric Causes of Blood Loss[12,13,15,16]

- Abruptio placenta[22]/placenta previa
- Incision of placenta at cesarean section
- Rupture of normal umbilical cord, rupture of anomalous insertion of the cord and hematoma of the cord containing large amount of blood, aneurysm of cord.
- Malformation of placenta and cord velamentous insertion, vasa previa.

The most common cause of anemia at or around the time of birth is due to fetal blood loss associated with conditions like placenta previa and accidental hemorrhage due to abruptio placentae.[14,15] When there is partial separation of the placenta, blood loss is predominantly maternal, however some fetal sinuses in the placenta may rupture causing fetal blood loss and anemia. Also, maternal blood loss and hypotension causes vasodilatation in placental vessels causing placental pooling of blood, thereby aggravating fetal hypovolemia. This abnormality is more common in multiple pregnancies and incidence of hemorrhage is seen between 1 and 2 percent.

- Umbilical cord anomalies like venous tortuosity or arterial aneurysm may lead to bleeding if injured.
- Unattended precipitous delivery may lead to rupture of normal umbilical cord. When cord ruptures, the tear generally occur in fetal third and bleeding is immediate and profuse.[12,15]

Other Causes of Hemorrhage Includes

- Fetomaternal hemorrhage[15-19,21,22]
- Fetoplacental hemorrhage[14,15]
- Fetofetal hemorrhage[23,24]

Fetomaternal Hemorrhage[15-19,21,22]

The passage of fetal erythrocytes in maternal circulation occurs commonly during pregnancy.

- In 50 percent of pregnancies some fetal cells are passed in maternal circulation at some times during gestation or during birth process.
- In about 8 to 10 percent of pregnancies transplacental blood loss ranges from 0.5 cc to 40 mL of blood.
- In about 1 percent of cases the loss may be even greater as much as 100 mL.
- It may be acute or chronic in nature.
- Fetal hemorrhage may also occur in substances of placenta or may result in retroplacental hemorrhage.

More common type of fetomaternal hemorrhage occurs when infant is held above placenta as during cesarean delivery. Anemia has been reported when infant is held above the placenta before clamping the cord. Blood is continuously returned through the umbilical arteries to the placenta, while hydrostatic pressure prevents continued venous return to the infant.

When infant is held above the introitus the placental transfusion is either markedly reduced or completely prevented.

In infants born by cesarean section, it is advisable to keep the baby at least 20 cm below the placenta for approximately 30 seconds before clamping the cord.

Other causes of fetomaternal hemorrhage

- Diagnostic amniocentesis—10 to 32%.[16,17]
- When infant is held above placenta during delivery—(particularly after cesarean section) before cord is clamped
- Traumatic injury to mother during pregnancy secondary to
  - Vehicular accidents
  - Fall
  - Abdominal trauma
- Application of fundal pressure during 2nd stage of labor.
- Maternal toxemia.
- Erythroblastosis fetalis.
- Placental chorioangioma and choriocarcinoma seen in 1 percent of placenta.

Chronic causes of fetomaternal hemorrhage:[18,19] Anemia in fetus occurs slowly if hemorrhage occurs repeatedly during course of pregnancy. Fetus tries to compensate and adjust hemodynamically and infant when born may present only with pallor, unexplained anemia and mild hepatosplenomegaly. Chronic blood loss may lead to iron deficiency anemia with hemoglobin values ranging from 4 to 6 gm/dL.[18,19,22]

High index of suspicion in an anemic neonate without jaundice and with negative direct Coomb's test will help in suspecting diagnosis of acute hemorrhage. Degree of anemia depends upon whether acute or chronic.

Diagnosis of fetomaternal hemorrhage:[16-19] It is confirmed by demonstrating the presence of fetal blood in maternal circulation by

- Kleihauer Betke's test (Fig. 1)
- Differential hemagglutination
- Mixed agglutination
- Fluorescent antibody technique.

All tests are sensitive and are capable of detecting as little as 0.1 cc of fetal blood in mother's circulation.

Kleihauer Betke's test may be false positive in

- Maternal thalassemia minor
- Sickle cell anemia
- Hereditary persistence of fetal hemoglobinopathy.

If ABO blood group incompatibility is associated diagnosis can be missed as infant's A or B cells are rapidly cleared from maternal circulation by maternal anti-A and anti-B antibodies. The amount of blood lost in a feto-maternal hemorrhage, can be calculated by the following formula:

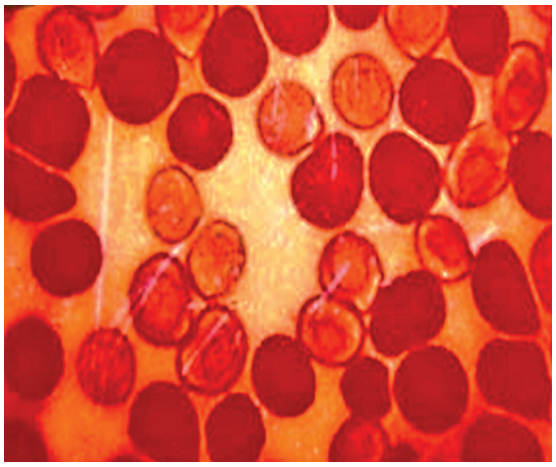


Fig. 1 *Kleihauer Betke's test:* Dark pink color fetal cells in mother's smear in a case of fetomaternal transfusion

$$\text{Cc of fetal blood} = \frac{\text{Fetal RBC} \times 240}{\text{Maternal RBCs}}$$

Or 1 fetal RBC in 1000 maternal RBCs indicates 2 cc of feto-maternal hemorrhage.

Causes of fetoplacental hemorrhage:[14-19,21] Multi-lobed placenta may be associated with vasa previa—(anomalous vessels crossing the os) vessels may be well compressed as well as lacerated during the 2nd stage of labor. The prenatal mortality rate range varies 50 to 80 percent.

Fetofetal hemorrhage (Twin-to-twin transfusion):[23,24] Simultaneous occurrence of anemia in one of the twins and polycythemia in other should always arouse a suspicion of twin-to-twin transfusion. Fetal transfusion is seen in monozygotic twins with monochorial placentae. Seventy percent of monozygotic twins have monochorial placentae. Fifteen to thirty-three percent of such pregnancies have feto-fetal transfusion.

Accidental Incision of Placenta or Umbilical Cord During Cesarean Section

Lower segment cesarean section with anterior placenta can result in direct placental injury. The placenta and membrane should always be examined for the damage from the fetal side following cesarean section. Hemoglobin of the infant should be estimated at birth and again 12 to 24 hours later. Hemoglobin should also be estimated in all neonates born to mothers with unusual vaginal bleeding, placenta previa or abruptio placenta. Anemia following obstetric accidents may not be realized if proper attempt is not made to examine the placenta and cord in time before disposing off the placenta and evidence of cause of anemia may be lost.

Anemia in the newborn may follow hematoma of the cord containing large amount of blood.[25] Rupture of umbilical cord, or of aneurysm of cord or aberrant vessels and due to velamentous insertion. This abnormality is more common in multiple pregnancies and incidence of hemorrhage is seen between 1 and 2 percent. Umbilical cord anomalies like venous tortuosity or arterial aneurysm may lead to bleeding if injured. The condition is 10 times more common in twin than in single term pregnancy. Perinatal death rates in such situations are about 50 to 80 percent. Many are stillborn.

Hemorrhage in the postnatal period[25]

- Unattended precipitous delivery may lead to rupture of normal umbilical cord. When cord ruptures tear generally occurs in fetal third and bleeding is immediate and profuse. Severe bleeding may result in stillbirth; may manifest with severe respiratory distress and asphyxia.
- Hemorrhage may be due to birth trauma resulting in intracranial bleeding cephalhematoma, subgaleal hemorrhage, retroperitoneal hemorrhage.

Other common causes

- Slipped ligature.
- Bleeding disorder in the neonate.
- Hemorrhagic disease of newborn, now called vitamin K deficiency bleeding.
- Sepsis with DIC.
- Intrauterine TORCH infections.
- Iatrogenic anemia due to excessive blood sampling.

Various studies have shown that blood withdrawal for investigations during first few weeks can range from 5 to 45 percent of total blood volume or 50.5 mL/kg per 28 days period hospitalization.

- 1 mL of blood represents 1 percent of total blood volume particularly in premature neonates
- Blood lost during the sampling may be estimated by recording the amount of blood collected in mL and by weighing the cotton swab on an electronic weighing machine, as two drop of blood in the cotton used to stop bleeding represents 100 μ of blood loss. Ten percent of blood loss during sampling for laboratory monitoring is "hidden" and represents blood on cotton swabs, in the dead space of syringe or tubing of the butterfly needle.
- Blanchette and Alvin Zupersky[7] in their study of 52 premature infants studied during first 6 weeks of life reported mean blood loss through sampling 22.9 ± 10 mL of packed cells. Forty-six percent of infants studied had cumulative losses that exceeded their circulating red cell masses at birth.

Therefore anemia in neonates from intensive care unit may be caused by frequent blood sampling. It should be remembered that removal of 8 to 10 cc of blood from 1500 gm neonate constitute 8 percent of the blood volume which is equivalent to about 400 cc blood from the adult.

Vitamin K deficiency bleeding: Vitamin K is necessary for synthesis of coagulation factors II (prothrombin), VII, IX and X in the liver. Newborns are relatively vitamin K deficient. Poor placental transfer of vitamin K, low vitamin K stores at birth, low levels of vitamin K in breast milk, and sterility of the gut are contributory factors. Bleeding can occur from different sites, though GI bleeding is the most common manifestation as hematochezia, hematemesis. Hematuria, oozing around the umbilical cord, bleeding from circumcision, and venipunctures may occasionally occur. Hematomas at sites of trauma, such as large cephal-hematomas and bruising, are also common findings. Intracranial bleeding may occasionally occur. Significant bleeding due to vitamin K deficiency bleeding and other inherited bleeding disorders may result in severe anemia in the newborn.

Diagnostic tools for complete blood count (CBC) for diagnosing anemia

- Manual counting techniques. Not reliable, not reproducible
- Electronic impedance principles/light scanner principles.

Advantages of electronic methods

- Accurate
- Reproducible
- Number of indices available in a short time.

Peripheral smear examination: In acute hemorrhage, peripheral smear (PS) may show normochromic, normocytic RBCs, whereas in chronic blood loss hypo-chromic, microcytic anemia.

Increased retic count and number of nucleated RBCs are seen in both acute and chronic hemorrhage.

In chronic hemorrhage, the serum iron values are decreased and in acute may be normal.

- Hyperbilirubinemia is differentiating feature in anemia of hemolysis versus hemorrhage. However, when there is bleeding in tissues or body cavities, hyperbilirubinemia may also occur in the latter situation.
- Bone marrow aspiration if done may show no stainable iron. However, bone marrow aspiration is not indicated for diagnosis.

CLINICAL FEATURES

Acute Blood Loss

Clinical features depend on the rapidity of blood loss and amount of loss of blood. Degree of anemia depends upon whether blood loss is acute or chronic. Complete obstetrical history gives clue to diagnosis.

In history of vaginal spotting during last trimester or prior to delivery, suspect placenta previa.

- Following acute hemorrhage Hb may not drop in first 6 to 24 hours. Several hours may elapse before profound anemia is documented. Even if Hb is initially normal, neonate should be repeatedly followed up closely during next 12 to 24 hours and falling Hb may be noticed after some time due to hemodilution that accompanies. If the neonate is in shock, Hb determination should be performed on venous blood as capillary Hb may be misleadingly high. The infant looks pale, sluggish, gasping and with features of circulatory shock. If 20 percent or more blood is lost acutely, the signs and symptoms of shock are present.
- Jaundice is absent and bilirubin levels do not increase.
- Mother may present with shaking chills as consequence of transfusion reactions when there is blood group incompatibility. This may result in stillbirth.
- Examination of placenta and cord should be performed before it is thrown to ascertain the site of blood loss.

Traumatic deliveries result in:

- Subdural hemorrhage
- Subarachnoid hemorrhage
- Cephalhematoma
- Blood loss in subaponeurotic area of the scalp. Subaponeurotic hemorrhage usually extends throughout the soft tissue of the scalp and covers entire calvaria and this blood loss can lead to exsanguination and death. Boggy swelling of the head extending from frontal region to nape of the neck may be present and may be associated with the swelling of the eyelids. Hb may drop as low as 2.2 g/dL at 48 hours of age and infant may be in shock. It can be estimated that for each centimeter of increase in head circumference above that expected, 38 mL loss of blood and may also develop hyperbilirubinemia.[12,13]

Other Hemorrhages[12,13,25]

Adrenal hemorrhage: Clinical picture including sudden collapse, cyanosis, limpness, irregular respiration, presence of flank mass accompanied by bluish discoloration of overlying skin should suspect of adrenal hemorrhage. Presence of the free fluid can be demonstrated by clinical examination as well as an ultrasound evaluation. Jaundice may appear due to breakdown of RBCs from these entrapped hemorrhages.

Splenic hemorrhage: Splenic hemorrhage should be kept in mind in large babies with pallor, abdominal distension, scrotal swelling and radiographic evidence of peritoneal effusion following difficult delivery or in babies with erythroblastosis fetalis. Umbilical venous pressure is decreased rather than increased.

Hemorrhage may be subcapsular or free blood may be present in the peritoneal cavity.

An infant with ruptured liver is generally normal for first 24 to 48 hours and suddenly goes into shock, as hematoma ruptures the capsule, causing hemoperitoneum. Mass in the right hypochondrium may be palpable.

Severe fetal bleeding may result in stillbirth; or may manifest with severe respiratory distress and asphyxia in the newborn. It is important to differentiate pallor resulting from severe anemia from perinatal asphyxia.

ETIOLOGY

Anemia Due to Increased RBC Destruction (Hemolytic Anemia)

Anemia as a consequence of hemolytic process is common in the newborn period. A hemolytic process is defined as a pathologic shortening of the life span of the red blood cell. The normal life span of adult RBC is 120 days. However, red cell life survival in term infants may be 60 to 80 days and in 32 to 36 weeks gestation preterm babies cells may survive only 35 to 70 days. Since destruction of 1 g/dL of hemoglobin results in production of 35 mg of bilirubin, hemolytic anemia in the newborn is always associated with significant hyperbilirubinemia.

Causes of Hemolytic Anemia of Newborn[27-37]

Immune Hemolysis[26-31]

- *Allo/isoimmunization:* Rh, ABO, minor group
- *Autoimmune:* Autoimmune anemia due to passive transplacental transfer of maternal antibodies.

Nonimmune Hemolysis[32-36]

- RBC membrane defects[32,33] (Hereditary spherocytosis, elliptocytosis, stomatocytosis, etc.)
- Enzyme defects (G6PD deficiency, pyruvate kinase deficiency, glucose phosphate isomerase deficiency)[34,35]
- Hemoglobinopathies[36] (alpha thalassemia/structural defects); beta thalassemia.

Hemolytic Disease of Newborn

Hemolytic anemia of newborn should be suspected when there is

- A rapid fall of hemoglobin concentration in the absence of evidence of hemorrhage in early neonatal period
- *Evidence of increased red cell production:* Reticulocytosis and increased normoblasts in prognostic signs
- Jaundice during first 24 to 48 hours of life
- Abnormal erythrocyte morphology
- Hemoglobinuria
- Positive direct Coomb's test.

Causes of Hemolytic Disease of Newborn

- Isoimmunization, ABO/Rh (minor) blood group incompatibility[26-31]
- Congenital or acquired defects of RBCs[32-36]

Isoimmunization:[26-31] Hemolytic disease of newborn as a consequence of isoimmunization is caused by passage of fetal red cells into the maternal circulation where they stimulate the production of antibodies. Placental transfer of these maternal antibodies directed against fetal red cell antigens is the most common cause of neonatal hemolysis. These antibodies of IgG class return to fetal circulation, attach to the antigenic site on the fetal red cells leading to hemolysis of these cells.

In this group of disorders, fetal and/or neonatal red blood cell hemolysis results from the presence of maternal antibodies to the red cell antigens in fetal circulation.

Rh isoimmunization: When an Rh negative mother conceives an Rh positive baby, fetal red cells may cross the placenta and sensitize the maternal immune system. On subsequent exposure to Rh positive cells across the placenta, the antibodies produced by the maternal immune system may cross the placenta and cause hemolysis of fetal red cells. As few as 0.2 mL of fetal cells are sufficient to cause maternal anti-D sensitization.[26,27,29]

The initial IgM response is slow and weak. As IgM does not cross placenta, no fetal effects are seen. On second exposure to RhD, IgG antibodies formed readily cross the placenta, into fetal circulation and binds to the RhD antigen on fetal RBC membrane. Antibody coated fetal red cells adhere to macrophages and lead to eventual destruction of the cell. This hemolytic process can take place starting *in utero*, resulting in marked compensatory over production of nucleated red cells. During pregnancy fetomaternal transfusion may occur spontaneously in about 7 percent of cases in the first trimester,16 percent in the second trimester and 29 percent in the third trimester and in as much as 50 percent in the peripartum period, leading to the formation of anti-D IgG antibodies in the mother. Hence, the second and the subsequent pregnancies have a higher chance of being affected.

In severely sensitized pregnancies, fetal marrow cannot keep up with red cell destruction and extra-medullary erythropoiesis resulting in hepatosplenomegaly occurs, nucleated cells are poured into the circulation, giving this disorder the name "Erythroblastosis Fetalis".

Though Rh isoimmunization was the most common cause of hemolytic anemia in the newborn in the past, this condition is fast disappearing due to adequate immunization of Rh negative mothers with anti-D.

ABO incompatibility:[26-31] ABO incompatibility is common and occurs in approximately in 20 percent of all pregnancies. In only 1:1000 births, severe disease occurs. ABO incompatibility has a similar pathophysiology but is a relatively milder disease. Group O mothers have a predilection for producing IgG antibodies against antigens A and B as against the normally produced IgM type. Antibody titers are usually >1:64. This is predilection may run in families. Mothers may be produced as an immune response to A and B antigens contained in food, bacteria and vaccines. The presence of naturally occurring antibodies in the maternal serum explains the frequent occurrence of ABO hemolytic disease in first born infants in contrast to Rh D disease in subsequent gestations.

- A direct Coomb's test usually positive in Rh incompatibility and may be weakly positive in ABO incompatibility.
- Peripheral blood smear shows polychromasia, increased number of erythroblasts.
- In ABO incompatibility increased number of microspherocytosis may be seen on peripheral blood smear examination.
- Laboratory investigations will reveal blood group incompatibility.
- Levels of IgG, anti-A, or anti-B antibodies in the mothers of babies with ABO hemolytic disease are significantly higher than in the mother whose infants do not have the disease increased.
- Alloimmunization due to Minor Group Incompatibility.[29,31] Alloimmunization can occur due to a variety of other fetal red blood cell antigens.
- Rh blood group system, cc, ee, ec, ce, of the among the antigens Rh group alloimmunization with E and C occurs most frequently after anti-D sensitization
- The principal antibodies found are anti-E, anti-C, anti-Kell, Duffy, Kidd, Fu, MNSs, etc.

Alloimmunization to the accounts for up to 10 percent of severely affected fetuses. As the Kell antigen is expressed on the surface of erythroid progenitors, whereas D antigen is not, Kell sensitization in addition to causing hemolysis, results in suppression of erythropoiesis.

While *in utero*, excess of bilirubin produced by hemolysis is removed by the placenta hence at the time of birth, child may not be jaundiced. Jaundice usually appears in the first 24 to 48 hours of life as neonatal immature liver is not in a position to conjugate excessive bilirubin load. Significant hyperbilirubinemia is evident. It may be accompanied by anemia and hepatosplenomegaly.

Hemolytic disease due to minor blood group incompatibility should be suspected when Coomb's test is positive and there is no evidence of major blood group incompatibility in mother and child. The principal antibodies found are anti-E, anti-C, anti-Kell, Duffy, Kidd, Fu MNS systems.

Management:[28,29] Management of severely affected iso-immunized fetus consists of early delivery with or without fetal transfusions depending upon gestation of fetus. Management of neonate depends on degree of hemolysis and level of indirect hyperbilirubinemia.

All Rh negative mothers with Rh positive fetus, should be given Rh immunoglobulin at 28 to 30 weeks of gestations and within 72 hours after delivery and after spontaneous or therapeutic abortion. Hyperbilirubinemia must be aggressively treated to prevent kernicterus with phototherapy, exchange transfusion. Whenever required top-up transfusion with packed red blood cells may be given for symptomatic anemia.

Autoimmune hemolytic anemia

- Anemia may be due to 'warm' or 'cold' antibodies which signify the temperature at which antibodies become active. The rare combination of autoimmune hemolytic anemia (AIHA) and pregnancy carries great risks to both the woman herself and the fetus.
- The degree of hemolysis in the fetus depends mainly on the amount and avidity of the transferred antibody for the fetal red cells. Fifty percent of women with this condition are reported to improve during pregnancy, especially in the third trimester. The diagnosis rests on the demonstration of auto-antibodies directed against red cell surface antigens. In practice, this is detected by a positive direct antiglobulin Coombs' test. Most of these antibodies detected at 37°C are of the IgG subclass.
- Administration of prednisone, 2 mg/kg/day, to the mother with prenatally active AIHA may both reduce maternal hemolysis and reduce neonatal morbidity. Management is to monitor carefully for hemolysis, hyperbilirubinemia, jaundice, to prevent kernicterus.[28,29]
- Acquired hemolytic anemias in the newborn can be caused by infection, drugs or toxins. Congenital infections like syphilis, cytomegalovirus, rubella, 20 toxoplasmosis, mycoplasma pneumoniae, bacterial and viral infections may cause hemolytic anemia.
- IgM antibodies can cause disease and usually are active between 0 and 30 degrees celsius, hence are referred to as cold agglutinins. These antibodies with complement coat RBCs and cause hemolysis.
- Rapid onset of anemia, hyperbilirubinemia with splenomegaly and hepatomegaly occur. Occasionally a severe anemia requires treatment with corticosteroids, IV IgG or and immunosuppressive agent.[31]

Management of affected fetus

- Antibodies from the mother cross the placenta and result in fetal red cell hemolysis. Presence of autoimmune hemolytic anemia in mother may result in hemolytic anemia in the newborn infant. Anemia may be associated with bacterial and viral infections in the newborn period and is often associated with jaundice (both conjugated and unconjugated fraction) and hepatosplenomegaly.
- Intrauterine, viral infections like congenital CMV, toxoplasmosis, congenital syphilis, herpes, all may be associated with hemolytic anemia in the neonatal period.

Nonimmune hemolytic anemia in the newborn may occur due to

Conditions associated with defects of
- Red cell membrane[32,33]
- Red cell metabolism[34,35]
- Hemoglobin synthesis[37]
- Red cell under production[38-43]

Other causes include
- Disseminated intravascular coagulation
- Congenital infections.

Abnormalities of red cell membrane

Hereditary spherocytosis (Fig. 2)[32,33]

In approximately 50 percent of patients with hereditary spherocytosis, history of neonatal jaundice can be obtained and may be of a sufficient magnitude so as to need phototherapy and exchange transfusion and may lead to kernicterus if left untreated. A family history of chronic anemia, splenectomy, cholecystectomy, pain in abdomen, unhealed ulcers may be present. Physical examination of parents may reveal mild to moderate splenomegaly.

Examination of peripheral blood smear (Fig. 3) reveals characteristic microspherocytes and the osmotic fragility of erythrocytes may be increased.

Spherocytes however may be seen in the newborn period in other conditions like ABO incompatibility, septicemia, red cell enzyme deficiency, etc.

Defects of red cell metabolism include

G6PD (Glucose 6 phosphate dehydrogenase) deficiency:[34,35]

With G6PD enzyme deficiency, oxidation of membrane protein leads to precipitation of denatured hemoglobin (Heinz bodies) thus shortening the RBC life span.

- X-linked recessive disorder. History of anemia, jaundice may be elicited in maternal cousins, maternal uncles, maternal grandfather and grand uncles.
- Hyperbilirubinemia in G6PD deficient males can be very severe.
- In India, G6PD deficiency is most commonly seen in Parsis, Bhanushalis, Sindhis, Punjabi, Khoja communities.

Because of the diminished capacity of neonatal RBCs to deal with oxidative stress, as a result of lower glutathione peroxidase, catalases as well as relative deficiency of Vit E, newborn infants with G6PD deficiency are at a greater risk of developing hemolytic anemia than are adults. This particularly is the case in more severe types affecting Asians and Mediterranean group. In this group of patients, jaundice in the newborn period may be severe leading to kernicterus. Jaundice that occurs, appears to be due to accentuation of physiologic jaundice of newborn, although jaundice may appear in some during first 24 hours.

- Abnormal RBC morphology with evidence of "Heinz bodies" in the RBC (Fig. 4).
- Intravascular hemolysis may be associated.
- Normal G6PD levels during acute hemolysis may not rule out G6PD deficiency as younger RBCs contain high level of enzymes. Hence, the test may have to be repeated after 6 weeks.

Fig. 2 Hereditary spherocytosis showing jaundice in mother and child

Pyruvate kinase deficiency:[35] The other common red cell enzyme deficiency leading to hemolytic anemia in newborn during first week of life is "pyruvate kinase deficiency".[35] This disorder is generally characterized by evidence of hemolysis with increased retic count, few or no spherocytes on the peripheral smear, no blood group incompatibility and a negative Coomb's test. In some instances abnormal RBC morphology with evidence of Heinz bodies, intravascular hemolysis may be associated.

Furthermore, there is a tendency for jaundice to occur more frequently in particular families and communities, indicating that the genetic and environmental factors may influence the presentation. In this group of patients, jaundice may be severe leading to kernicterus.

Characterized by

- Evidence of hemolysis
- Increased retic count
- Few or no spherocytes on the peripheral smear

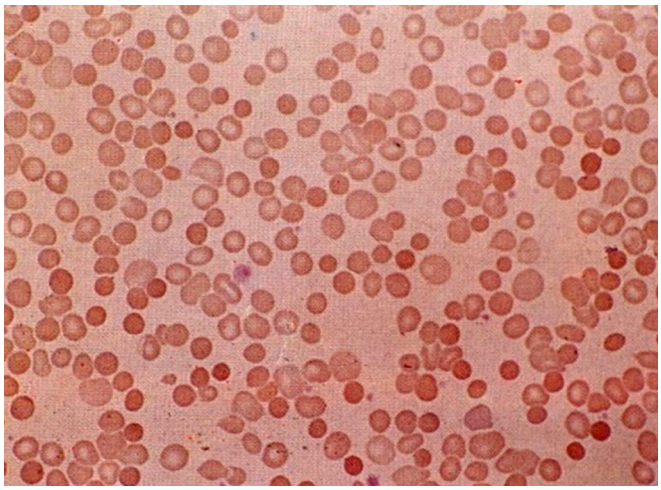


Fig. 3 Spherocytes on peripheral smear

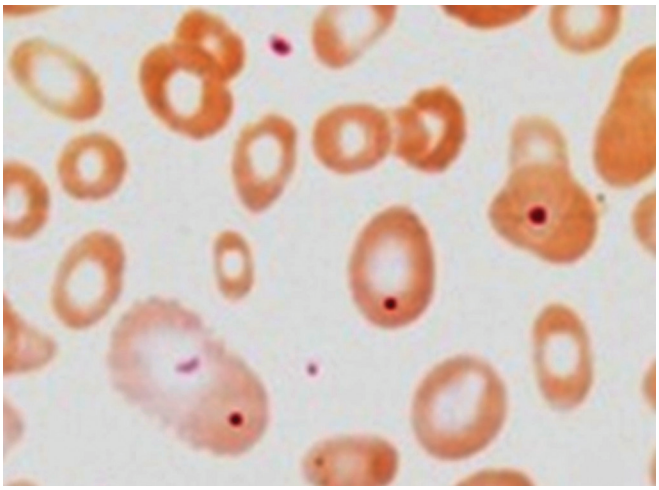


Fig. 4 Heinz bodies in RBCs in G6PD deficiency

- No blood group incompatibility
- Negative Coomb's test
- Enzyme analysis is not easily available
- It may need treatment like phototherapy/exchange transfusion.

Hemoglobinopathies: Alpha thalassemia syndrome.[36]

Of the 4 varieties of alpha thalassemia the homozygous alpha thalassemia which results from the absence of all four genetic loci for alpha chain synthesis, presents in the neonatal period. In the absence of alpha chains, cord blood contains Bart's hemoglobin (Gamma 4) and Hemoglobin H (Beta 4). Most affected infants are stillborn, although some may live for a few hours after birth. These infants are hydropic at birth and thus are similar in appearance to neonates with severe erythroblastosis due to Rh incompatibility.

Gamma thalassemia syndrome: The production of gamma polypeptides is regulated by four genes. The complete absence of gamma chains is incompatible with fetal life. Intermediate reduction of gamma-polypeptide synthesis may produce a mild to moderate neonatal anemia characterized by a reduced percentage of fetal hemoglobin. This type of anemia resolves when significant beta chain synthesis begins. It is important to note that beta thalassemia does not present in the neonatal period.

Other unusual causes of neonatal anemia include congenital dyserythropoietic anemia, leukemia, microangiopathic, hemolytic anemia, DIC, etc.

Anemia due to red cell under production:[37-42] Impaired red cell production is an unusual cause of anemia in the newborn period. Congenital pure red cell anemia (Diamond Blackfan syndrome) is an uncommon disorder in which red cell precursors in the bone marrow are markedly reduced or virtually absent while white blood cell and platelet production remains normal.

It occurs either due to a lack of erythroid stem cells or immune suppression of stem cell differentiation.

- Inheritance seems to follow an autosomal recessive inheritance. The disorder should be suspected in any newborn with anemia and reticulocytopenia, normal platelets and leukocytes.
- Musculoskeletal abnormalities—triphalangeal thumbs may occur in a third of patients. The diagnosis is confirmed by examination of bone marrow aspirate which reveals a virtual absence of erythroid precursors. Mothers of affected children may have increased incidence of miscarriages/abortion and premature birth. Associated physical abnormalities are triphalangeal or duplicated thumb, cleft palate, epicanthal folds, hypertelorism, ptosis, short or webbed neck, congenital heart diseases and short stature.

Infections that cause anemia due to reduced red cell production include parvovirus B1, CMV, toxoplasmosis, congenital syphilis, rubella, herpes simplex. Maternal infection with parvovirus B19 causes fetal anemia which is severe enough to lead to intrauterine death due to hydrops In addition to anemia, parvovirus infections cause marked reticulocytopenia. Thrombocytopenia may also occur.[41]

Laboratory abnormalities include[49]

- Macrocytic anemia
- Absent/reduced reticulocytes
- Elevated HbF
- Absence of erythroid precursors in the bone marrow
- Erythroid—myeloid ratio ranges from 1:6 to 1: 240
- Pancytopenia, accompanied by reticulocytopenia, leukopenia and thrombocytopenia may be seen in severe septicemia and TORCH group of infections. Transplacental transmission of parvovirus B19 cause hypoplastic anemia, which when severe may lead to intrauterine death. Those that survive intrauterine infection may be born with hydrops fetalis. Osteopetrosis/marble bone disease may present with anemia in the newborn period, which could be due to hemolysis or non-production. The disease is associated with hydrocephalus, hepatosplenomegaly and marked increase in the density of the bones particularly of long bones, ribs and base of the skull.
- Other rare causes of anemia include transcobalamin II Fanconi's anemia usually does not manifest in the newborn period and often presents with anemia around the age of 5 to 8 years.

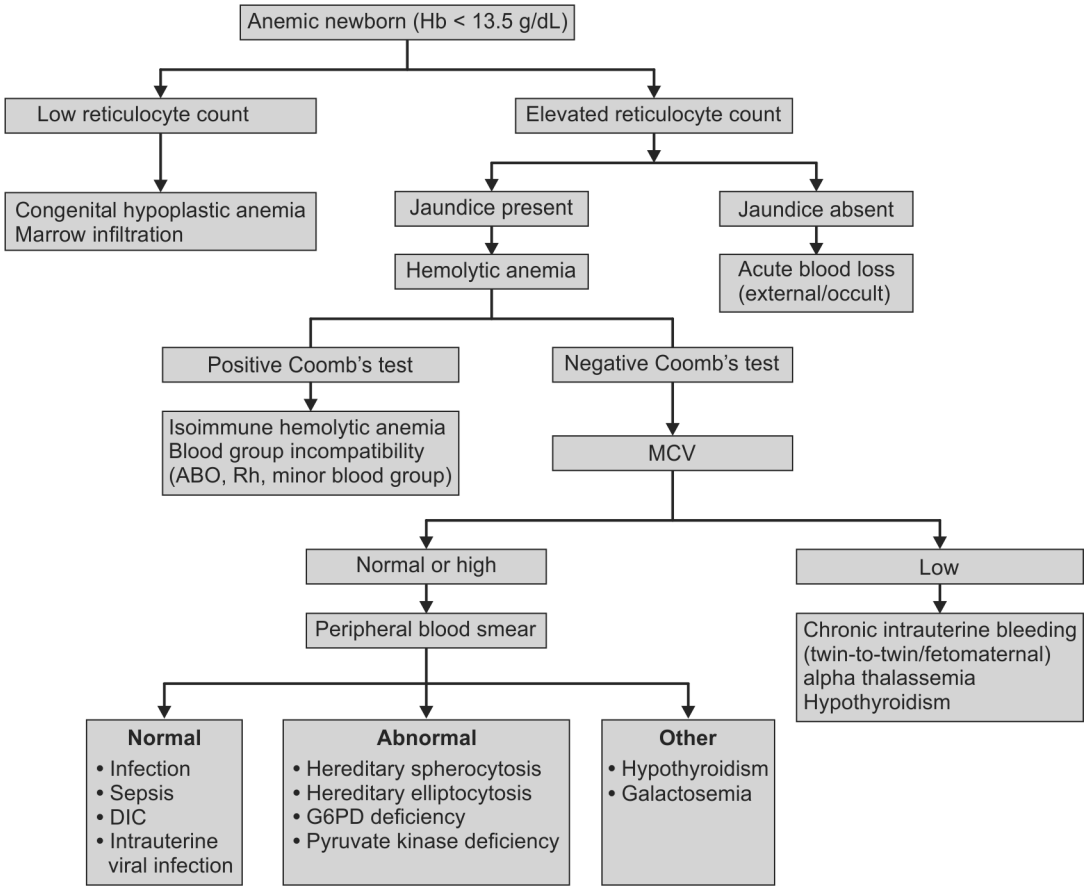
MANAGEMENT OF NEONATAL ANEMIA[49]

The treatment of a neonate with anemia due to blood loss depends on the degree of hypovolemia or anemia. Whether the blood loss has been acute or chronic. Baby born pale at birth should be differentiated from an asphyxiated baby. Besides transfusion for hemorrhagic shock, adoption of transfusion protocols take a variety of factors into account, including hemoglobin levels, degree of cardiorespiratory disease and traditional signs and symptoms of pathologic anemia (Flow chart 1).

Anemia with Shock

Pale babies usually will have tachycardia with minimal or no cyanosis, decreased central venous pressure (CVP) and a rapid fall in hemoglobin with circulatory collapse.

Flow chart 1 Diagnostic approach to neonatal anemia[49]



Abbreviations: DIC: Disseminated intravascular coagulation; G6PD: Glucose 6-phosphate dehydrogenase deficiency; Hb: Hemoglobin; MCV: Mean corpuscular volume.

Guidelines for management

- Clear the airway
- Administer oxygen and intubate if necessary
- Insert the catheter in the umbilical vein and measure CVP
- Obtain blood specimen for investigation
- Rapid expansion of the vascular space with 20 mL/kg of isotonic saline or ringer's lactate, followed by either type specific cross matched whole blood or packed red cells resuspended with saline.

In infants with severe anemia or hypoxia, O, Rh negative RBCs are an acceptable alternative.

- In infants with anemia with congestive heart failure. Furosemide 1 mg/kg followed by a packed cell transfusion of 10 to 15 cc/kg may be given (3 mL/kg of packed cells or 6 mL/kg of whole blood rises the hemoglobin by 1 g/dL).

In severely anemic babies with CHF, a partial exchange transfusion with packed red cells may be carried out to reduce circulatory overload.

Anemia due to Chronic Hemorrhage/Hemolysis

- Iron therapy is initiated for the neonates with anemia who are stable without any signs and symptoms of failure or hyperbilirubinemia even though Hb is low and do not require blood transfusion.
- Phototherapy and double volume exchange transfusion for hyperbilirubinemia followed by a top up packed cell transfusion if required is the main stay of treatment for neonates with isoimmune hemolytic anemia depending upon levels of bilirubin and Hb levels and day of life (age).

- In severely anemic babies with CHF, a partial exchange transfusion with packed red cells may be carried out to reduce circulatory overload.
- Even though neonate has received the blood transfusion, iron therapy is also needed because the transfusions are not sufficient to totally replace the iron lost due to hemorrhage.
- Elemental iron in the dose of 2 mg/kg body weight daily for three months is required to replenish iron stores and return the hemoglobin to normal.
- *Recombinant erythropoietin treatment:*[43-48] Premature infants respond to exogenously administered recombinant human EPO with reticulocytosis, modest decreases in the frequency of PRBC transfusions have been documented primarily in premature infants. The cost-benefit ratio for EPO has yet to be clearly established.
- Babies on erythropoietin therapy should receive oral iron therapy.
- Provision of adequate amounts of vitamin E, vitamin B_{12}, folate, and iron are important in the management of anemia in premature infant.[10,11]
- Diamond Blackfan syndrome can be managed by corticosteroids and repeated blood transfusions and supportive line of treatment.[38-42]
- Anemia of transcobalamin II deficiency requires weekly intramuscular injections of 100 micro grams of vitamin B_{12}.

PREVENTION OF ANEMIA

Newborn, particularly in premature infants reducing the amount of blood taken for investigation purposes diminishes the need to replace blood. The use of noninvasive monitoring devices, such as transcutaneous oxygen saturation, partial pressure of oxygen, and partial pressure of carbon dioxide, may allow decreased blood drawing.

Iron prophylaxis to adolescent girls, antenatal iron administration during pregnancy, oral iron therapy 2 to 6 mg/kg/day in preterm babies, starting by 4 to 6 weeks and continued till weaning has been adequately achieved is an important preventive measure to prevent anemia in new born period and early infancy.

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CHAPTER 9

Polycythemia and Hyperviscosity Syndrome

MMA Faridi, Sriram Krishnamurthy

Polycythemia is defined as an abnormal increase in the red blood cell mass. In neonates the hematocrit rises soon after birth, peaks at around 2 hours of age and falls to about 57 percent in next 12 to 18 hours of age. The most widely accepted definition of neonatal polycythemia is a venous hematocrit greater than 65 percent (capillary hematocrit >70 percent) or a venous hemoglobin concentration in excess of 22.0 g/dL.[1,2] This cut off value has been chosen based on the observation that blood viscosity exponentially increases above a hematocrit of 65 percent.[3] Other definitions of polycythemia include a venous hematocrit of 64 percent or more at 2 hours of age,[4] or an umbilical venous or arterial hematocrit of 63 percent or more.[5]

Hyperviscosity is defined as thickness of the blood greater than 14.6 centipoise at a shear rate of 11.5 sec^{-1}. The normal blood viscosity (mean ± SD) in the neonate is 1.18 ± 0.17 centipoise at a shear rate of 11.5 sec^{-1}.[2-6] Hyperviscosity and polycythemia have a linear relationship up till a hematocrit of 60 percent. Hyperviscosity starts rising exponentially after a hematocrit of 65 percent and markedly increases at a hematocrit of 70 percent or more.[7] Hyperviscosity is influenced not only by the red cell mass, but also by other factors such as plasma fibrinogen and local blood flow.

The terms ***polycythemia*** **and** ***hyperviscosity*** are often used interchangeably although they are not equivalent as is evident from the description. Polycythemia is significant only because it increases the risk of hyperviscosity syndrome. Hyperviscosity syndrome comprises symptoms and signs caused by tardy flow and sludging of blood within the blood vessels especially in the smaller arterioles and capillaries. Sludging of blood occurs because increased red blood cell mass causes a relative decrease in the plasma volume and a relative increase in the proteins and platelets. The sludging of blood within blood vessels can also lead to thrombosis and infarcts in the territory supplied by them.

Viscosity is difficult to measure. It is usually measured by **Wells-Brookfield cone-plate microviscometer.** Since instruments to measure viscosity are not readily available in most neonatal intensive care units, hyperviscosity is usually suspected on the basis of clinical symptoms and signs in the presence of abnormally high hematocrit (polycythemia).

INCIDENCE OF NEONATAL POLYCYTHEMIA AND HYPERVISCOSITY

The true incidence of polycythemia and hyperviscosity is not known since majority of infants are likely to be asymptomatic normal newborns. Hematocrit is not routinely recommended or drawn in this population, most likely due to the controversy surrounding the treatment of asymptomatic infant.[8] The reported incidence of polycythemia ranges between 0.4 and 12 percent; in USA 1 to 5 percent newborns reportedly suffer from polycythemia.[2,5,7,9] This wide variation may be due to different screening techniques, sampling sites (capillary versus peripheral, central venous or arterial), varied patient population, mode of delivery, method of measuring (Coulter counter or centrifuged capillary blood), and sampling time. Sampling time is the most important source of this variation. The hematocrit normally rises after birth, reaching a peak at two hours postpartum and then slowly decreases over the next 12 hours. At two hours of life the

upper limit (2 SD) of a normal capillary hematocrit is 71 percent while it is 64 percent for a venous hematocrit.[5] Hyperviscosity occurs in 6.7 percent of neonates.

- Only 47 percent of infants with polycythemia exhibit hyperviscosity and 24 percent infant with hyperviscosity have polycythemia.[4,10,11]
- It is more common in infants.
- Small-for-gestational age (SGA).
- Large-for-gestational age (LGA).
- Infants born to diabetic mothers have predilection for developing polycythemia. Thirty percent and forty percent infants suffer from polycythemia if mothers have diabetes or suffer from gestational diabetes respectively.

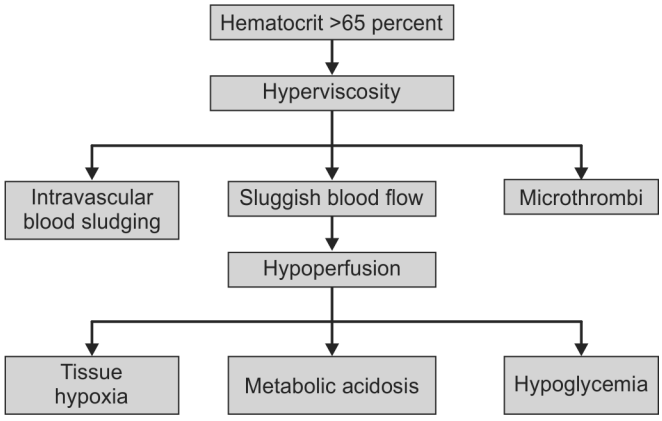
PATHOPHYSIOLOGY

The mean venous hematocrit in term infants is 52 percent at 12 to 18 hours of age[5] though values up to 71 percent have also been described as normal at 2 hours of age by many authors.[12,13]

The initial rise in the hematocrit is related to transudation of fluid out of the intravascular space. As the venous hematocrit increases, the viscosity rises. When the hematocrit increases to more than 65 percent, there is exponential elevation in the blood viscosity that, coupled with decreased deformability of the fetal erythrocytes, in comparison to adult red blood cells, leads to sluggish peripheral circulation, formation of microthrombi, fall in the oxygen transport and tissue hypoxia (Flow chart 1).

Tissue hypoxia leads to increased glucose metabolism, to generate adequate amounts of ATP by anaerobic respiration, hypoglycemia and metabolic acidosis. Hypoxia and acidosis further aggravate hyperviscosity. Increased viscosity of blood, in general, mimics symptoms and signs of hypoperfusion. Polycythemia, therefore, creates a pathophysiological situation analogous to shock.

Flow chart 1 Pathophysiology of polycythemia-hyperviscosity



Microthrombi formation may occur in the blood vessels supplying cerebral cortex, kidneys, intestines and adrenal glands, with potential long lasting sequelae.

INCREASED FETAL ERYTHROPOIESIS (PRIMARY POLYCYTHEMIA)

Neonatal polycythemia can occur by two mechanisms.

1. *Active form:* Fetus/newborn produces excess number of red blood cells due to physiological triggers like perinatal hypoxia, uncontrolled maternal diabetes and chromosomal defects in the fetus. It is also referred as primary polycythemia.
2. *Passive form:* Fetus/newborn can acquire large blood volume as a result of delayed cord clamping or maternofetal transfusion. Increase in the red blood cells by hypertransfusion is also called secondary polycythemia.

The causes of neonatal polycythemia can be divided according to temporal happenings like:

- Before birth (antenatal)
- During birth (intranatal)
- After birth (neonatal).

These are summarized in Table 1.

It can be secondary to pre-eclampsia, primary renovascular disease, chronic or recurrent abruptio placentae, maternal cyanotic congenital heart disease including Eisenmenger syndrome[14] postdated pregnancy and maternal smoking. Awonusonu et al. (2002) have reported that the risk of symptomatic polycythemia requiring partial exchange blood transfusion is 2.5 times more in mothers who smoke (1.59%) during pregnancy than those who do not (0.64%).[15] Most of these maternal conditions may also be associated with intrauterine growth restriction (IUGR).

The incidence of polycythemia increases with increasing severity of fetal growth retardation. In severely growth retarded fetuses, a hematological syndrome of polycythemia, thrombocytopenia, leukopenia and increased numbers of nucleated red blood cells has been described.[16,17]

- *Endocrine abnormalities:* Conditions associated with increased fetal oxygen consumption may result in fetal hypoxia and subsequent polycythemia like congenital thyrotoxicosis and Beckwith-Wiedemann syndrome or infants of diabetic mother (IDM) with poor glycemic control. Polycythemia in IDMs correlates with macrosomia and neonatal hypoglycemia.
- *Chromosomal disorders:* Trisomy 13, trisomy 18 and trisomy 21 may be associated with polycythemia. The exact mechanism is not well understood. However, it has been observed that in trisomy 13, 18 and 21, placentae show trophoblastic hypoplasia and

Table 1 Etiology of polycythemia

Antenatal	*Intranatal*	*Neonatal*
Hypoxia	Hypoxia	Genetic causes • Trisomy[13, 18, 21] • Beckwith-Wiedemann syndrome
IUGR, SGA infants	**Intrapartum asphyxia due to etiologies such as** • Obstructed labor • Prolonged labor • Abruptio placentae	**Endocrine causes** • Infants of diabetic mothers • Neonatal thyrotoxicosis • Congenital hypothyroidism • Congenital adrenal hyperplasia
Infants of diabetic mothers	Delayed clamping of umbilical cord	
Placental insufficiency	Perinatal asphyxia	
Pre-eclampsia	**Hypertransfusions**	
Maternal smoking	Oxytocin use during labor	
High altitude	Holding the baby below the introitus at the time of delivery	
Postmaturity	Milking of the umbilical cord	
Infants born to mothers with chronic cardiopulmonary conditions		
Hypertransfusions		
Primary renovascular disease		
In utero asphyxia		
Twin-to-twin transfusion		
Maternofetal transfusions		
Genetic syndromes Trisomy Beckwith-Wiedemann syndrome		
Miscellaneous congenital hypothyroidism, Congenital adrenal hyperplasia Maternal use of propranolol		

hypovascularity, which has been attributed to low levels of vascular endothelial growth factor (VEGF) and placental growth factor (PLGF).[18,19] VEGF and PLGF are considered to play important roles in angiogenesis and vascular permeability during placental development. Therefore, low levels of these factors could cause chronic fetal hypoxia, resulting in increased erythropoietin levels and polycythemia.[20]

- *High altitude:* Babies born at high altitude are found to have polycythemia due to relative paucity of oxygen.

HYPERTRANSFUSION (SECONDARY POLYCYTHEMIA)

- *Delayed cord clamping:* It allows increased blood volume to be delivered to the infant. When cord clamping is delayed to more than 3 minutes after birth, neonatal blood volume increases by 30 percent. Gravity may facilitate transfer of large blood volume to the newborn because of the position of the delivered infant in relation to the maternal introitus before cord clamping. Oxytocin may enhance blood flow to the infant via umbilical vessels in the event of delayed cord clamping.
- *Twin-to-twin transfusion syndrome:* It occurs in approximately 10 percent of monozygotic twin pregnancies due to a vascular communications between twin babies. The recipient twin suffers from polycythemia and hypervolemia.
- *Maternofetal transfusion:* It has been recognized as a cause of blood transfer from the uterine vessels via placenta to the fetal circulation due to placental vascular malformations leading to hypervolemia, polycythemia and hyperviscosity syndrome in the newborn infant.[21]
- *Intrapartum asphyxia:* Perinatal asphyxia during delivery, due to any cause, may shift blood volume from the placenta to the fetus to maintain cerebral perfusion. This may lead to increased blood volume in the perinate followed by polycythemia and hyperviscosity.

CLINICAL FEATURES

Majority of appropriately grown term polycythemic newborn infants have no symptoms, particularly if the polycythemia is found on routine neonatal screening. Symptoms, when present, are usually attributable to hyperviscosity and poor tissue perfusion or to associated metabolic derangements such as hypoglycemia. About 50 percent of polycythemic infants develop one or more symptoms. Clinically the baby may manifest skin color from red (polycythemia), blue (cyanosis resulting from peripheral stasis), yellow (jaundice due to breakdown of large amount of RBCs; 34.5 mg bilirubin is produced from 1g of hemoglobin) to pale when shock sets in.

- *Common early symptoms* include plethora, lethargy, hypotonia, poor suck and feeding, and tremulousness. Serious complications include cardiorespiratory distress (with or without congestive heart failure), seizures, peripheral gangrene, necrotizing enterocolitis, renal failure (occasionally resulting from renal vein thrombosis), hyperbilirubinemia and priapism. Most of these symptoms are non-specific and may be related to the underlying causes rather than polycythemia *per se*.
- *Central nervous system:* It is the most common system to be affected. Early effects include lethargy, poor feeding, easy startle, hypotonia, difficult arousal, tremors, irritability, jitteriness and seizures. Long term sequelae include developmental delay and poor fine motor control.
- *Metabolic derangement:* Hypoglycemia is the most common metabolic abnormality in the infant, reagent glucose strips frequently give falsely low values Therefore, blood sugar should always be reconfirmed by a laboratory test. Hypocalcemia and hypomagnesemia are also known to occur in polycythemia. The elevated red blood cell mass increases catabolism of the hemoglobin so hyperbilirubinemia is common and even gall stones occasionally occur.
- *Cardiopulmonary system:* Tachycardia, tachypnea, congestive cardiac failure and cyanosis may be found. Rarely persistent fetal circulation may develop with poor prognosis. Polycythemia should be considered as a differential diagnosis for transient tachypnea of the newborn. Chest radiography may reveal cardiomegaly, pulmonary plethora, hyperaeration and pleural effusion. Echocardiographic findings include increased pulmonary resistance, bidirectional shunt and decreased cardiac output.
- *Gastrointestinal system:* Features of gastrointestinal affliction are poor suckling, vomiting, feed intolerance, prefeed aspirates, abdominal distension, paralytic ileus and necrotizing enterocolitis (NEC).
- *Renal system:* Hyperviscosity affects renal perfusion. Oliguria, acute renal failure, renal vein thrombosis and decreased urinary sodium may occur in polycythemic infants.
- *Miscellaneous:* Thrombocytopenia, coagulation defects, stroke, peripheral gangrene, thrombosis, priapism and testicular infarction are well known complications of polycythemia.

Laboratory Diagnosis

- Certain high-risk groups such as small for gestational age (SGA) infants, infants of diabetic mothers (IDMs), monochorionic twins, large for gestational age (LGA) infants or presence of risk factors (enlisted in Table 1) should be screened for polycythemia.[2]
- Screening is usually done in high-risk neonates at 2 hours of age. A normal value at 2 hours of age (venous hematocrit <65%) does not warrant further screening unless the neonate is symptomatic.
- Hematocrit values >65 percent at 2 hours of age merit repeat screening at 12 and 24 hours.
- Polycythemia is diagnosed when venous hematocrit is >65 percent.[2]
- In the presence of clinical features suggestive of poor circulation with plethora and/or cyanosis and tachypnea, a venous hematocrit measurement is used as a surrogate for diagnosing hyperviscosity because former can be readily done and the latter exponentially increases after a hematocrit of >65 percent.
- Capillary or venous hematocrit—which one to measure? Capillary hematocrit measurements depend upon the blood flow and are significantly higher than venous hematocrits and are therefore, unreliable. However, capillary samples may be used for screening but all high values should be confirmed by a venous hematocrit for the diagnosis of polycythemia.

Hematocrit Measurement

Two methods are available:

1. *Automated hematology analyzer:* This calculates hematocrit from a direct measurement of mean cell volume and the hemoglobin. Hematocrit (%) is approximately three times the hemoglobin concentration in g/dL.
2. *Microcentrifuge:* Blood is collected in heparinized microcapillaries (110 mm length and 1–2 mm internal diameter) and centrifuged at 10,000 to 15,000 revolutions per minute (rpm) for 3 to 5 minutes. Plasma separates and the packed cell volume is measured to give the hematocrit. An automated analyzer gives lower values as compared to hematocrits measured by the centrifugation methods. Most of the reported literature on polycythemia is based on centrifuged hematocrits.[8]

Other laboratory tests to be done in a case of polycythemia:

- *Kidney function tests:* Renal functions should always be evaluated in a case of symptomatic polycythemia. Blood urea (BUN) and serum creatinine may increase. There may be dilutional hyponatremia and serum potassium may rise. Judicious fluid and electrolytic intake is warranted for good prognosis.
- Serum glucose and calcium levels should be determined in all symptomatic polycythemia and infants and vigorously treated if the patient has abnormal levels.

- Serum bilirubin rises rapidly in babies who have polycythemia due to increased RBC destruction much beyond the neonatal hepatic conjugation capacity. Serum bilirubin must be checked serially. If nomograms are available then transcutaneous bilirubinometry can be a very useful modality to screen for hyperbilirubinemia as it is a noninvasive technique. However, when transcutaneous bilirubin index suggests a higher serum bilirubin level, it should always be confirmed by laboratory method before instituting therapy for hyperbilirubinemia.
- *Arterial blood gases (ABG):* Consider measuring ABG values to assess oxygenation in the symptomatic infant with respiratory distress and cyanosis.
- *Platelet count:* Platelet count must be checked at base line. Thrombocytopenia is present if thrombosis or disseminated intravascular coagulation (DIC) has set in. Thrombocytopenia may also be found in babies born to pre-eclamptic mothers, who are prone to develop symptomatic polycythemia.
- *Urinalysis:* Proteinuria and casts may be present.

MANAGEMENT

- Possible ways of avoiding polycythemia include early cord clamping and holding the baby at the level of the introitus at the time of delivery to minimize hypertransfusion.
- A good glycemic control and management of growth retardation in the antenatal period may prevent development of polycythemia and in turn hyperviscosity after birth.
- It is essential to exclude dehydration before a diagnosis of polycythemia is made. A clue to dehydration could be excessive weight loss. If this is present, increasing the fluid intake would be the appropriate therapeutic measure. The hematocrit should be measured again after correction of dehydration.
- Associated metabolic problems like hypoglycemia, hypocalcemia and acidosis should be treated simultaneously.

The principles of management of neonatal polycythemia are:

- To decrease red cell mass below threshold level.
- To remove excess blood volume.
- To maintain metabolic and blood gas homeostasis till the condition reverts back to normal.

The following modes of therapy have been employed for the treatment of polycythemia.

- *Conservative management with additional fluid intake:* This mode of therapy may be tried in cases of asymptomatic polycythemia when the hematocrit reaches 70 to 75 percent. An extra fluid aliquot of 20 mL/kg may be added to the daily requirements.[2] Extra fluid intake may be ensured either by enteral route (supervised feeding) or by parenteral route (IV fluids). The rationale for this therapy is hemodilution and resultant decrease in viscosity. However, liberal and extra fluid therapy may be associated with problems especially in preterm babies. Hence conservative management by using extra fluids should be reserved for hemodynamically stable babies with asymptomatic polycythemia.
- *Partial exchange transfusion:* Partial exchange transfusion (PET) is traditionally used as the method of choice for the treatment of symptomatic polycythemia to lower hematocrit as well as hyperviscosity. This method is also employed to treat asymptomatic polycythemia if hematocrit is >75 percent. PET aims to decrease the hematocrit to a target packed cell volume of 55 percent. PET is performed with either crystalloid (normal saline or Ringer's lactate) or colloid (5% albumin or fresh frozen plasma) solutions. Crystalloids are preferred because they are economical, easily available, produce similar reduction in the hematocrit as colloids[22,23] and do not have the risk of transfusion associated infections (e.g. HIV, hepatitis B, hepatitis C, CMV). Additionally, adult plasma may potentially increase the blood viscosity when mixed with fetal erythrocytes.

The blood volume to be partially exchanged is calculated by the following formula:

Volume to be exchanged (V mL) = infant's blood volume × (observed hematocrit—desired hematocrit)/ observed hematocrit.

The desired hematocrit is kept as 55 percent. Blood volume is estimated to be 80 to 90 mL/kg in term and 90 to 100 mL/kg in preterm babies. As a rough guide, the volume of blood to be exchanged is usually 20 mL/kg.

PET normalizes cerebral hemodynamics and improves clinical status of the infants with polycythemia.[1] PET has also been shown to reduce pulmonary vascular resistance[24] and increase cerebral blood flow velocity.[25, 26] PET is a relatively simple procedure, but has numerous potential complications. Unfortunately, there are no data regarding the incidence of complications of PET; one can only extrapolate from the data on full exchange transfusions performed for neonatal hyperbilirubinemia.[8] Reported complications in whole blood exchange include apnea, cardiac arrhythmia, decreased PR interval,[27] embolism, vessel perforation, accidental hemorrhage, hypothermia, reduction in blood pressure, cerebral blood flow fluctuations, sepsis, necrotizing enterocolitis and portal vein thrombosis. Hypernatremia and raised osmolality following whole blood exchange transfusion have also been reported by Jain, Puri and Faridi (1997).[28]

However, whole blood exchange transfusion is expected to have a higher incidence of complications than PET, since the amount of blood to be exchanged is almost nine times higher and the product utilized for the exchange is donor's blood.

The statement of the committee of the fetus and newborn, American Academy of Pediatrics[8] regarding the treatment of neonatal polycythemia with PET reflects both the concern and uncertainty—"The accepted treatment of polycythemia is partial exchange transfusion. However there is no evidence that exchange transfusion affects the long term outcome. Universal screening for polycythemia fails to meet the methodology and treatment criteria and also, possibly the natural history criterion". Despite this ambivalent statement, the standard practice in most nurseries is to perform PET in symptomatic babies with a hematocrit greater than 65 percent or in asymptomatic babies with a hematocrit greater than 70 percent.[29,30]

PHLEBOTOMY

Michael and Mauer[21] have described phlebotomy as a successful treatment modality in cases suffering from maternofetal transfusion. It seems logical to reduce hypervolemia in cases of hypertransfusion state. Therefore, phlebotomy can be employed in cases where polycythemia is a result of passive or secondary polycythemia.

Routes for Partial Exchange Transfusion

PET may be done through a

- *Peripheral or central route:* A peripheral route avoids umbilical vessel cannulation and is done by using a peripheral arterial and venous line. Blood is withdrawn from the arterial line and replaced simultaneously through the venous line.
- *A central route* requires umbilical vein cannulation. The umbilical venous catheter may be used for withdrawing blood while the same amount of saline is replaced through a peripheral vein. Alternatively the umbilical venous catheter may be used both for the withdrawal of blood and replacement with saline.
- Dempsey et al.,[31] in a recent systematic review have shown that PET through umbilical route may be associated with increased risk of necrotizing enterocolitis (Relative risk 8.68, 95 percent CI 1.06, 71.1).

Short-term and long term outcome with polycythemia: PET reverses the short term pathophysiological abnormalities associated with polycythemia hyperviscosity syndrome. It improves capillary perfusion, cerebral blood flow and cardiac function.[1,24-26] However, there is very little data to suggest that PET improves long-term (neurodevelopmental) outcome in patients with polycythemia. Studies by Black et al.[16] and Goldberg et al.[17] did not demonstrate improvement in the long-term outcome with the use of PET in symptomatic polycythemia. Similarly PET has not shown any beneficial effect on long-term outcome in neonates with asymptomatic polycythemia.[1]

The recent systematic review by Dempsey et al.[31] also supports these findings. In this review, randomized or quasi-randomized trials in term infants with polycythemia and/or documented hyperviscosity were considered. Clinically relevant outcomes included were short-term (resolution of symptoms, neurobehavioral scores, major complications) and long-term neurodevelopmental outcome. There was no evidence of an improvement in the long-term neurological outcome (Mental Developmental Index, incidence of mental delay and incidence of neurological diagnoses) following PET in symptomatic or asymptomatic infants. Also, there was no evidence of improvement in early neurobehavioral assessment scores (Brazelton Neonatal Behavioral Assessment Scale). It was concluded that PET may be associated with an early improvement in symptoms, but there are insufficient data to calculate the size of the effect.

It is probable that the underlying etiology of polycythemia is a more important determinant of the ultimate outcome. However, definitive data on long-term outcome with treatment is still unavailable in infants with symptomatic polycythemia and in asymptomatic infants with hematocrit >70 percent. Therefore, it may be advisable to perform a PET or consider plasma expansion with additional fluids based on the presence or absence of symptoms in these polycythemic neonates.

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CHAPTER 10

Vitamin K Deficiency: Bleeding in Newborns

Arvind Saili, Ajay Kumar

Vitamin K deficiency bleeding (VKDB) refers to bleeding that occurs as a consequence of vitamin K deficiency during first six months of life. Previously known as the hemorrhagic disease of the newborns, it has been renamed to emphasize that bleeding problems during the neonatal period are not confined to those arising from vitamin K deficiency alone and that bleeding secondary to vitamin K deficiency may occur beyond the first month of life.

DEVELOPMENT OF HEMOSTATIC SYSTEM

Hemostasis develops in an orderly way during intrauterine life. The most basic reaction-contraction of blood vessels in response to injury-is present from eight weeks, though its strength does not become normal until much later. Platelets appear in the circulation by 11 weeks and can form aggregates by 12 to 15 weeks; they approximate to adult numbers by 30 weeks. Clotting and fibrinolytic plasma proteins are found from 10 to 11 weeks; the concentrations of some clotting factors reach adult values *in utero*, but of others, mainly those dependent on vitamin K (II, VII, IX and X) are still low at term.

Normal neonatal hemostasis reflects a highly complex process dependent on interactions between endothelial cells, platelets and hemostatic proteins. It is now recognized that the traditional extrinsic pathway involving tissue factors and factor VIIa, is the major pathway where by coagulation is initiated and the thrombin plays a crucial role in coagulation as well as platelet activation.

At birth concentrations of vitamin K dependent factors (II, VII, IX and X) and contact factors (XI and XII) are reduced to about 50 percent of normal adult values. Similarly, concentration of the naturally occurring anticoagulants, antithrombin, protein C and protein S are low at birth and as a consequence, both thrombin generation and thrombin inhibition are reduced in the newborn period.

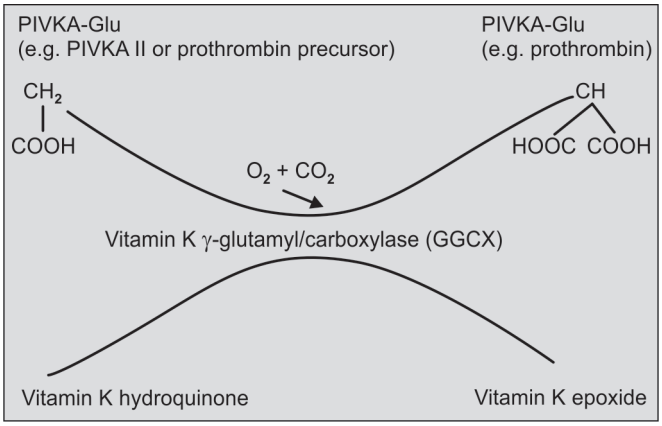
Biology of vitamin K: Vitamin K is a generic name for several molecules sharing a 2 methyl-1,4 naphthoquinone ring but differing regarding the side chain at the 3-position. Phylloquinone or vitamin K_1 is from plant origin and has a phytyl side chain. The group of menaquinones or vitamin K_2 differs in the number of isoprenyl units in the side chain and are synthesized by the bacteria in humans and animal intestine. Menadione or vitamin K_3 is a synthetic and water soluble vitamin K without a side chain. This preparation is not preferred as it has been shown to cause hemolytic anemia, indirect hyperbilirubinemia and kernicterus. In our country, only vitamin K_3 preparations are available. Vitamin K acts as a cofactor for gamma glutamyl carboxylase (GGCX) serving as an electron donor for the post-translational conversion of protein bound glutamate into Gamma-carboxyglutamate. During this process, it is oxidized to vitamin K_2, 3-epoxide. Gla residues are calcium binding groups which are essential for the biological activities of proteins in which they are found. Gla containing proteins are the coagulation factors II, VII, IX and X but also protein C, protein S, protein Z, osteocalcin, etc.

Vitamin K deficiency leads to the synthesis of undercarboxylated proteins unable to bind calcium and hence inactive. In vitamin K deficient individuals, undercarboxylated forms of vitamin K dependent coagulation proteins (proteins induced by vitamin K absence PIVKA) are released from the liver into the blood. PIVKA are inactive in the coagulation cascade. PIVKA II or

undercarboxylated prothrombin is a marker of subclinical vitamin K deficiency.

CHEMICAL STRUCTURE OF VITAMIN K

Vitamin K Cycle



Vitamin K deficiency bleeding (VKDB): As a consequence of limited stores at birth, neonates are prone to vitamin K deficiency if no sufficient intake is provided. Vitamin K deficiency has been traditionally classified as early, classical and late depending on timing of the presentation.

Early VKDB: Presents within 24 hours of birth and is almost exclusively seen in infants of mothers taking drugs which inhibit vitamin K. These drugs include anticonvulsants (carbamazepine, phenytoin and barbiturates but not valproic acid), antitubercular drugs (isoniazid, rifampicin), some antibiotics (cephalosporins) and vitamin K antagonists (coumarin, warfarin).

Clinical presentation is often severe with cephalic hematoma, intracranial and intra-abdominal hemorrhage. The incidence in an at-risk group without vitamin K supplementation is 6 to 12 percent.

Classical VKDB: Occurs between 24 hours and 7 days of life and is associated with delayed or insufficient feeding. Clinical presentation is often mild, with bruises, gastrointestinal blood loss or bleeding from the umbilicus and puncture sites. Blood loss, however, can be significant, and intracranial hemorrhage, although rare, has been described. Without vitamin K supplementation, incidence estimated is 0.01 to 0.44 percent.

Late VKDB: It is associated with exclusive breastfeeding. It occurs between the ages of 2 and 12 weeks. Clinical presentation is severe, with a mortality rate of 20 percent and intracranial hemorrhage occurring in 50 percent. Persistent neurological damage is frequent in survivors. In fully breastfed infants who did not receive vitamin K at birth, the incidence is between 1/15,000 and 1/20,000. Infants with cholestasis or malabsorption syndromes are at particular risk. Often, VKDB is the first sign of this underlying condition. Vitamin K epoxide is recycled to vitamin K by vitamin K epoxide reductase (VKOR). This recycling process is inhibited by coumarin and warfarin.

CLINICAL FEATURES

Diagnosis

The diagnosis of vitamin deficiency may be suspected from the results of coagulation screening where initially, there is isolated prolongation of the prothrombin time followed by prolongation of the APTT, in association with the normal concentrations of fibrinogen and normal platelet count. Confirmation of the diagnosis requires measurements of PIVKA II.

Treatment

Once the diagnosis is confirmed, intravenous vitamin K should be administered to correct the existing deficiency. In suspected cases, vitamin K can be given while factor concentrations are pending. In the presence of major bleeding, factor replacement therapy may also be required with fresh frozen plasma, prothrombin complex concentrate (FII, FIX, FX), or a four factor concentrate containing all the vitamin K dependent factors.

No formal studies were ever performed to establish what dose might be appropriate before it became standard practice to give every infant a 1-mg dose at birth and to give it intramuscularly simply because that was the only product available. In 1990, an epidemiological study described an association between intramuscular (IM) vitamin K at birth and childhood cancer and leukemia. In response to these findings, several European countries, Australia and New Zealand changed their policy to oral prophylaxis. In the following years, new studies failed to confirm the association between IM vitamin K at birth and childhood cancer. A risk of solid tumors can now almost definitely be ruled out, but a small risk of leukemia cannot be excluded. When cases of late VKDB started to reappear, Denmark, Canada, Australia and New Zealand responded by reintroducing universal IM prophylaxis, offering oral prophylaxis with repeated doses to those parents refusing the IM injection at birth. Oral prophylaxis with repeated doses has remained the policy in the Netherlands and in Germany, using different products and dosing schemes. The American Academy of Pediatrics has always endorsed the IM route.

ORAL VITAMIN K

Oral vitamin K administration would appear to offer several advantages for routine VKDB prophylaxis. In addition to the concerns raised about a link with childhood cancer, other disadvantages with IM administration

include the trauma and complications associated with this route of administration (hematoma, vessel or nerve injury, abscess, or osteomyelitis) and the higher cost of therapy. While no oral liquid preparation is available, the injectable product has been found to be safe and effective when given by the oral route. Unfortunately, the rise in the use of oral vitamin K prophylaxis has led to an increase in reports of late VKDB. Several countries currently use an alternative mixed micellar preparation of vitamin K (Konakion MM®; Roche) for multidose oral prophylaxis. This formulation is expected to provide greater absorption than traditional preparations and may make oral administration more effective. Unfortunately this preparation is not available in most of the countries including India.

The Cochrane Review

All trials using random or quasi-random patient allocation, in which methods of vitamin K prophylaxis in infants were compared to each other, placebo or no treatment, were included. Two eligible randomized trials comparing a single dose of intramuscular vitamin K with placebo or nothing, assessed effect on clinical bleeding. One dose of vitamin K reduced clinical bleeding at 1 to 7 days, including bleeding after circumcision, and improved biochemical indices of coagulation status. Eleven additional eligible randomized trials compared either a single oral dose of vitamin K with placebo or nothing, a single oral with a single intramuscular dose of vitamin K, or three oral doses with a single intramuscular dose. None of these trials assessed clinical bleeding. Oral vitamin K improved biochemial indices of coagulation status at 1 to 7 days. There was no evidence of a difference between the oral and intramuscular route in effects on biochemical indices of coagulation status. A single oral compared with a single intramuscular dose resulted in lower plasma vitamin K levels at two weeks and one month, whereas a 3-dose oral schedule resulted in higher plasma vitamin K levels at two weeks and at two months than did a single intramuscular dose. It was concluded that a single dose (1.0 mg) of intramuscular vitamin K after birth is effective in the prevention of classic HDN. Either intramuscular or oral (1.0 mg) vitamin K prophylaxis improves biochemical indices of coagulation status at 1 to 7 days. Neither intramuscular nor oral vitamin K has been tested in randomized trials with respect to effect on late HDN. Oral vitamin K, either single or multiple doses, has not been tested in randomized trials for its effect on either classic or late HDN.

The American Academy Recommendation

The vitamin K Ad Hoc Task Force of the American Academy of Pediatrics (AAP) recommends: (1) Vitamin K, should be given to all newborn as a single, intramuscular dose of 0.5 to 1 mg. (2) Additional research should be conducted on the efficacy, safety, and bioavailability of oral formulations and optimal dosing regimens of vitamin K to prevent late VKDB. (3) Health care professionals should promote awareness among families of the risk of late VKDB associated with inadequate vitamin K prophylaxis from current oral dosage regimens, particularly for newborns who are breastfed exclusively.

Vitamin K and Preterm Newborn

Ever since the discovery of vitamin K, it has been clear that premature infants are at particular risk of VKDB. Although there is consensus on the fact that all premature infants should receive vitamin K, neonatology units use a variety of doses, dosing schedules, routes and formulations. Reports have shown very high plasma vitamin K levels in preterm infants receiving 0.5 to 1 mg at birth. Although no toxic effects of these excessively high serum levels have been recognized, caution is warranted because the functions of some Gla proteins are not fully understood. A recent randomized trial shows adequate serum vitamin K levels in preterm infants receiving 0.2 mg at birth. In this trial, preterm infants receiving 0.5 mg have elevated levels of vitamin K epoxide, suggesting inefficient recycling of vitamin K by VKOR in the immature liver. These findings support current empirical dosage recommendations for preterm infants advising a reduced dose of 0.3 mg for birth weights <1,000 g and 0.5 mg for those >1,000 g and <1,500 g.

Vitamin K and Cholestatic Disorders

Due to fat malabsorption and inadequate intake, infants with cholestatic liver disease are especially at risk for vitamin K deficiency. Some of the current standard regimens of oral vitamin K prophylaxis are mostly insufficient in cholestatic patients making them extremely vulnerable for VKDB. More than 80 percent of breastfed infants with biliary atresia who received oral vitamin K prophylaxis (1 mg oral vitamin K at birth followed by 25 microgram daily) developed a VKDB at the time of diagnosis. Forty three percent presented with an intracranial hemorrhage. The empirical dosing guideline for oral vitamin K_1 in infants and children with chronic cholestasis is 2.5 to 5 mg given two to seven times per week. Nevertheless, with this regimen, subclinical vitamin K deficiency seems prevalent despite normal prothrombin time (PT). In a group of 43 cholestatic children supplemented following this schedule, 23 (54%) had elevated plasma PIVKA II levels (>3 ng/mL) with normal PT. Vitamin K doses sufficient to maintain normal coagulation may not be sufficient to maximize carboxylation of coagulation factors. Based on the above-mentioned data, it is thus of utmost importance

that, as soon as the diagnosis of cholestasis is made in an infant, extra vitamin K supplementation should be given to prevent VKDB with its serious consequences. However, the best strategy for vitamin K supplementation in chronic childhood cholestasis still remains a critical issue. Current regimens may be underestimating the optimal dosage of vitamin K.

Current International Scenario

The various schemes for vitamin K administration being followed world over with risk of late HDN has been summarized in Table 1.

Table 1 Various schemes for vitamin K administration

	Administration scheme	*Incidence per 1,00000*
The Netherlands	1mg oral at birth followed by 25 µg daily for 2 weeks	3.2
Germany	2 mg oral at birth followed by 2 mg at 1 and 4 weeks	0.44
Denmark	2 mg oral at birth followed by 1 mg weekly till 12 weeks	0
Great Britain	1 mg IM	0.1
	1 mg oral, continuing after 1 week	0.43
	1 mg oral, not beyond 1 week	2.9
	Nil	6.2

CONCLUSION

There is no doubt that all newborns need vitamin K. Classic VKDB is prevented by the administration of 0.3 to 1 mg vitamin K at birth; IM administration is the preferred route in at-risk groups. IM administration of vitamin K at birth is effective in preventing both classic and late VKDB. In exclusively breastfed infants, oral vitamin K administration should be continued. Weekly oral administration of 1 mg vitamin K is more effective in preventing late VKDB than daily administration of 25 µg. Infantile cholestasis needs extra vitamin K supplementation. Current regimens may be underestimating the optimal dosage of vitamin K.

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CHAPTER 11

Bleeding Neonate: Approach and Management

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Normal hemostasis, the process that arrests bleeding after blood vessel injury, is achieved through normal functioning of platelets and coagulation proteins along with vascular integrity. These functions are delicately balanced so that blood may freely circulate within the intact vessels and if bleeding occurs, the site of bleeding can be effectively sealed. Disruption of one or more of these factors results in bleeding. Blood is in a dynamic equilibrium between fluidity and coagulation. This is maintained by balance between coagulation mechanism on one hand and fibrinolysis as well as anticoagulation on the other hand. Failure of this balance makes the neonate susceptible for both hemorrhagic as well as thrombotic tendencies. Hemorrhage and thrombosis may result from variety of pathological processes.[1-10]

Hemostatic functions in infants and newborns differ from those in children and adults. However, abnormalities in some of these functions predispose the neonate to bleeding, especially the preterm and sick neonates in the neonatal intensive care units. Bleeding in a neonate can be one of the important causes of morbidity and mortality and can be a life threatening situation due to the small blood volume of the neonate. Hence, any bleeding neonate requires prompt attention and a rapid diagnosis and immediate institution of therapy.

INCIDENCE

It is estimated that 1 percent of all nursery admissions and 25 to 30 percent of neonatal intensive care unit admissions are complicated by disorder of bleeding. This problem is accentuated in preterms and low birth weight babies and with their increasing survival the incidence of encountering bleeding disorder has risen particularly in neonatal intensive care units.[1-13]

NORMAL NEONATAL HEMOSTASIS

Normal neonatal hemostasis is a highly complex process dependent on interactions between:

- Endothelial cells of the blood vessels
- Platelets
- Hemostatic proteins (coagulation factors) resulting in a stable clot. Disruptions of one or more of these factors results in bleeding. This is in balance with the natural inhibitors of coagulation factors present in blood like anti-thrombin III, protein C and protien S.

MECHANISM OF HEMOSTASIS[3-8]

Hemostasis can be considered in two phases—primary and secondary.

Primary Hemostasis

Vessel Wall Contractions and Platelet Plug Formation

- It is characterized by vessel wall contractions and platelet plug formation in smaller vessels. Following vascular endothelial disruption, a complex series of biochemical reaction set into motion.
- The exposed subendothelial structures attract platelets and they adhere to the exposed collagen with the help of von Willebrand factor and Fibronectin.
- Following the platelet adhesion substances like ADP, Thrombaxane A2 and platelet factor III are released.
- This leads to primary platelet aggregation which attracts more platelets to aggregate and to release

ADP and Thrombaxane A2 from its dense granules, ultimately expanding the hemostatic plug.

Secondary Phase of Hemostasis[3,12,14]

It involves sequential activation of circulating coagulation factors by intrinsic and extrinsic pathways ultimately to form a secondary stable fibrin clot. This controls hemostasis in large vessels.

Fibrinolytic Activity

Under normal hemostatic mechanisms, where fibrin is deposited upon the vessel wall or in the tissues, fibrinolytic processes are simultaneously stimulated so that fibrin is slowly broken down into fibrin split products by plasmin which is activated from its precursor plasminogen. Normally fibrinolytic mechanism is also balanced by its inhibitors present in the blood.[8-10]

Hemostasis in Newborn: Salient Features

Due to physiological immaturity there are both quantitative as well as qualitative differences in hemostatic functions in newborn as compared to older children.

Primary Phase of Hemostasis

- Though capillary fragility is normal in term infants it is increased in preterm. Vasoconstriction following injury is therefore incomplete in preterms[2,3,6,8,12] and hence intracranial hemorrhage is more common in premature babies.
- Platelet count in both term and preterm babies are similar to that in older children. However platelet function such as adhesion, aggregation and release of factors like ADP and Thrombaxane A2 are abnormal.

Secondary Phase of Hemostasis[5,6,8,10,13,15]

- Extrinsic pathway involving tissue factor V and factor VIIa, is the major pathway where by coagulation is initiated and the thrombin plays a crucial role in coagulation as well as platelet activation.
- The concentrations of some clotting factors reach adult values *in utero*, but concentrations of vitamin K dependent factors (II, VII, IX and X) and contact factors (XI, XII, prekalliekrien, high molecular weight kininogen) are reduced to about 50 percent of normal adult values. Levels of factors II, VII, IX, X are decreased more so in preterm babies due to hepatic immaturity and poor availability of vitamin K. Hence hemorrhagic disease of newborn is more common in premature babies.[5,17] More immature the infant, lower is the factor XII activity. Reduced concentrations of factor XII, XI are partly responsible for prolongation of activated partial thromboplastin time (APTT) that is observed in low birth weight infants.
- Factor VII levels reaches adult range by 5 days, while other factors increase gradually over the 1st 6 months of life.
- von Willebrand factor (vWF) levels are increased at birth and although they decline slightly, they remain high. Neonatal VWF is made up of multimers which have increased platelet aggregation in response to Ristocetin.
- Factor XIII level in cord blood is 50 percent of that in adults, but as only small amount of factor XIII is required for its activation or clot stabilization, this low values in newborns have no clinical significance.[5,16,17]
- The plasma level, molecular weight, amino acid composition and immunological properties of fibrinogen in the newborn is comparable to that of adult. But it is found to coagulase more slowly and has different chromatogram on DEAE cellulose. The term fetal fibrinogen is applied to this factor and hence often newborn babies have prolonged thrombin time.[5,18]

Inherited Permanent Abnormality of Coagulation Factors

- Hemophilia A (Factor VIII def.)
- Hemophilia B (Factor IX def.)
- von Willebrand disease, etc.
- Other rare deficiency of coagulation factors
- Coagulation factors are synthesized in the fetus from around the tenth week of gestation,[16] and are not transferred transplacentally from mother to the baby. Hence, the values of coagulation factors estimated in newborn reflect the synthesis of the various factors in them.[16]
- At term, levels of factor V, VIII are equivalent to older children and adults and hence if deficiency of these factors is present during newborn period then it suggests inherited factor deficiency in them.[5]

In healthy neonates this transient deficiency of clotting factors does not play an important role as levels of 20 to 30 percent of coagulant activities are adequate for clot formation. However, stresses like prematurity, sepsis, asphyxia, apneic spells, hypoxia, acidosis, RDS, etc. can tilt the balance leading to bleeding episodes.

Fibrinolytic Activity

In newborn fibrinolytic activity is transiently increased as compared to adults or older children. It declines to adult level by 6 hours in term neonate. Plasminogen levels are only half that of an adult and FDP is normally absent in healthy preterm and term infants. This low level

of plasminogen along with physiological deficiency of circulating anticoagulants like antithrombin III, protein-C promotes thrombotic tendencies in neonates. Deficiency or low level of plasminogen (around 50% of adult value-reaches normal adult value by 6 months) along with physiological deficiency of circulating anticoagulants like antithrombin III and protein C and S, promotes thrombotic tendencies in neonates.

- In addition plasminogen is present in fetal form with both reduced functional activity and decreased binding to cellular receptors.
- C4b binding protein is absent in neonates and protein S therefore circulates in active free form.
- Tissue factor pathway inhibitors (TFPI) or external pathway inhibitors are around 65 percent of adult values.

Antithrombin III, Protein C and Protein S

Similarly concentration of the naturally occurring anticoagulants, antithrombin III, protein C and protein S are low at birth and as a consequence, both thrombin generation and thrombin inhibition are reduced in the newborn period. Antithrombin III (ATIII) heparin co-factor II (HCII), beta-2 macroglobulin, are increased at birth, continue to rise till the age of 6 months and reach the twice normal adult values at this time.

Neonates are thus susceptible to hemorrhage and thrombotic tendencies. This paradox is due to a combined deficiency of coagulation factors along with defective platelet function on one hand and decreased levels of natural inhibitors of coagulation and fibrinolysis on the other.

Local Pathological Lesion

- Trauma
- Slipped ligature
- Cephalhematoma, etc.

Combined Factors Deficiencies

- Disseminated intravascular coagulation (DIC).
- Hepatic dysfunction.

Etiology of bleeding in neonate:[11-13,15]

Bleeding in a neonate may be due to:

- Vascular abnormalities, e.g. in prematurity–intracranial hemorrhage.
- Platelets abnormalities

Quantitative:

- Congenital infections (CMV, Rubella, HIV)
- Thrombocytopenia with absent radius (TAR syndrome)
- Certain syndromes
- Fanconi's anemia
- Amegakaryocytic thrombocytopenic purpura`
- Sepsis
- Increased platelet consumption
- Immune thrombocytopenic purpura
- Auto immune—Child born to mother with SLE or ITP
- Neonatal alloimmune thrombocytopenia–PIA antigen +ve child born to PIA -ve mother
- Asphyxia, shock
- Sepsis, polycythemia, hyperviscocity
- Thrombosis due to catheter, hemangioma
- IUGR with toxemia of pregnancy
- Heparin induced thrombocytopenia.

Qualitative:

Drugs like aspirin given to mother and inherited disorders of platelet function like

Glanzmann's thrombasthenia, Bernard Soulier syndrome.

- *Exaggeration of transient deficiency of coagulation factors:* Hemorrhagic disease of newborn.
- Transitory disturbances of coagulation mechanism as a result of associated systemic disease process, e.g. sepsis, liver disease, DIC, etc.
- *Inherited permanent abnormality of coagulation factors:* Hemophilia A and B, von Willebrand disease, etc.
- Trauma alone or often associated with other factor deficiencies Slipped ligature, cephalhematoma, etc.[5-8]

Approach to Bleeding Disorders in Neonate

- Detail history—antenatal, perinatal, postnatal, family history
- Complete physical examination
- Selected laboratory investigations
- Confirmatory tests.

History

Maternal History

- Presence of an underlying maternal systemic diseases like pre-eclampsia, cardiovascular diseases, viral infection
- Recent drugs taken like aspirin, anticonvulsants like phenobarbitone and phenytoin Na and anticoagulants
- History of collagen vascular disorder, past history of ITP in mother.

Detailed Birth History

- Type of delivery, birth asphyxia, trauma.
- Gestational age should be noted.
- History of vitamin K given to neonate, use of antibiotics and whether neonate is receiving only breastfeeds.

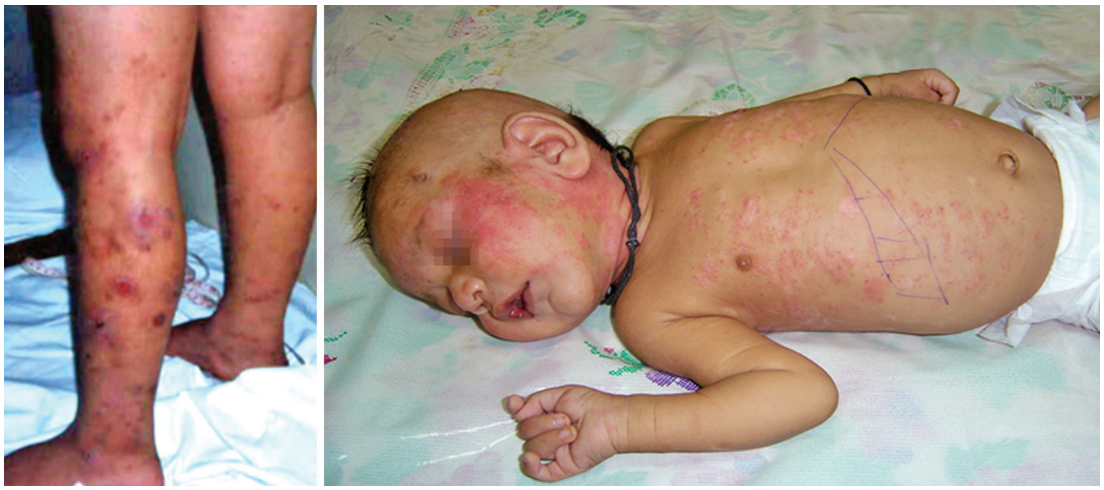

Fig. 1 Wiskott-Aldrich syndrome

Family History

History should include:

- Family history suggestive of bleeding disorder, e.g. history of excessive bleeding after injury or history of menorrhagia in female members.
- Proper pedigree charting of any affected memebers, both living and expired will help to know the type of inheritance of the disorder.

X-linked inheritance: Factor VIII, IX deficiency—enquire similar history of bleeding episodes in male siblings, maternal cousins, maternal uncles, etc.

Autosomal dominant: von Willebrand's disease, dysfibrinogenemia, hemorrhagic telangiectasia

Autosomal recessive: Other factor deficiencies—enquire history of consanguinity.

Physical Examination

A rapid and thorough physical assessment of the bleeding neonate should include:

- General examination.

Well Baby—A 'Healthy' Baby with Bleeding Indicates

- Hemorrhagic disease of newborn
- Inherited coagulation factor deficiency
- Isoimmune thrombocytopenia
- Platelet function disorders
- Vascular causes, slipped ligature, etc.

Sick Baby with Bleeding Indicates

Sepsis, asphyxia, RDS, hypothermia, apnic spells, acidosis, hypoglycemia, seizures, prematurity, hypovolemia, shock, etc. In such babies bleeding is likely to be secondary phenomenon such as DIC, consumption platelet coagulopathy, liver dysfunction, etc.[3-5,12]

Site of Bleeding

- Bleeding from umbilicus in a healthy child without any evidence of umbilical sepsis or slipped ligature, suspect factor XIII deficiency or hypodysfibrinogenemia.
- Bleeding from circumcision or hematoma at injection site in a healthy child-suspect factor deficiency or hemorrhagic disease of newborn.
- Bleeding from GIT is probably due to swallowed maternal blood or vitamin K deficiency.
- In a sick child-suspect DIC.
- Big cephalhematoma following normal delivery (without prolonged or difficult labor) should lead to suspicion of inherited bleeding disorders.
- Petechiae or ecchymosis on presenting part, secondary to congestion and birth trauma may be seen soon after birth and they gradually disappear and are not associated with bleeding anywhere else.
- Skin bleeds like purpura or petechiae in a healthy child-suspect immune thrombocytopenia and differentiate it from mosquito bites (Fig. 2).

Associated Findings

If associated hepatosplenomegaly jaundice or chorioretinitis present, it may suggest:

- Congenital/acquired infections
- Leukemia
- Erythroblastosis fetalis

If associated with eczema—Wiskott-Aldrich syndrome (Fig. 1):

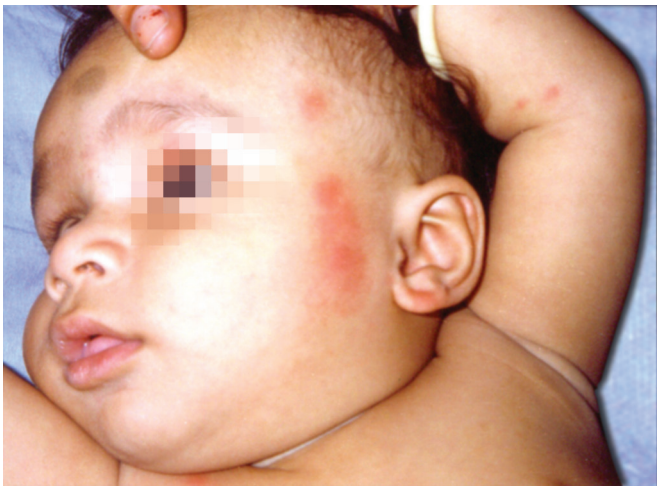

Fig. 2 Mosquito bite

Associated Congenital Anomalies

- TAR syndrome—absent radius with thrombocytopenia
- Large hemangioma with DIC suggest Kasabach–Merritt syndrome
- *Syndactyly with bleeding:* Factor V deficiency
- *Ehler-Danlos syndrome:* Ecchymosis, bruises, purpura with hyperelastic skin.

Laboratory Approach (Table 1)

Though thorough history and clinical evaluation help in suspecting the nature and type of bleeding disorders, laboratory investigations are required to identify the precise nature of the underlying cause of bleeding disorder. It is necessary to confirm whether it is bleeding disorder or not, particularly in a newborn baby with GI bleeding as maternal blood swallow syndrome is seen during early newborn period due to swallowing of maternal blood by baby during the delivery or from the cracked nipple of mother while feeding. Simple bedside test like Apt test will differentiate these two as fetal hemoglobin is resistant to denaturation by alkali where as adult hemoglobin present in mother's RBCs denaturates.

Apt Test

1 part of vomitus is mixed with 5 parts of saline and centrifuged at 2000 rpm for 10 minutes. Add 4 cc of 10 percent NaOH to 1 cc of supernatant centrifuged fluid. Brown color indicates maternal blood and pink color fetal blood.[5]

In a suspected case of bleeding disorder further laboratory tests need to be carried out. They can be divided into:

- Screening tests
- Special tests.

Screening Tests (Table 2)

They are applied to know the presence and nature of bleeding disorder so that relevant special tests can be done to confirm the diagnosis thus avoiding unnecessary battery of tests in each case. They include:

- CBC
- Peripheral smear examination
- PT, APTT, BT and clot retraction.

Table 1 Laboratory tests

Platelets	*PT*	*PTT*	*BT*	*CR*	*Likely diagnosis*
Sick infants					
Decreased	Increased	Increased	Increased	Decreased	DIC
Decreased	Normal	Normal	Increased	Decreased	Early sepsis
Normal	Increased	Increased	Normal	Normal	Liver disease
Normal	Normal/L	Normal/L	Normal	Normal	Compromised vascular intergrity associated hypoxia, increased prematurity, acidosis hyperosmolality
Healthy infants					
Decreased	Normal	Normal	Increased	Decreased	Occult infection or immune thrombocytopenia; thrombosis
Normal	Increased	Increased	Normal	Normal	Hemorrhagic disease of newborn (vitamin K deficiency) or common pathway defect
Normal	Normal	Increased	Normal	Normal	Hereditary intrinsic clotting factor deficiencies
Normal	Increased	Normal	Normal	Normal	Factor VII deficiency
Normal	Normal	Normal	Normal	Normal	Bleeding due to local factors (trauma, anatomic abnormalities) Factor XIII deficiency
Normal	Normal	Normal	Increased	Decreased	Platelet abnormalities (rare)

Abbreviations: PT: Prothrombin time; PTT: Partial thromboplastin time; BT: Bleeding time; CR: Clot retraction time.

Table 2 Screening tests for bleeding disorders[18-20]

Test	*Adult*	*Full term*	*Preterm*
Prothrombin time (sec)	12 ± 1	14 ± 1.3	14 ± 1.3
Partial thromboplastin time	42 ± 4	51 ± 10	57 ± 10.5
Thrombin clotting time (2U)	25 ± 2	23 ± 2.9	23 ± 2.4
Factor II (%)	81 ± 17	50 ± 14.5	31 ± 8.6
Factor V (%)	90 ± 19	79 ± 17	70 ± 22
Factor VII-X (%)	93 ± 20	54 ± 12.2	37 ± 11
Factor VIII (%)	87 ± 27	126 ± 56	116 ± 73
Factor IX (%)	99 ± 23	35 ± 12.6	28 ± 11
Factor X (%)	89 ± 23	45 ± 12	31 ± 9.0
Antithrombin III (%)	99 ± 10	58 ± 9.6	33 ± 9.0
Fibrinogen (mg/dL)	315 ± 60	215 ± 35	256 ± 20

The following table shows the interpretation and the likely causes of bleeding in a given case and further confirmatory tests required to be done in such newborn.

Laboratory screening tests in differential diagnosis of the bleeding infant.

Confirmatory Tests

Platelet Disorders

- Thrombocytopenia with normal PT/APTT in a healthy neonate suggest allo- or autoimmune thrombocytopenia
- If autoimmune thrombocytopenia mother's platelet count study for thrombocytopenia to rule out chronic ITP/Test for collagen disorder should be done.
- If alloimmune thrombocytopenia mother's platelet count study is normal. Platelet study in the mother and child—mother will be PLA1 antigen negative and a child PLA1 positive.
- Low platelet count with normal PT/APTT in a sick child suspect platelet consumption as in septicemia and if associated with prolonged PT/APTT it suggest DIC. Do peripheral smear examination for burr cells, broken RBCs, helmet cells, serum fibrinogen which is decreased and FDP is increased.

Coagulation Factors Defects

- Normal platelet count with prolonged PT/APTT in a healthy child suspect vitamin K deficiency or defect in the common pathways and hence do thrombin time and serum fibrinogen estimation. Therapeutic trial with vitamin K will normalize PT/APTT in hemorrhagic disease of newborn.
- Low serum fibrinogen with prolonged thrombin time suggest hypofibrinogenemia. If fibrinogen level is normal and thrombin time is prolonged then it suggests dysfibrinogenemia or presence of inhibitors, heparin, etc.
- In a sick child normal platelet count and prolonged PT and APTT suggest liver disorder—liver function test are needed.
- Normal platelet count with normal PT and increased PTT in a healthy child suggest intrinsic pathway defect like hemophilia A, B or factor XI deficiency. Correction studies are required and estimation of factor levels.
- Normal platelet count with normal APTT with increased PT suggest extrinsic pathway defect due to deficiency of factor VII—correction studies and factor assay are needed for establishing diagnosis.

If all screening tests are normal including normal platelet count, PT and APTT following conditions should be kept in mind.

- *Local factors:* Slipped ligature, umbilical sepsis, compromised vascular integrity, etc.
- *Qualitative platelet disorders:* Bleeding time will be prolonged with poor clot retraction. Do aggregation study.
- *Factor XIII deficiency:* Do urea solubility test.
- *Ehler-Danlos syndrome:* Clinical examination for skin elasticity.
- *Hemorrhagic telangiectasia:* See for telangiectasia in mucous membrane of nose, bulbar conjuctiva, tongue and tips of the fingers.

Vitamin 'K' and Neonatal Hemostasis (Fig. 3)[12-17]

Biology of Vitamin K

Vitamin K deficiency bleeding (VKDB) also known as the hemorrhagic disease of the newborns refers to bleeding that occurs as a consequence of vitamin K deficiency during first year of life.

Vitamin K

- Phylloquinone or vitamin K_1 is from plant origin.
- The group of menaquinones or vitamin K_2 differ in the number of isoprenyl units in the side chain and are synthesized by the bacteria in humans and animal intestine.
- Menadione or vitamin K_3 is a synthetic and water soluble vitamin K without a side chain. This preparation is not preferred as it has been shown to cause hemolytic

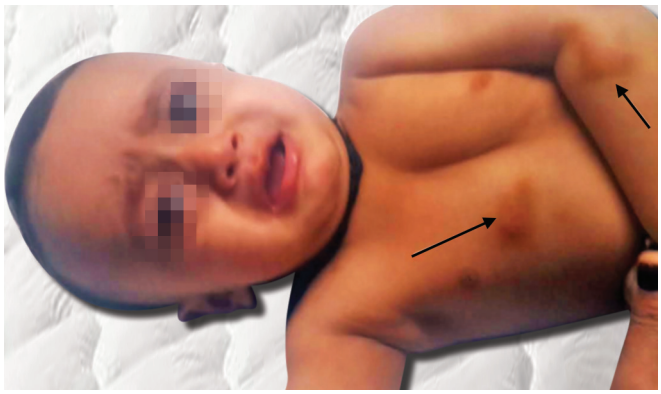

Fig. 3 Vitamin K deficiency

anemia, indirect hyperbilirubinemia and kernicterus.

Vitamin K acts as a cofactor for gamma glutamyl carboxylase (GGCX) serving as an electron donor for the post-translational conversion of protein bound glutamate into gamma carboxyglutamate. During this process it is oxidized to vitamin K_2, 3-epoxide. Gla residues are calcium binding groups which are essential for the biological activities of proteins in which they are found. Gla containing proteins are the coagulation factors II, VII, IX and X and procoagulants like proteins C, protein S, protein Z, osteocalcin, etc.

- Vitamin K deficiency leads to the synthesis of under-carboxylated proteins unable to bind calcium and hence inactive. In vitamin K deficient individuals, undercarboxylated forms of vitamin K dependant coagulation proteins (proteins induced by vitamin K absence PIVKA) are released from the liver into the blood. PIVKA are inactive in the coagulation cascade. PIVKA II or undercarboxylated prothrombin is a marker of subclinical vitamin K deficiency. As a consequence of limited stores at birth, neonates are prone to vitamin K deficiency if no sufficient intake is provided.

Early Vitamin K Deficiency Bleeding

- Presents within 24 hours of birth.
- Seen in infants of mothers taking drugs which inhibit vitamin K
- Anticonvulsants (carbamazepine, phenytoin and barbiturates)
- Anti-tubercular drugs (isoniazid, rifampicin)
- Antibiotics (cephalosporins)
- Vitamin K—antagonists (coumadin, warfarin).
- In preterms the liver is immature and incapable of optimal synthesis of many of precursor proteins. The action of vitamin K, as cofactor for gamma glutamyl carboxylase is therefore limited in preterm because precursor proteins themselves are deficient, often below 30 percent of adult value.
- Though colostrums contain adequate amount of vitamin K, lesser colonization by bacterial flora of the gut of exclusively breastfed infants contribute to lower plasma level of vitamin K. Cow's milk contains 6 ug/dL of vitamin K_1 as compared to breast milk which contains 1.5 ug/dL vitamin K deficiency is classified based on timing of presentation as follows:
  - Early VKDB
  - Classical VKDB
  - Late VKDB.

Clinical Presentation

Early VKDB: Hemorrhagic disease of newborn.

Clinical presentation is often mild, with bruises, gastrointestinal bleeding. Severe manifestations include cephalohematoma, intracranial and intra-abdominal hemorrhage (Fig. 4). The incidence in an at-risk group without vitamin K supplementation 6 to 12 percent. Occurs between 24 hours and 7 days of life.

Classical VKDB: Associated with delay or insufficient feeding.

Clinical presentation—without vitamin K supplementation incidence estimated is 0.01 to 0.44 percent.

- Bruises, gastrointestinal blood loss or bleeding from the umbilicus and puncture sites
- Blood loss, however, can be significant, and intracranial hemorrhage, although rare, has been described.

Late VKDB: Occurs in breastfed infants after 2nd month of life.

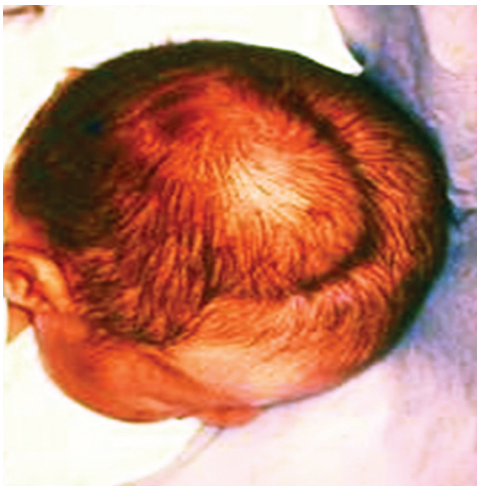

Fig. 4 Cephalohematoma

Predisposing Factors

- Prolonged antibiotic use
- Prolonged/recurrent diarrhea
- Cholestasis
- Malabsorption syndromes
- Clinical presentation may be severe with mortality rate as high as 20 percent. Intracranial hemorrhage is seen in 50 percent of these infants with residual neurological damage in survivors.
- In fully breastfed infants who did not receive vitamin K at birth, the incidence is between 1/15,000 and 1/20,000.

Diagnosis of HDN

The diagnosis of vitamin K deficiency may be suspected from the results of coagulation screening. Initially there is isolated prolongation of the prothrombin time followed by prolongation of the APTT, in association with the normal concentrations of fibrinogen, normal CBC and platelet count.

- Confirmation of the diagnosis requires measurements of PIVKA II.

Treatment

- Intravenous vitamin K should be administered to correct the existing deficiency.
- Factor replacement therapy may also be required with fresh frozen plasma or prothrombin complex concentrate (FII, FIX, FX).

Prevention

- *Routine prophylaxis with 1 mg vitamin K at birth.* (Daily requirement of 5 to 10 μg in infants) and given intramuscularly simply because that was the only product available.
- In 1990 an epidemiological study described an association between intramuscular (IM) vitamin K at birth and childhood cancer and leukemia. In response to these findings, several European countries, Australia and New Zealand changed their policy to oral prophylaxis. In the following years, new studies failed to confirm the association between IM vitamin K at birth and childhood cancer. A risk of solid tumors can now almost definitely be ruled out.[13,14]

The American Academy of Pediatrics has always endorsed the IM route.

- No oral liquid preparation is available, the injectable product has been found to be safe and effective when given by the oral route.
- Several countries currently use an alternative synthetic preparation of vitamin K_1 (Konakion® MM; Roche) for multidose oral prophylaxis. Unfortunately this preparation is not available in most of the countries including India.

Single dose (1.0 mg) of intramuscular vitamin K after birth is effective in the prevention of classic HDN. Vitamin K, should be given to all newborn as a single, intramuscular dose of 0.5 to 1 mg. All premature infants should receive vitamin K. Reports have shown very high plasma vitamin K levels in preterm infants receiving 0.5 to 1 mg at birth and adequate levels in those receiving 0.2 mg at birth.

Current empirical dosage recommendations for preterm infants:

- Dose of 0.3 mg for birth weights <1,000 g
- 0.5 mg for those >1,000 g and <1,500 g.

CONCLUSION

As a child is not a miniature adult so also a neonate is not a miniature child. It is important to realize and to keep in mind the normal physiological variations of hematological parameters in term and preterm neonates. The causes of bleeding in neonates are much different from that in adult or an older child and hence the approach to bleeding neonate is different than that in older children.

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CHAPTER 12

Approach to Neonatal Thrombocytopenia

Nitin K Shah

After birth the platelets are produced by megakaryocytes in the bone marrow and have a life of 9 to 10 days. In the fetus they are produced predominantly in the liver. Platelets appear in the circulation in a fetus by 5 to 6 weeks of gestational age and platelet count steadily rises to $159 \pm 34 \times 10^9$/L by 10 to 17 weeks of gestation and $240 \pm 60 \times 10^9$/L by 18 weeks of gestation, remaining constant at that level thereafter until birth and beyond. Hence the lower limit of the platelet count in a newborn, irrespective of the gestational age, remains at the adult level, i.e. $> 150 \times 10^9$/L.

Thrombocytopenia in a newborn is defined as platelet count lower than 150×10^9/L, though clinical bleeding is usually seen with platelet count less than 50×10^9/L or even lower. Spontaneous and severe bleeding is usually seen when the platelet count drops to $<20 \times 10^9$/L. Presence of significant bleeding at higher platelet counts should arouse suspicion of associated platelet dysfunction as is seen in cases with inherited thrombocytopenia, e.g. Bernard-Soulier syndrome.

A minimum platelet count of 7×10^9/L is required for maintenance of the vascular integrity, and in that sense a normal platelet count of $> 150 \times 10^9$/L provides a lot of reserve functional capacity. Similarly, 30 percent of the circulating platelets are stored in the spleen which can be brought in to the circulation during the need or stress. Normal bone marrow contains 6×10^6 megakaryocytes/kg body weight.

The normal daily platelet production is approximately 35,000 ± 4,300 platelets per microliter of blood to maintain steady levels of about 2,50,000 ± 1,00,000 platelets/mL. Normal bone marrow can respond by increasing the platelet production by 7 to 8 fold during periods of thrombocytopenia.

Platelets are required at each stage of hemostasis.

With the help of von Willebrand factor, they adhere to the exposed endothelial cells at the site of injury and help to form the primary platelet plug.

They secrete granules that contain the factors to help aggregate further platelets and coagulation factors that help form a clot locally. They secrete vasoactive amines that lead to vasospasm to prevent further blood loss.

Platelet membrane provides the surface required for the coagulation to proceed.

There are several growth factors that are required for the formation of platelets from megakaryocytes in the bone marrow. These include IL_3, IL_6, IL_{11}, stem cell factor, leukemia inhibitory factor, erythropoietin and the most important, thrombopoietin (Tpo). Tpo, discovered in 1994, is a polypeptide glycoprotein that binds to its receptor c-mpl (named after its cell of discovery, i.e. acute myeloproliferative leukemic cell) expressed on the megakaryocytes, megakaryocytic precursors, hematopoietic precursor cells and platelets. Tpo acts to commit early precursor cells to lineage specific differentiation and enhances production and release of platelets in the circulation. It acts via further signal conduction pathway and prevents apoptosis in the target cells. It also appears to play a role as pan-hematopoietic growth factor which explains why defects in its metabolism ultimately leads to total bone marrow failure, as seen in rare cases of congenital amegakaryocytic thrombocytopenia. Tpo levels are inversely proportional to the total megakaryocytic mass. High levels of Tpo indicate aregenerative type of thrombocytopenia.

The bone marrow in neonates is very sensitive to insults like hypoxia which leads to suppression of the megakaryocytes more than other precursor cells. This explains why thrombocytopenia is more common than other cytopenias in neonate, especially following hypoxic stress.

Incidence

There very few prospective studies which have specifically looked at the incidence of thrombocytopenia in newborns.

> Recent prospective studies have shown that 0.5 to 4.1 percent of the neonates have thrombocytopenia. 30 percent of the babies admitted to the NICU have platelet counts < 150×10^9/L and around 10 percent of the babies will have counts < 100×10^9/L.

Fortunately most of the episodes of thrombocytopenia in NICU are mild to moderate but 20 percent of them (6 percent of the neonatal admissions) will have severe thrombocytopenia with counts < 50×10^9/L who are at a risk of severe bleeding including intracranial bleeds. 80 percent of these babies with severe bleeds are sick, preterms who have sepsis or necrotizing enterocolitis (NEC).

Causes of Neonatal Thrombocytopenia

While traditionally one can classify the causes of neonatal thrombocytopenia in to those caused by (Table 1).

Reduced Production

- Increased destruction or consumption
- Sequestration
- Dilutional
- A combination of these.

It does not help while approaching a case of neonatal thrombocytopenia as most of the causes listed are rare.

It is easy to group the cases as per their onset, the nadir of counts, the type of recovery, their mechanism of disease, their associated findings, presence of physical anomalies, presence of immunodeficiency and syndromes which helps to narrow down the differential diagnosis.

Patterns of Neonatal Thrombocytopenia

While there are many etiological causes of neonatal thrombocytopenia, this helps us narrow down the etiology. Most of the patients fall into two types of patterns.

Early onset type 75 percent of the neonatal thrombocytopenia. Most babies have low platelet at birth or soon after birth, in the first 72 hours of life. The causes include thrombocytopenia due to chronic or acute hypoxia like in a preterm, IUGR baby risk factors like perinatal asphyxia, maternal diabetes, placental insufficiency, maternal hypertension or pre-eclampsia. The platelet counts in low normal in the first 2 to 3 days, falls to a nadir at 4 to 5 days and recovers by 7 to 8 days. The counts rarely drop below 50×10^9/L. If the pattern of recovery does not follow this path one has to think of other causes of early onset thrombocytopenia like chromosomal anomalies, congenital or perinatal infections, inherited causes or immune causes.

Late onset type: This accounts for the remaining 25 percent of the cases and usually associated with late onset sepsis

Table 1 Causes of neonatal thrombocytopenia

• *Perinatal hypoxia:*
– Maternal diabetes – Maternal hypertension, pre-eclampsia – Intrauterine growth restriction (IUGR)
• *Immune causes:*
– Alloimmune – Autoimmune
• *Infections:*
– *Perinatal infections:* Bacterial, TORCH, HIV – Late onset sepsis/NEC
• *Disseminated intravascular coagulation (DIC):*
– Asphyxia, infections – Mis-matched transfusions – Congenital thrombotic thrombocytopenic purpura (TTP)
• *Aneuploidy:* Trisomy 18, trisomy 13, trisomy 21, Turner's syndrome
• *Inherited*
Giant platelets: Bernard-Soulier syndrome, May-Hegglin anomaly, Sebastian syndrome, Fechtner syndrome, Epstein syndrome, Alport's syndrome, Montreal platelet syndrome, Quebec syndrome, Gray platelet syndrome
• *Immunodeficiency:* Wiskott-Aldrich syndrome • X-linked thrombocytopenia • Hemophagocytic lymphohistiocytosis • *Bone marrow failure:* Fanconi's anemia • Thrombocytopenia with absent radius (TAR) syndrome • Congenital amegakaryocytic thrombocytopenia • *Premalignant:* Monosomy 7
• *Others*
• *Consumption:* Kasabach-Merritt syndrome, vascular thrombosis, hepatic hemangioendothelioma
– *Metabolic:* Propionic academia, methylmalonic academia – *Miscellaneous* – Congenital leukemia – Exchange transfusions (i) Rh disease of newborn (ii) Subcutaneous fat necrosis of the neonate

with or without NEC. The counts drop to a low level and there may be significant bleeding in these babies.

Important Causes of Neonatal Thrombocytopenia

Neonatal Alloimmune Thrombocytopenia

This is the platelet equivalent of the Rh disease where alloantibodies are produced by the mother against the paternal platelets antigens inherited by the newborn from the father which are missing in the mother. These antibodies pass across the placenta and destroy the baby's platelets resulting in often moderate to severe thrombocytopenia. Unlike in Rh disease, in more than 50 percent of cases the first born babies are also affected. In 80 percent of cases the missing platelet antigen in the mother is HPA-1a (also known as Pl^{A1}), in 10 to 15 percent it is HPA-5b and in the rest it is HPA-3a, HPA-1b or some other unknown and rare antigen. In Asian communities, it is usually the HPA-4 antigen. Development of the alloantibodies in HLA-1A negative women is strongly associated with HLA DRB3 0101 (odds ratio of 140).

1:350 pregnancies are associated with maternal antiplatelet antibodies, but only 1:1000 livebirths are associated with neonatal alloimmune thrombocytopenia proving that a good number of them are silent or unnoticed. The affected neonates, who otherwise look well, present with purpura, bruising, mucosal bleeds and thrombocytopenia at birth or immediately after birth. The platelet count is usually < 30×10^9/L. The 20 percent of the affected neonates develop severe bleeding including intracranial bleeds. 20 to 50 percent of the intracranial bleeds start in the intrauterine life and 20 percent of the survivors develop long-term neurodevelopmental sequelae. The diagnosis is done by laboratory testing for the antiplatelet antibodies.

Treatment

- Treatment of the mildly affected babies without mucosal bleeds or with platelet count above 30×10^9/L is conservative.
- Those with significant mucosal bleeds, evidence of intracranial bleeds or platelet count < 30×10^9/L need specific treatment including platelet transfusions, IVIg and steroids. HPA compatible platelets need to be transfused. While one can use washed mother's platelets (which obviously will be negative for the missing platelets), HPA-1a negative platelets are usually stored and easily available in major centers in the west.
- If severe thrombocytopenia persists in spite of the platelet transfusions, one need to give IVIg in the dose of 2 gm/kg body weight over 2 to 5 days and or IV methylprednisolone in the dose of 1 mg/kg 8 hourly. The treatment is required for 1 to 6 weeks till the platelet counts is in the safe range.

The severity depends on the maternal antibody titers. The second born neonate is usually severely affected, if the first baby was symptomatic or had intracranial bleeds especially with intrauterine onset. In such cases, fetal monitoring of the platelet counts is advocated and the mother is given IVIg in the dose of 1 to 2 gm/kg/week with prednisolone in the dose of 0.5 to 1 mg/kg/day. The fetus can also be given *in utero* HPA compatible platelet transfusions if the maternal treatment alone dose not help. Elective LSCS is also advocated to prevent trauma to head.

Neonatal Autoimmune Thrombocytopenia

This occurs due to transplacental transfer of the antiplatelet autoantibodies. It is suspected because of the prior history of thrombocytopenia in the mother due to ITP, SLE or some such auto-immune disease. The platelet count in the mother may be normal even when the antibodies persist, especially following splenectomy. The antibodies are formed against the common platelet antigens like gp1b/IX or gpIIb/IIIa. The disease is generally milder than the alloantibody mediated neonatal thrombocytopenia. The newborn is usually normal at birth with even normal platelet count which drops after few days. Less than 10 to 15 percent of the babies have platelet counts < 50×10^9/L and the bleeding is usually milder. Intracranial bleeds occur in < 1 to 2 percent of babies. In most the platelet counts recover in the next 2 to 3 weeks though rarely they may take a longer time. There is no role of antenatal treatment or LSCS. After birth, treatment is required in presence of significant bleeding or platelet counts < 30×10^9/L. The treatment consists of IVIg and/or steroids as in the case of alloimmune thrombocytopenia. Like in ITP, there is no role of platelet transfusions as the autoantibodies will react with all the platelets as the target antigens are common to all the donors. The prognosis is usually excellent.

Thrombocytopenia following hypoxia: This is the most common cause of early onset pattern of thrombocytopenia.

The newborn is usually a preterm or an IUGR baby who has risk factors like maternal diabetes, maternal hypertension or pre-eclampsia or perinatal asphyxia. The counts are 100×10^9/L in first few days, fall to a nadir of 50×10^9/L at 4 to 5 days and recover by 7 to 8 days. The counts often recover above the normal levels. They may also have neutropenia and polycythemia besides thrombocytopenia. Though platelet destruction is an important cause, especially in acute hypoxia where DIC with thrombocytopenia is seen in 50 percent of such

babies, in most with chronic hypoxia there is decreased production too as the major contributing factor. Less than 10 percent of such babies have evidence of DIC and the Tpo levels are high suggestive of aregenerative type of thrombocytopenia. There is evidence of increased erythropoiesis with increased levels of EPO and circulating normoblasts. This with increased Tpo levels suggests preferential differentiation towards erythropoiesis with depressed megakaryopoiesis. This is because the megakaryocytes are sensitive to hypoxia and are suppressed with hypoxia temporarily. The bleeding is usually mild. Most do not need any specific treatment. Those who are symptomatic need platelet transfusions.

Thrombocytopenia due to Neonatal Infections

- *Perinatal sepsis:* Perinatal bacterial sepsis can occur in 1 to 2/1000 livebirths. The common organisms include group B sepsis or *E. coli* in the west and gram-negative organisms like *Klebsiella* or *E. coli* in our country. The 50 percent of the sepsis cases develop thrombocytopenia and DIC is an important cause of low platelet counts. The platelet counts are low early in life and recover with the treatment of the primary cause.
- *Congenital infections:* TORCH group of infection can lead to thrombocytopenia. Most, but not all, will have other signs like jaundice, anemia, congenital defects, hepatosplenomegaly, etc. Acute cytomegalovirus (CMV) is a common cause of such thrombocytopenia. 0.5 to 1 percent of all newborns develops congenital CMV infection and though only 10 to 15 percent of them are otherwise symptomatic, 75 percent of them have low platelet counts. There may be associated neutropenia and the thrombocytopenia may persist for several months. Similarly, 40 percent of those infected with toxoplasma can develop low platelet counts. Other common cause in our country is perinatal HIV infection and rubella infection as MMR is still not a part of our national schedule.
- *Late onset sepsis:* A common cause of late onset of thrombocytopenia is late onset of sepsis with or without NEC. Thrombocytopenia may be the first sign of infection, though usually other signs of infection are also present. The platelet counts falls after the first 72 hours and may take around 7 to 8 days to recover after the treatment for sepsis is started and effective. Less than 10 percent of these patients have DIC. The Tpo levels are high suggesting aregenerative type of thrombocytopenia. This is also evident by the fact that thrombocytopenia persist even after the infection is under control. This suggests that the cause of low platelet is less production due to suppression of the megakaryocytes. The counts are usually < 30×10^9/L and 15 to 20 percent of the patients have significant bleeding. Platelet transfusions are required for the symptomatic patients.

Consumption of Platelets

- *Disseminated intravascular coagulation (DIC):* Consumption and destruction of platelets occur in DIC which usually occurs following perinatal asphyxia or neonatal infection. The newborn will have the signs of the primary disease and will have severe thrombocytopenia with significant bleeding. The patient may have the signs of thrombosis like gangrene. The prothrombin time and activated partial thromboplastin time will be prolonged and D-dimers or the fibrinogen split products (FDP) will be raised. The peripheral smear will show evidence of microangiopathic hemolytic anemia along with low platelet counts. Treatment will include use of platelets along with fresh-frozen plasma besides treatment of the primary cause.
- *Kasabach-Merritt syndrome:* Large hemangioma can occur over extremities, trunk, neck or in internal organs. Platelets can get trapped in the slow circulation within the hemangioma and can lead to local consumption or localized DIC with consumption of other coagulation factors too. There may be multiple afferent and feeders to the hemangioma. The mass may not be restricted to the defined anatomical layers and usually infiltrates deep in to the tissues making it difficult to excise the hemangioma. The diagnosis is obvious when the hemangioma is seen externally, whereas it can be difficult if the hemangioma is in some organ. Very low platelet counts, evidence of microangiopathy, raised D-dimers or FDP levels and high retic count will suggest the diagnosis. Imaging and vascular studies will help define the extent of the lesion which may be actually much widespread than appearing visibly. Medical treatment includes use of FFP followed by anti-fibrinolytic agents hoping to induce thrombosis of the important feeding vessels. Interferon therapy and vincristine can help shrink the lesion permanently; however, they have their own side effects. Surgery often is mutilating and may land up into amputation.

Congenital and Inherited Thrombocytopenia

This includes causes like chromosomal anomalies and rare inherited causes of low platelet count. The counts are low at or soon after the birth. In most, but not all, there are other features of the basic disease. The platelet count can be very low in some of them needing platelet

transfusions and other treatment. The counts take a long to recover and may be low for months. Those with associated thrombasthenia bleed a lot at relative higher platelet counts. Some of them will have typical platelet morphology on smear examination.

- *Chromosomal anomalies:* The incidence of thrombocytopenia in various aneuploidy cases varies from 86 percent in trisomy 18 to 31 percent in trisomy 13, 6 percent in trisomy 21 and 31 percent in Turner's syndrome. Usually the thrombocytopenia is not severe and many a times, it is associated with neutropenia and polycythemia as seen in placental insufficiency. The mechanism of thrombocytopenia may also be similar to that seen in placental insufficiency.
- *Inherited thrombocytopenia:* Inherited thrombocytopenias are a rare but interesting group of disorders which provided dome insight in to the molecular mechanisms for megakaryopoiesis. For some of these disorders, the molecular defects have been identified, like mutation of the CMPL in congenital amegakaryocytic thrombocytopenia, defect in transduction pathway after binding of Tpo to CMPL in TAR syndrome, mutations in CBFA2 transcription factor gene *RUNX1* (*AML1*) in familial platelet syndrome with predisposition to AML, mutation in GATA-1 transcription factor (xp 11) in X-linked thrombocytopenia with microcytosis, mutation in myosin heavy chain A gene (*MYH9*) in giant platelet syndromes including May-Hegglin anomaly and mutations in the WASP gene in Wiskott-Aldrich syndrome and X-linked thrombocytopenia. Most, but not all, have associated congenital anomalies to point to the possible diagnosis. Many of them have abnormal platelet morphology like giant platelet on peripheral smear examination. Some have associated platelet dysfunction leading to more bleeding than expected for the platelet count.
- *Thrombocytopenia with giant platelets:* This group includes the disorders like Bernard-Soulier syndrome; May-Hegglin syndrome and its variants like Sebastian syndrome, Fechtner syndrome, Epstein syndrome and Alport syndrome; Montreal platelet syndrome; Quebec syndrome and gray platelet syndrome.
- *Bernard-Soulier syndrome:* It is an autosomal recessive disorder. The platelet count is moderately low but the bleeding is quite significant. The platelets can be as large as 20 microns in diameter with cytoplasmic vacuoles. The defect is in the demarcation membrane system. This results in defect in gpIb-V-IX complex leading to poor adhesion. Diagnosis is made by absent response to Ristocetin in platelet function study. A close differential with similar results is von Willebrand disease which can be differentiated by normal von Willebrand factor levels and pattern. Treatment includes platelet transfusions for significant bleeding. Stem cell transplant will be curative in this condition.
- *May-Hegglin anomaly:* This is an autosomal dominant condition characterized by thrombocytopenia, giant platelets, large inclusions in granulocytes and monocytes known as Dohle bodies and presence of physical defects like hearing loss, renal failure and cataract. The platelet count is moderately low in the range of 80 to 1,00,000/cumm but with significant bleeding due to associated platelet dysfunction. The defect is in the MYH9 gene that codes for Myosin II-A which is the only protein expressed in platelets and neutrophils, which explains the characteristic defects seen in platelets and neutrophils. DDAVP, anti-fibrinolytic agents help control bleeding. Platelet transfusions are rarely required.
- *Gray platelet syndrome:* It is an autosomal recessive disorder with mild thrombocytopenia and significant bleeding. The platelets are unable to store alpha granule proteins. This leads to poor aggregation especially with thrombin, ADP and collagen. The platelets appear large, gray and bland with vacuolations. The treatment is platelet transfusion for significant bleeding.
- *Inherited thrombocytopenia with immunodeficiency:* This includes Wiskott-Aldrich syndrome and hemophagocytic lymphohistiocytosis.
- *Wiskott-Aldrich syndrome:* This is an X-linked disorder characterized by thrombocytopenia, eczema and immunodeficiency. It is caused by mutations in the WASP gene located at Xp11-12 band. The Wiskott-Aldrich syndrome protein is known to be involved in the signal transduction and it regulates actin filament assembly in platelets as well as lymphocytes affecting their cytoskeleton. This explains the association of thrombocytopenia and immunodeficiency. Patients present with thrombocytopenia early in life with more bleeding than expected based on the platelet counts. The platelets are small in volume and the mean platelet volume (MPV) is characteristically low (as is also seen in patients with TORCH group of infection). Patient usually has lower GI bleeding. Eczema and immunodeficiency develop as the child grows. Some patients have milder phenotype with minimal immunodeficiency as the defect is restricted only to the platelets even when they have the same WASP gene mutations (though restricted only to the exon 2 of the gene), these patients are grouped as X-linked thrombocytopenia. Often these patients are mistaken as ITP later in the life.

The treatment of Wiskott-Aldrich syndrome includes control of bleeding with platelet transfusions and treatment of infections. In those with difficulty, splenectomy will also help as also IVIg at times. Bone marrow transplantation

is the only curative treatment possible at present. The 10 percent of the patients can progress to develop lymphoma later in their life.

Hemophagocytic Lymphohistiocytosis (HLH)

This is a rare but interesting disease caused by mutations in the genes for perforin or granzymes involved in the killing of phagocytosed material by the phagosomes of the macrophage-monocyte series and NK cells. Absence of effective killing by the phagosomes leads to unnecessary stimulation of the macrophages and monocytes leading to production of cytokines by these cells leading to what is described as cytokine storm which leads to all the symptoms like hemophagocytosis, cytopenias, liver dysfunction, CNS changes, fever, DIC, increased triglycerides levels, etc. It is an autosomal recessive disorder which can present even at or soon after the birth with fever, thrombocytopenia, convulsions, hyperbilirubinemia and cytopenias. The diagnosis is made by high index of suspicion and demonstrating hemophagocytes in the bone marrow or organs like liver, spleen or lymph nodes. Sometimes, hemophagocytes are also seen on the peripheral blood smears. The treatment includes use of steroids and VP-16 to induce remission. Bone marrow transplant will cure those who do not respond to conservative management.

Inherited thrombocytopenia with bone marrow failure: This includes three disorders that is:

1. Congenital amegakaryocytic thrombocytopenia,
2. Thrombocytopenia with absent radius syndrome (TAR syndrome)
3. Fanconi's anemia.

Congenital Amegakaryocytic Thrombocytopenia

This is a rare autosomal recessive disorder presenting as neonatal thrombocytopenia which progresses over the time to total bone marrow failure. It presents with bleeding and thrombocytopenia in the first few days to weeks of life where it may be confused with many other common etiologies especially alloimmune thrombocytopenia. 20 percent of the patients can develop intracranial hemorrhage. 10 to 30 percent of them can have associated orthopedic or CNS anomalies. It is only when the patient dose not recover that a bone marrow is ordered which will pick up absent or grossly reduced megakaryocytes with normal granulopoietic and erythropoietic elements. It is caused by mutations in the CMPL genes which results in marked maturation of the megakaryocytes and its precursors. As the action of Tpo via CMPL is vital for maturation of other hemopoietic cells, even the other series will get affected with time including the stem cells due to lack of preservation of apoptosis induced by Tpo. This explains why there is total bone marrow failure with time. While platelet transfusions help controlling the bleeding, the only curative treatment is bone marrow transplant. The 50 percent of them will progress to aplastic anemia over next 5 years. And 5 percent of them can develop leukemia. IL3 and GM-CSF have limited role and Tpo has no role what so ever in the treatment of these patients.

Fanconi's Anemia and TAR

Both are autosomal recessive disorders characterized by inherited bone marrow failure. Both have characteristic physical anomalies. Both are caused by known mutations. Mutations in HOXA10, HOXA11 and HOXD11 genes are implicated for TAR whereas more than 3 different types of Fanconi's anemia gene mutations are identified for Fanconi's anemia.

The TAR presents with thrombocytopenia, bleeding and characteristic forearm deformity due to absence of radius. Isolated abence of radii is seen in only 10 percent of the patients and 50 percent of the patients also have associated defects in ulna, knees or humorous, besides absent radii. The thumb on the affected side is always present in TAR, which differentiates it from Fanconi's anemia (which rarely can present in neonatal period) where such radial deformities are seen but the thumb is always absent from the affected side. Besides this, other anomalies seen in Fanconi's anemia seen include short stature, microcephaly, mental retardation, hyperpigmentation, hypogonadism, renal anomalies, other skeletal anomalies like rudimentary thumb, absent thumb, triphalangeal thumb, etc. Fanconi's anemia usually presents as anemia in later life though it can presents in neonatal period in 10 percent of the cases. While TAR tends to improve after the age of one year, Fanconi's anemia usually progresses with time in to complete bone failure and has high chances of developing malignancies. The only curative treatment is bone marrow transplant with proper conditioning regime avoiding radiation. Another syndrome of congenital thrombocytopenia with radioulnar synostosis (CTRUS) is described where the patient has absent pronation-supination due to fusion of radius and ulna. It behaves similar to TAR with improved platelet counts after the age of 1 year. Defect in HOX 11a is implicated in this condition.

Miscellaneous Causes

There are some rare but important and distinct causes of neonatal thrombocytopenia like osteopetrosis which leads to thrombocytopenia and later bone marrow failure due to calcification of marrow spaces; congenital leukemia and metastatic neuroblastoma which cause thrombocytopenia due to bone marrow infiltration by the malignant cells and organic acidemias and other metabolic disorders which all can lead to bone marrow suppression.

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SECTION 3

RBC and WBC Disorders

CHAPTERS OUTLINE

CHAPTER 13

Introduction and Classification of Anemias in Children

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Anemia can be defined as a reduction in the hemoglobin concentration, hematocrit, or the number of red blood cells (RBC) per cubic millimeter. Conventionally, the lower limit of the normal range is set at two standard deviations below the mean for the normal population. Thus, 2.5 percent of the normal population will be mistakenly classified as anemic. The primary function of red blood cells is to deliver adequate quantities of oxygen to meet the body's metabolic demands. Thus, a measure of oxygen metabolism and accompanying cardiovascular compensation should be considered in defining anemia. Children with cyanotic congenital heart disease, respiratory insufficiency, or hemoglobinopathy that alters oxygen affinity can be functionally anemic with hemoglobin in the normal range.[1,2]

Hemoglobin (Hb) concentration varies with age, with higher values being present in the newborn and adolescent male. The high Hb level at birth occurs in response to the low fetal ambient oxygen tension (PO_2 of 30 mm Hg). Immediately after birth, the rise in arterial PO_2 (around 90 mm Hg) decreases erythropoietin production, producing the ''physiologic anemia'' seen in the first 2 months of life (i.e. Hb concentration of around 10 g/dL at 2 months of age). This period is followed by a steady rise in Hb, reaching a maximum at about 14 years of age. The zenith of hemoglobin concentration occurs earlier in preterm infants and may be more in terms of lower Hb levels as well as prolonged anemia. Table 1 gives the approximate values of some vital hematologic parameters in pediatric age group.

PHYSIOLOGY OF HEMOGLOBIN PRODUCTION

Erythropoietin is the primary hormone regulator of red blood cell (RBC) production. In the fetus, erythropoietin comes from the monocyte/macrophage system of the liver. Postnatally, erythropoietin is produced in the peritubular cells of the kidneys. Key steps in red blood cell differentiation include condensation of red cell nuclear material, production of hemoglobin until it amounts to 90 percent of the total red blood cell mass and the extrusion of the nucleus that causes loss of RBC synthetic ability. Normal RBCs survive an average of 120 days, while abnormal RBCs can survive as little as 15 days. The hemoglobin molecule is a heme-protein complex of two pairs of similar polypeptide chains. There are six types of hemoglobin in developing humans: the embryonic, Gower 1, Gower 2, Portland, fetal hemoglobin (HbF) and normal adult hemoglobin (HbA and HbA_2). HbF is the primary hemoglobin found in the fetus. It has a higher affinity for oxygen than adult hemoglobin, thus increasing the efficiency of oxygen transfer to the fetus. The relative quantities of HbF rapidly decrease to trace levels by the age of 6 to 12 months and are ultimately replaced by the adult forms, HbA and HbA_2.

CLASSIFICATION OF ANEMIAS

Anemias can be classified according to their appearance in the microscope, which is the morphologic. The former methodology classifies anemias into:

Morphologic classification:

- Microcytic
- Macrocytic
- Normocytic types.

The latter classification categorizes anemias into three main categories (Table 2)[3]:

Table 1 Age-specific blood cell indexes

Age	*Hemoglobin g/dL (g/L)*	*Hematocrit (%)*	*MCV, μm³ (fL)*	*MCHC, g/dL (g/L)*	*Reticulocytes*
• 26–30 weeks' gestation*	13.4 (134)	41.5 (0.42)	118.2 (118.2)	37.9 (379)	–
• 28 weeks' gestation	14.5 (145)	45 (0.45)	120 (120)	31.0 (310)	(5 to 10)
• 32 weeks' gestation	15.0 (150)	47 (0.47)	118 (118)	32.0 (320)	(3 to 10)
• Term† (cord)	16.5 (165)	51 (0.51)	108 (108)	33.0 (330)	(3 to 7)
• 1–3 days	18.5 (185)	56 (0.56)	108 (108)	33.0 (330)	(1.8–4.6)
• 2 weeks	16.6 (166)	53 (0.53)	105 (105)	31.4 (314)	
• 1 month	13.9 (139)	44 (0.44)	101 (101)	31.8 (318)	(0.1–1.7)
• 2 months	11.2 (112)	35 (0.35)	95 (95)	31.8 (318)	
• 6 months	12.6 (126)	36 (0.36)	76 (76)	35.0 (350)	(0.7–2.3)
• 6 months–2 years	12.0 (120)	36 (0.36)	78 (78)	33.0 (330)	
• 2–6 years	12.5 (125)	37 (0.37)	81 (81)	34.0 (340)	(0.5–1.0)
• 6–12 years	13.5 (135)	40 (0.40)	86 (86)	34.0 (340)	(0.5–1.0)
• 12–18 years					
– Male	14.5 (145)	43 (0.43)	88 (88)	34.0 (340)	(0.5–1.0)
– Female	14.0 (140)	41 (0.41)	90 (90)	34.0 (340)	(0.5–1.0)
• Adult					
– Male	15.5 (155)	47 (0.47)	90 (90)	34.0 (340)	(0.8–2.5)
– Female	14.0 (140)	41 (0.41)	90 (90)	34.0 (340)	(0.8–4.1)

Abbreviations:
MCV: Mean corpuscular volume; MCHC: Mean corpuscular hemoglobin concentration.
* Values are from fetal samplings.
† Less than one month, capillary hemoglobin exceeds venous: 1 hour—3.6 gm difference; 5 days—2.2 gm difference; 3 weeks—1.1 gm difference. Adapted with permission from Siberry GK, Lannone R, Eds. The Harriet Lane handbook: a manual for pediatric house officers, 15th edn. St Louis: Mosby, 2000.

Table 2 Physiologic classification of anemia[3]

Classification of anemia according to underlying mechanism	
Mechanism	*Specific examples*
Blood loss	
Acute blood loss	Trauma
Chronic blood loss	Gastrointestinal tract lesions, gynecologic disturbances
Increased red cell destruction (hemolysis)	
Inherited genetic defects	
Red cell membrane disorders	Hereditary spherocytosis, hereditary elliptocytosis
Enzyme deficiencies	
Hexose monophosphate shunt enzyme deficiencies	G6PD deficiency, Glutathione synthetase deficiency
Glycolytic enzyme deficiencies	Pyruvate kinase deficiency, Hexokinase deficiency
Hemoglobin abnormalities	
Deficient globin synthesis	Thalassemia syndromes
Structurally abnormal globins (hemoglobinopathies)	Sickle cell disease, unstable hemoglobins
Acquired genetic defects	
Deficiency of phosphatidylinositol-linked glycoproteins	Paroxysmal nocturnal hemoglobinuria

Contd...

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Mechanism	*Specific examples*
Antibody-mediated destruction	Hemolytic disease of the newborn (Rh disease), transfusion reactions, drug-induced, autoimmune disorders
Mechanical trauma	
Microangiopathic hemolytic anemias	Hemolytic uremic syndrome, disseminated intravascular coagulation, thrombotic thrombocytopenia purpura
Cardiac traumatic hemolysis	Defective cardiac valves
Repetitive physical trauma	Bongo drumming, marathon running, karate chopping
Infections of red cells	Malaria, Babesiosis
Toxic or chemical injury	Clostridial sepsis, snake venom, lead poisoning
Membrane lipid abnormalities	Abetalipoproteinemia, severe hepatocellular liver disease
Sequestration	Hypersplenism
Decreased red cell production	
Inherited genetic defects	
Defects leading to stem cell depletion	Fanconi anemia, telomerase defects
Defects affecting erythroblast maturation	Thalassemia syndromes
Nutritional deficiencies	
Deficiencies affecting DNA synthesis	B_{12} and folate deficiencies
Deficiencies affecting hemoglobin synthesis	Iron deficiency anemia
Erythropoietin deficiency	Renal failure, anemia of chronic disease
Immune-mediated injury of progenitors	Aplastic anemia, pure red cell aplasia
Inflammation-mediated iron sequestration	Anemia of chronic disease
Primary hematopoietic neoplasms	Acute leukemia, myelodysplasia, myeloproliferative disorders
Space-occupying marrow lesions	Metastatic neoplasms, granulomatous disease
Infections of red cell progenitors	Parvovirus B_{19} infection
Unknown mechanisms	Endocrine disorders, hepatocellular liver disease

- Classification and on the basis of the underlying physiologic process.
- Anemias associated with decreased red cell production
- Anemias due to increased red cell destruction
- Anemias due to blood loss.

It is also important to realize that as in other issues the diagnosis of anemia can be easily made from the history and physical examination. Some of the salient features in the history as well as physical examination are given in Tables 3 and 4.

Role of complete blood counts (CBC) and peripheral smear (PS) for diagnosis of anemia: CBC, red cell indices and PS play an immensely vital role in helping a clinician for the differential diagnosis of anemia. It is imperative that care is taken in obtaining the sample. Venous samples are preferred. When capillary samples are obtained, it is important that the extremity is warm and that a free flow of blood is obtained. To ensure accurate results, an adequate volume of blood should be obtained to avoid excessive dilution by the anticoagulant. Analysis of the hemoglobin concentration is preferred as it is determined by direct spectrophotometry. In contrast, the hematocrit is determined indirectly by calculations using the red count and mean corpuscular volume (MCV).

The next step is to evaluate the red cell indices. Of these, the MCV is the most useful. MCV is the mean volume of single red cell expressed in femtoliters. It is a red cell index directly measured by the electronic counter and enables the classification of anemia by red blood cell size as microcytic, normocytic, or macrocytic (Flow charts 1 and 2) (Figs 1 and 2).[4]

While this classification is arbitrary and categories are not mutually exclusive, it provides a useful starting point for directing further evaluation. In children less than age 10 years and above 2 years, the lower limit for the MCV is approximately 70 fL + age in years. After 6 months of age, the approximate upper limit for the MCV is 84 + 0.6 fL per year until the upper limit of 96 fL in adults is reached. The mean corpuscular hemoglobin (mean Hb content of a single red cell expressed in pictograms, MCH) and mean corpuscular hemoglobin concentration (hemoglobin concentration within individual red cells expressed in %, MCHC) are calculated values and generally less diagnostic. The MCH usually parallels the MCV. Both the MCV and MCH have small measurement errors and biological variations. The MCHC is a measure of cellular hydration status. It remains relatively constant throughout development and in most clinical settings. A high value (> 35 g/dL) is characteristic of spherocytosis, while a low value is most commonly

Table 3 History of a child with anemia

Symptom	*Implications*
Maternal history	Anemia, blood loss during pregnancy, pica may predispose for iron deficiency
Family history	Anemia, splenomegaly, gallstones, splenectomy, leg ulcers, jaundice, transfusion dependency indicates hemolytic anemia. Look at the ethnicity and race as sickle cell anemia and thalassemia are more common in certain communities
Age	Iron deficiency is unlikely in term infants before 6 months of age. Anemia which manifests in the neonatal period is the result of recent blood loss, isoimmunization, or initial manifestation of congenital hemolytic anemia or congenital infection
Gender	G6PD and PK deficiency seen in males as inheritance is X linked
Neonatal history	Anemia and jaundice may point to a congenital hemolytic anemia such as HS, prematurity predisposes to an early iron deficiency state
Pica	Indicates iron deficient state
Diet history	Predominant cows milk leads to cows milk allergy and GI blood loss leading to iron deficiency
Infections	Hepatitis induced aplasia, infection assorted hemolysis, parvovirus induced red cell aplasia, worm infestation leads to blood loss and Fe deficiency
Drugs	Oxidant induced hemolytic anemias, phenytoin induced megaloblastic anemia, drug induced aplastic anemia
Hemoglobinuria	Indicates an intravascular hemolysis like autoimmune or G6PD deficiency
Bleeding manifestations	May indicate a second cell line being involved or a bleeding or clotting defect which may be causing the anemia
Diarrhea	Malabsorption or inflammatory bowel disease
Dactylitis or painful episodes	Sickle cell anemia, bony pains may indicate a infiltrative disorder of marrow
Blood loss	Acute or chronic

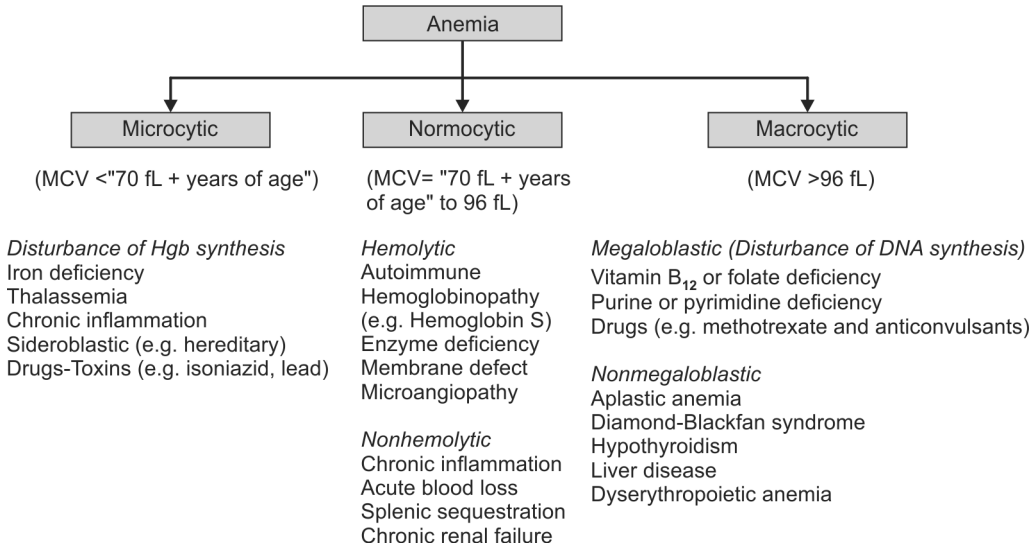
Table 4 Physical examination of a child with anemia

Physical findings	*Implications*
Tachycardia, respiratory distress	Acute anemia or decompensated state
Hemolytic facies	Chronic hemolytic anemia
Platonychia, koilonychia	Iron deficiency anemia
Glossitis	Iron deficiency or cobalamin deficiency
Blue sclera	Iron deficiency anemia
Vitiligo	Pernicious anemia
Telangiectasias on body	Similar lesions in the GIT
Splenomegaly, jaundice	Hemolytic anemia
Lymphadenopathy	Infiltrative disorder
Hepatomegaly	CCF, infiltrative disorder
Cardiomegaly	Chronic anemia
Bleeding manifestations	Bleeding diathesis or secondary to marrow infiltration
Knuckle pigmentation, loss of position or vibration sense	B_{12} deficiency
Failure to thrive	Chronic anemia, systemic illnesses
Facial or digital anomalies	Fanconi's anemia, Diamond-Blackfan syndrome
Rectal examination	Rectal varices or polyp

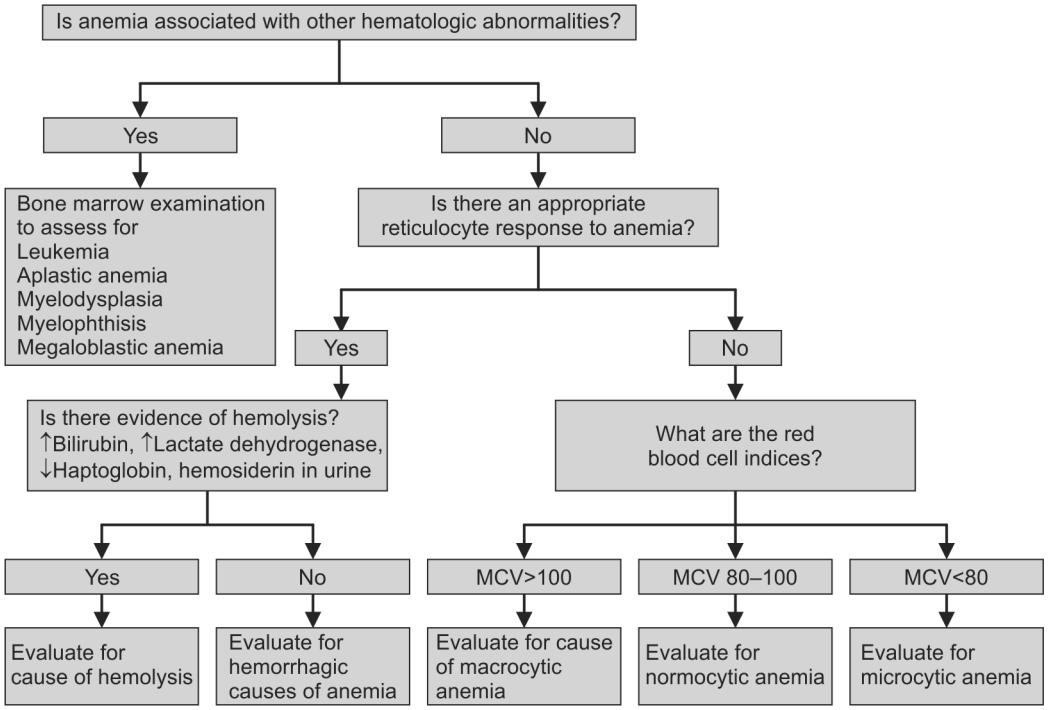
associated with iron deficiency. The red cell volume distribution width (RDW) reflects the variability in cell size and can be used as a measure of anisocytosis.[2,4] Some kind of anemias can easily be diagnosed on distinctive abnormalities seen on the peripheral smear (Table 5 and Figs 1 to 19).[1,5]

The next step is to assess the white blood cell (WBC) and platelet counts. Is the anemia isolated or are other cell lines affected? Pancytopenia results from disorders that are distinct from those causing simple anemia and generally mandate analysis of the bone marrow. Thus on the basis of morphology we can divide the anemias

Flow chart 1 Classification of anemia based on morphology[4]



Flow chart 2 Approach to anemia[6]



into microcytic hypochromic and an approach to this is given in Flow chart 3.[6] The anemias can be macrocytic and an algorithmic approach to macrocytic anemias is given in Flow chart 4.[6] The normocytic normochromic anemias are associated with many systemic disorders and an approach to them is delineated in Flow chart 5.[6] A leukoerythroblastic blood picture (nucleated red cells, reticulocytosis, a shift to the left in the neutrophil cell line, tear drop red cells) is characteristic of diseases in which the normal bone marrow is replaced by tumor or other diseases. Elevated WBC and/or platelet counts in children are most often due to reactive processes. While infection is the most common cause, other etiologies including iron deficiency anemia, autoimmune disorders, or hemolytic anemia, vitamin E deficiency, and postoperative states are possible. Microscopic examination of the PBS can

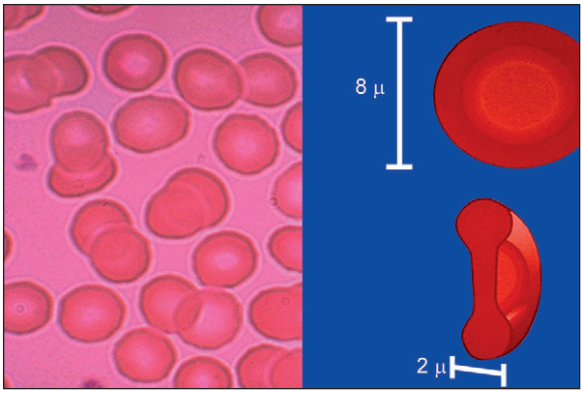


Fig. 1 Dimensions of a normal RBC

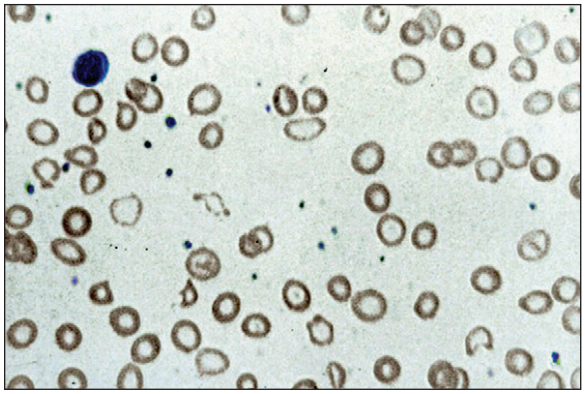


Fig. 2 Microcytic hypochromic anemia: RBC have a increased central pallor and the size of RBC is smaller than the nucleus of a lymphocyte

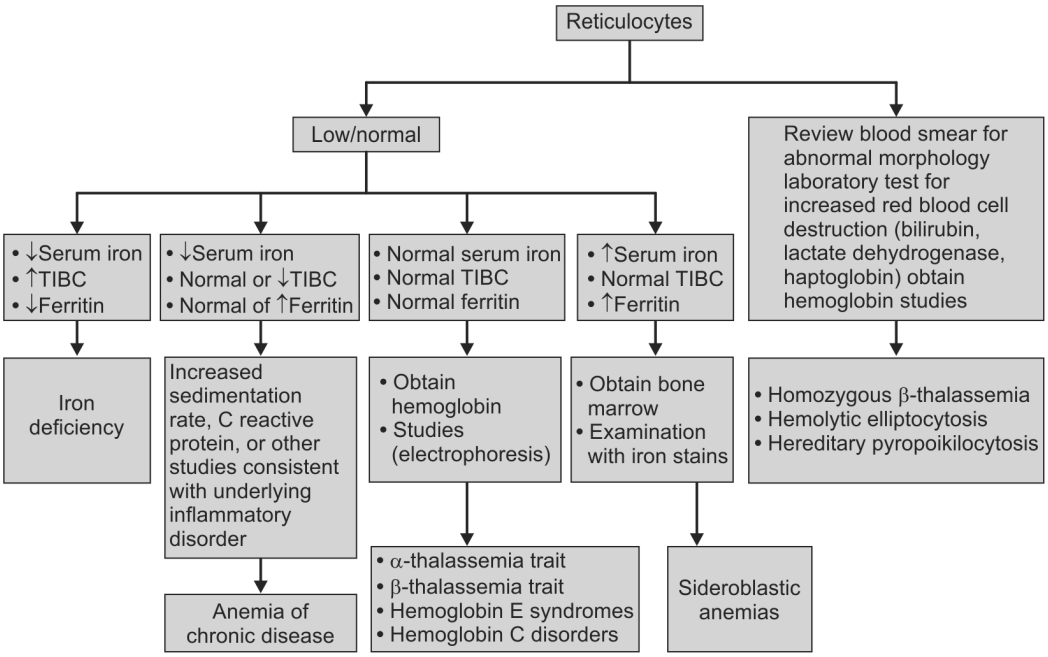
Table 5 Some peculiar red cell abnormalities on the smear[1,5]

Morphologic characteristic	*Basis of the abnormality*	*Comment*
Howell-Jolly bodies	RBC nuclear remnants	Increased with brisk hemolysis, increased following splenectomy, pernicious anemia, CDA
Basophilic stippling	RNA remnants/ aggregated ribosomes	Impaired globin chain production (thalassemia; lead intoxication), unstable hemoglobinopathies and iron deficiency
Pappenheimer bodies	Iron ferritin granules in cytoplasm	Increased following splenectomy increased with transfusional iron overload
Heinz bodies	Hemoglobin aggregates	Needs brilliant cresyl blue, crystal violet stains. Unstable Hb, enzymopathies, hemoglobin H and thalassemia syndromes
Burr cells	Membrane perturbance	Chronic renal failure, common smear preparation artifact
Acanthocytes (spur cells)	Membrane perturbance	Hepatic insufficiency
Nucleated RBCs	Normoblast nuclei	High with brisk hemolysis present with myelophthisis
Sickle cells	RBC distortion by hemoglobin polymers	Sickle cell disease
Target cells	Low ratio of hemoglobin to red cell membrane; RBC dehydration	Prominent in thalassemia Present with iron deficiency
Spherocytes	Defective membrane protein	Hereditary disorder Immune hemolysis

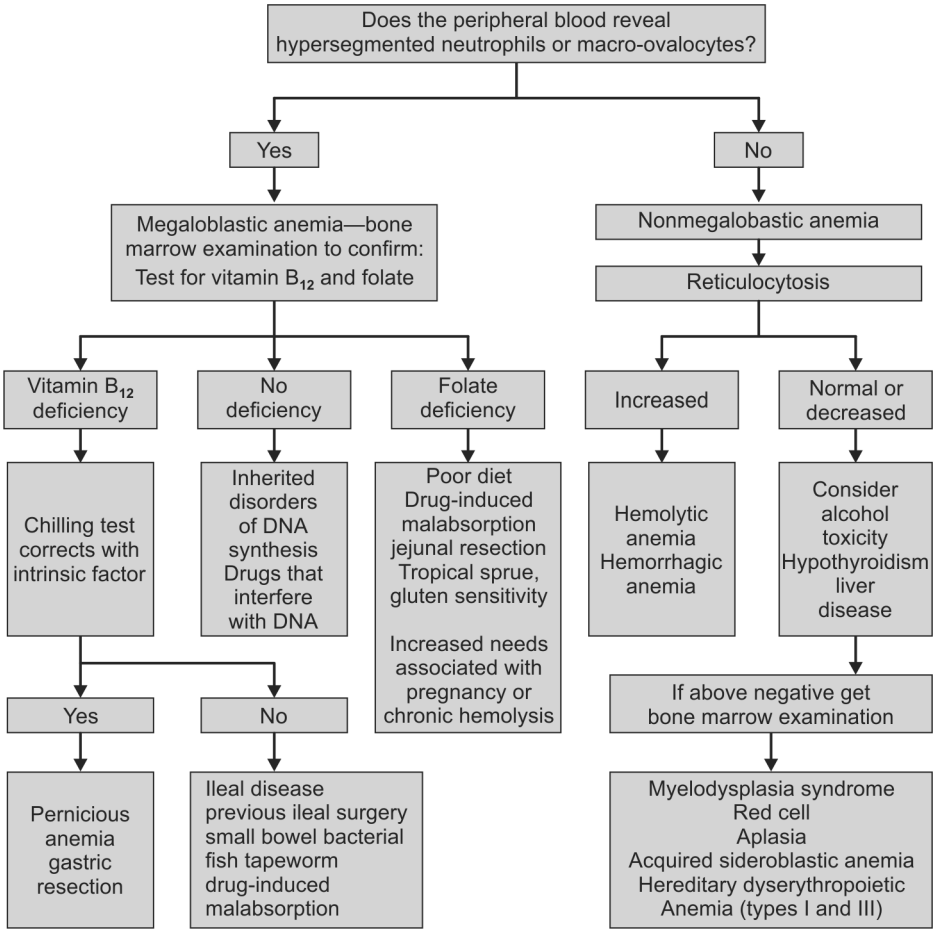
aid in further focusing the differential. Assess the size, color, and shape of the red cells. The normal red blood cell is about the size of the nucleus of a small lymphocyte. On a well-stained blood smear the area of central pallor in a normal erythrocyte has a diameter about one-third of that of the entire cell. Cells with excessive central pallor are hypochromic. Absence of central pallor is seen in spherocytosis. Polychromasia with large cells is indicative of reticulocytosis. Distinctive abnormalities in shape are suggestive of red cell membrane disorders (e.g. spherocytosis, stomatocytosis, or elliptocytosis) or hemoglobinopathies (e.g. sickle cell disease, thalassemia). Presence of inclusions such as basophilic stippling (as seen in thalassemia, lead poisoning) should be noted. Nucleated red blood cells are never normal, except in the newborn, and are indicative of a stressed marrow. The number and morphology of WBCs and platelets should also be assessed. Toxic granulation suggests an acute inflammatory state while hypersegmented neutrophils are characteristic of vitamin B_{12} and folate deficiency.

Reticulocytes are newly formed red cells with residual strands of nuclear material called "reticulin" that remain following extrusion of the nucleus from bone marrow normoblasts. These new erythrocytes are 10 to 20 percent

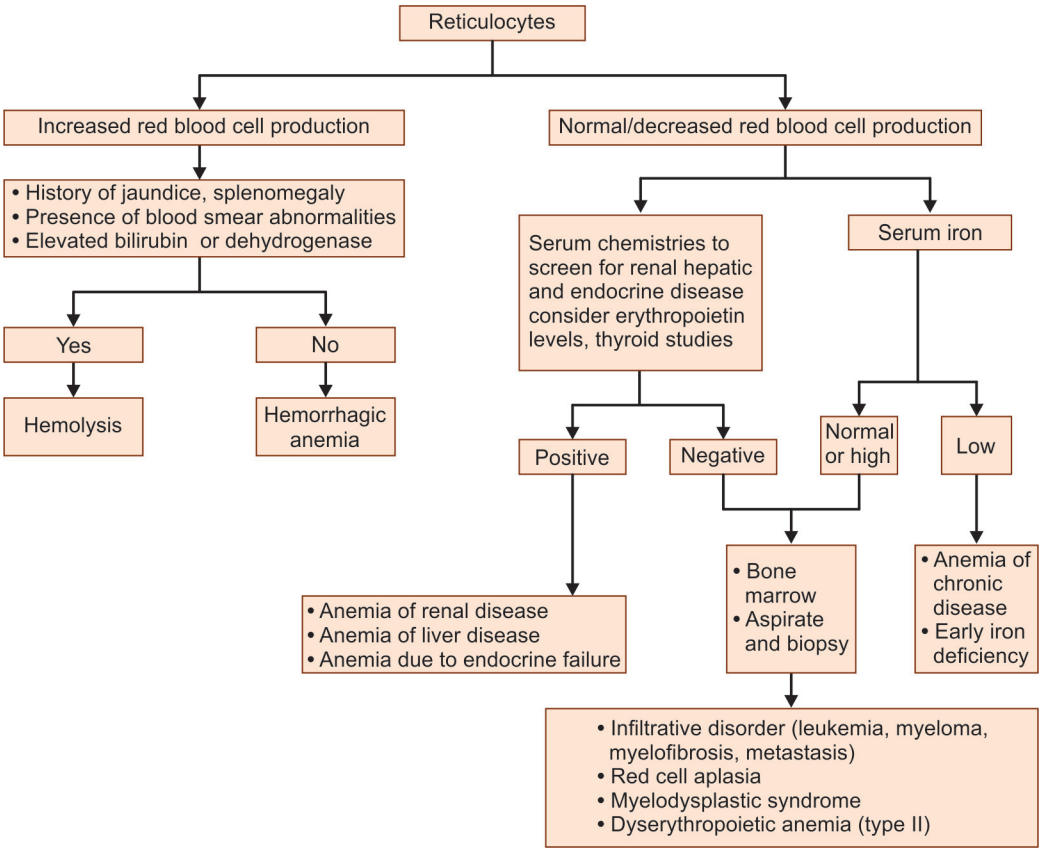
Flow chart 3 Approach to microcytic hypochromic anemia[6]



Flow chart 4 Approach to macrocytic anemia[6]



Flow chart 5 Approach to normocytic normochromic anemia



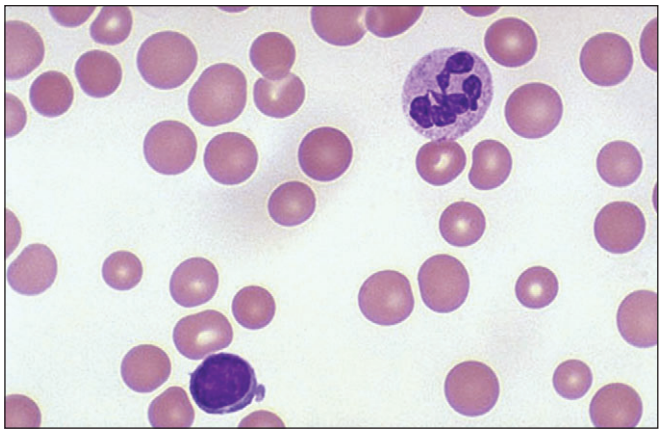


Fig. 3 Macrocytic anemia with RBC larger than the size of nucleus of lymphocyte

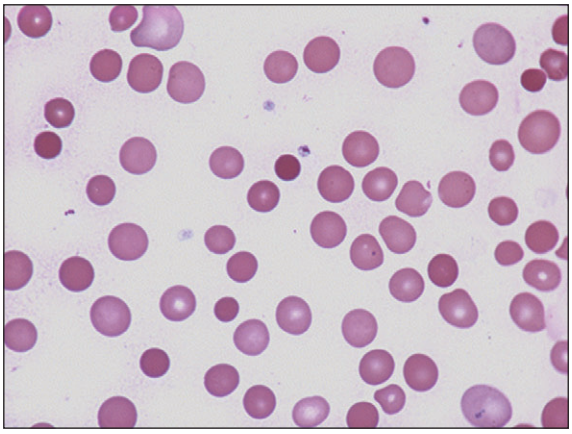


Fig. 4 Spherocytes lack central pallor and may appear smaller than typical red

larger than red cells on average and have a faint bluish tint with Wright-Giemsa stain. Staining with a supravital dye such as brilliant cresol blue highlights the residual nuclear material and definitively identifies reticulocytes. These staining characteristics persist for only 1 or 2 days following release from the bone marrow. Thereafter, the erstwhile reticulocytes are identical to other red cells already in the circulation. The pathophysiology of anemia involves either the production of too few erythrocytes or the production of large numbers of red cells in concert

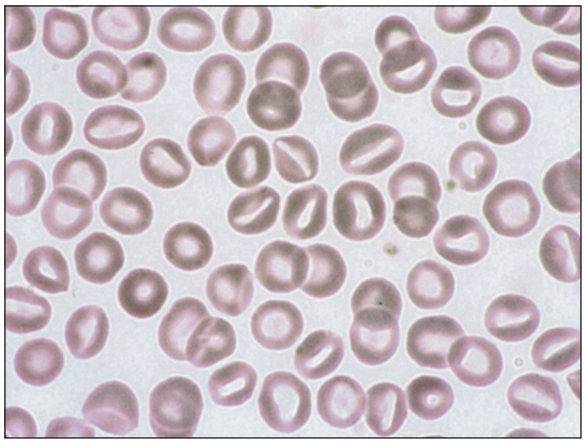


Fig. 5 Stomatocytes

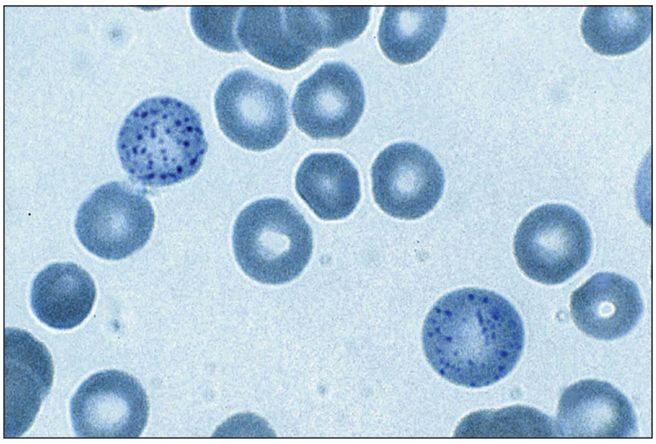


Fig. 8 Basophilic stippling: Numerous, small purple inclusions in RBCs. Aggregates of ribosomal RNA. Most commonly seen in lead poisoning

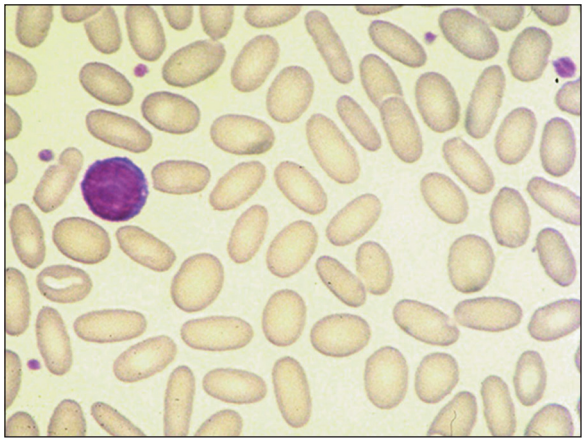


Fig. 6 Elliptocytes

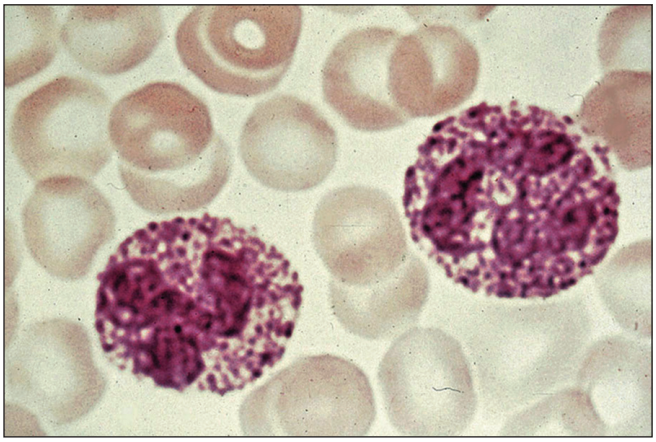


Fig. 9 Toxic granulation: Increased basophilic granules in neutrophils. Seen in severe infections, burns, malignancies, and pregnancy. Distinguish from basophils

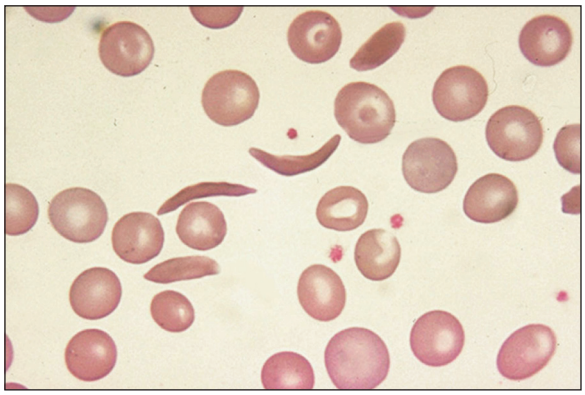


Fig. 7 Sickle cells

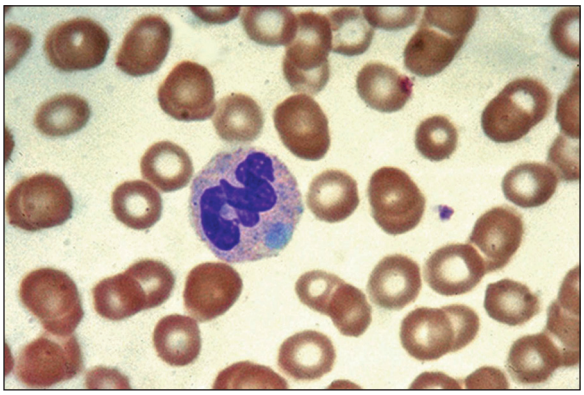


Fig. 10 Dohle bodies: Sky blue inclusions in cytoplasm of neutrophils. Seen in infections, burns, myeloproliferative disorders, and pregnancy. Composed of RER and glycogen granules

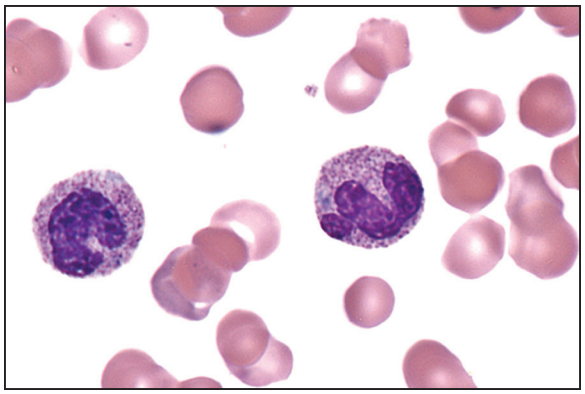


Fig. 11 Peripheral smear from a patient with infection shows granulocytes with Dohle bodies

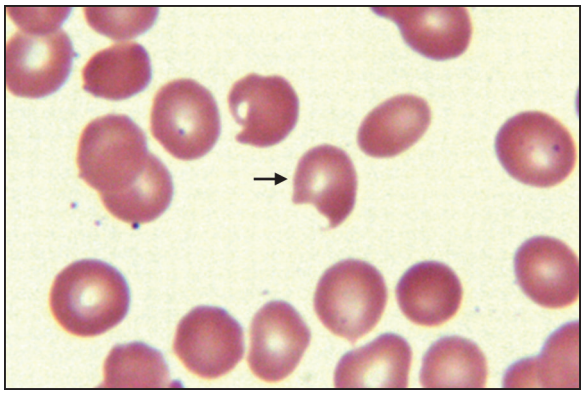


Fig. 14 The RBC deformity (arrow) shown in this image is referred to as a "bite" cell: G6PD deficiency

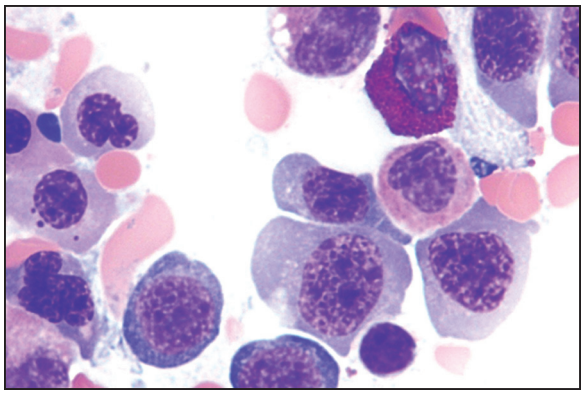


Fig. 12 The open, sieve-like chromatin (salt and pepper) pattern in nucleus of megaloblastic cells is apparent

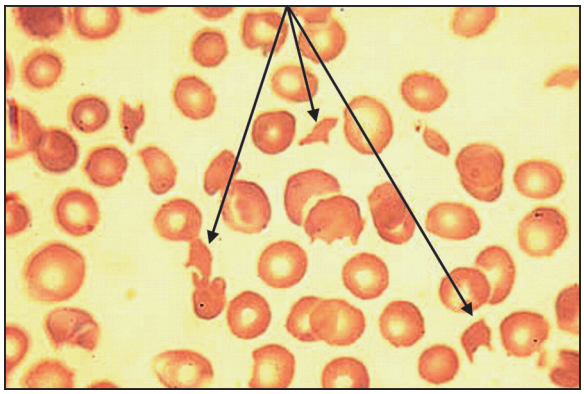


Fig. 15 Note the fragmented schistocytes, burr cells, and helmet cells

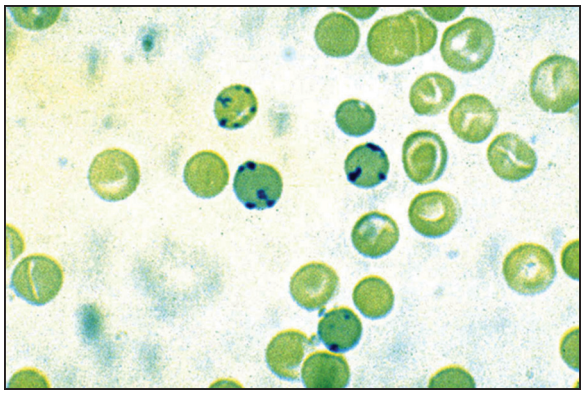


Fig. 13 Reticulocytes: Immature RBCs. Contain residual ribosomal RNA. Reticulum stains blue using a supravital stain (new methylene blue). Counted and expressed as % of total red cells

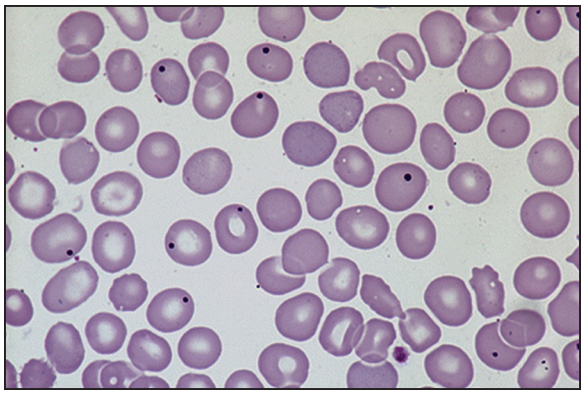


Fig. 16 Howell-Jolly bodies are red cell inclusions which are residual nuclear fragments

with their rapid destruction or loss. The reticulocyte count is the arbiter of the type of anemia, being low in the former instance and high in the latter. This basic distinction allows the clinician to home dramatically the search for the cause of the anemia. A terminological peculiarity sometimes confuses discussions of reticulocytes. The "reticulocyte count" reported by most clinical laboratories is given as a percentage of reticulocytes with respect to red

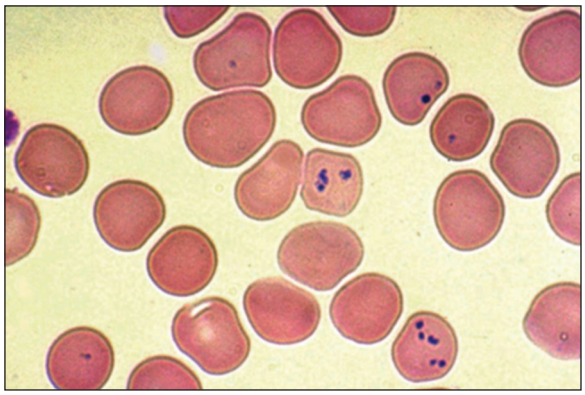


Fig. 17 Pappenheimer bodies: Clusters of dark blue granules, irregular in size and shape. Composed of iron and ribosomal RNA. Seen in sideroblastic and hemolytic anemias

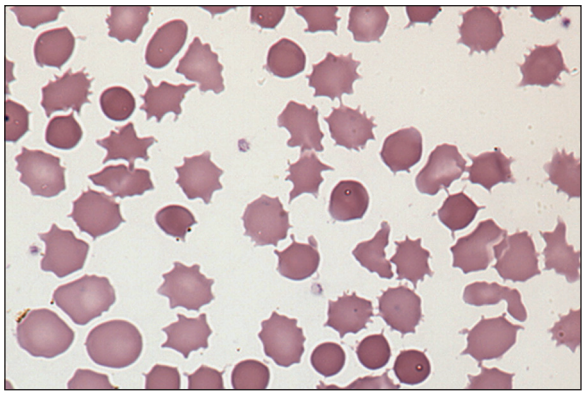


Fig. 18 Acanthocytes are red cells which retain a spherical shape but have a spiculated appearance

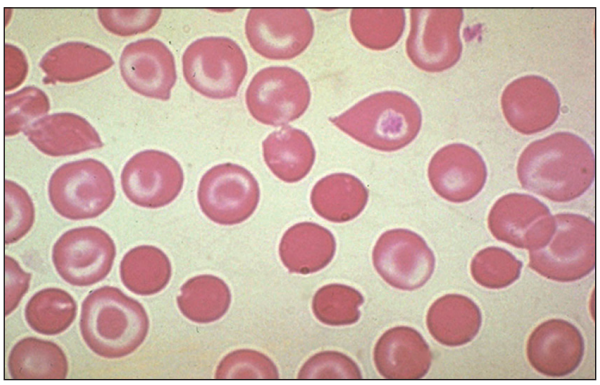


Fig. 19 Target cells

cells in the sample. The normal value is 1 to 2 percent. This reflects the practice of enumerating reticulocytes relative to erythrocytes in the early days of hematology when this work entailed manual counting of many microscopic fields. Expression of "reticulocyte count" as a percentage of red cells is a reasonable practice as long as the number of red cells is in the normal range. Anemias by definition have fewer red cells than normal. Consequently, the use of a percentage of red cells to determine reticulocyte production is invalid in the very circumstance where it is most needed. A normal "reticulocyte count" of 1 percent in the face of a hemoglobin value of 5 g/dL and 2.0×10^6 red cells/μL is patently abnormal. A number of formulae have been promulgated that purport to correct for the anemia and allow a clinician to assess whether the reticulocyte percentage value is elevated or depressed. This semantic and conceptual trap is best avoided by using the true reticulocyte count, which is the number of reticulocytes per microliter of blood. This value is also termed the "reticulocyte number" to distinguish it from the percentage reticulocyte count. When the hemoglobin level is normal, blood contains between 50,000 and 1,00,000 reticulocytes/μL. An anemia with a reticulocyte number below this level is prima facie evidence of a hypoproliferative anemia.[2,5] The reticulocyte percentage can be corrected to measure the magnitude of marrow production in response to hemolysis as follows:

Reticulocyte index = reticulocyte % × observed hematocrit/normal hematocrit × 1/μ where μ is a maturation factor of 1 to 3 related to the severity of the anemia. The duration of maturation as blood reticulocytes is taken as μ. The value for μ for various hematocrit is as follows: Hct 45%- μ 1.0, Hct 35%- μ 1.5, Hct 25%- μ 2.0, Hct 15%- μ 2.5. The normal reticulocyte index is 1.0; therefore, the index measures the fold increase in erythropoiesis. The usual marrow response in acute hemolytic anemia is reflected by a reticulocyte index of 2 to 3, whereas in long standing chronic hemolysis, the increase in erythropoiesis is approximately 6-fold.[7]

APPROACH TO HEMOLYTIC ANEMIA

Hemolysis is defined as an abnormally increased rate of red blood cell destruction. It may be acute or chronic, congenital or acquired, and intrinsic or extrinsic to the RBC. With chronic hemolysis, anemia may or may not be present depending on the rate of red cell destruction and the degree of bone marrow compensation. Moreover, the clinical signs and laboratory findings in hemolysis depend on both the rate and site of red cell destruction. If red cells are destroyed extravascularly in the reticuloendothelial system, the normal site of red cell catabolism, Hb is degraded to iron, bilirubin metabolites, and amino acids. Because hepatic clearance of bilirubin can increase substantially, a normal serum bilirubin does not exclude hemolysis. Unconjugated (indirect) bilirubin will be increased in more severe hemolysis resulting in jaundice. When hemolysis occurs intravascularly, free hemoglobin is released into the plasma where it is bound by haptoglobin and subsequently cleared in the liver or lost in the urine. An increase in plasma hemoglobin, a decrease in serum

haptoglobin, and the presence of hemoglobinuria suggest intravascular hemolysis.

Congenital hemolytic anemias include disorders of the red cell membrane (e.g. hereditary spherocytosis, stomatocytosis, or elliptocytosis), hemoglobinopathies (e.g. hemoglobin SS), and red cell enzyme deficiencies (e.g. glucose-6-phosphate dehydrogenase (G6PD) deficiency, pyruvate kinase deficiency). A family history positive for anemia, splenomegaly, jaundice, and/or gallstones supports a congenital hemolytic anemia. Ethnic background can also be helpful. Hereditary spherocytosis can occur in any ethnicity, but is most common in whites. An elevated MCHC and spherocytes on the PBS supports this diagnosis. An MCHC greater than 35.4 coupled with an RDW >14 is almost always diagnostic of hereditary spherocytosis. The osmotic fragility test or ektacytometry are confirmatory. Family members should also be screened. G6PD deficiency is most common in those of African or Mediterranean descent (with the latter tending to have more severe disease). Individuals with G6PD deficiency often present with acute hemolysis after an infection or after encountering an oxidant stress. Signs of acute intravascular hemolysis with tachycardia, jaundice, and hemoglobinuria will be observed. The PBS reveals schistocytes and spherocytes initially, then becomes normal after the enzyme deficient cells are hemolyzed. The sickle cell syndromes are usually detected by newborn screening in the United States and the pediatrician becomes aware of the diagnosis before anemia or other clinical manifestations occur. More detailed information about these syndromes can be found elsewhere in this volume. In any patient with congenital hemolytic anemia, an aplastic episode is the exception to the rule that hemolytic anemias are associated with reticulocytosis. While many viruses have been implicated, human parvovirus B19 is the most frequent cause. Complete suppression of erythropoiesis can last for 7 to 10 days after infection. Prompt intervention with red blood cell transfusions as needed can be life saving.

Acquired hemolytic anemias can be immune-mediated or secondary to factors that cause mechanical damage to the red cells such as toxins, mechanical or abnormal heart valves, and fibrin strands in disseminated intravascular coagulation (DIC) or hemolytic uremic syndrome (HUS).[8,9] Immune-mediated destruction is confirmed by a positive direct and indirect Coombs' test. Spherocytes may be seen on the PBS. Antibody-mediated hemolytic anemia can occur as part of a more generalized autoimmune process (such as lupus) or after exposure to a drug. However, in children it is most often a self-limited disease following a viral illness. In neonates, immune-mediated hemolysis is due to placentally-transferred maternal antibodies. Historically, this was most commonly due to sensitization to the Rh antigen in Rh negative mothers. The advent of anti-D serum in women who are a potential set-up for sensitization has caused a dramatic decrease in the incidence of this disease. Currently, immune-mediated hemolysis in the infant most often reflects a maternal response to other blood group antigens. Mechanical damage is usually intravascular and associated with red cell fragments on the PBS. The history and/or physical examination can often identify the source of damage.[2]

The following clinical situations warrant a specialist referral:

- Anemia requiring transfusion
- Acute or active bleeding resulting in anemia
- Moderate to severe anemia in a child or elderly or a pregnant lady
- Anemia showing no response or inadequate response to transfusions
- Anemia showing no response or poor response to medical treatment
- A chronic or recurrent anemia
- Anemia of chronic renal failure
- Recurrent or long standing bleeding disorders
- Anemia of systemic disease, if disproportionate to disease or of moderate severe degree
- Anemia secondary to bleeding disorders, leukemia or hematological malignancies
- Where the underlying causative disease is likely to result in progressive or persistent anemia
- Anemia where the underlying causative disease is undiagnosed or not clear
- Where surgery or other intervention procedure is contemplated
- When there are co-existent abnormalities of platelet or leukocyte
- When you are uncertain of your diagnosis or treatment.

Key Points

- There are numerous ways of classifying the causes of anemia, and no one way is necessarily superior to another.
- Regardless of the specific algorithm followed in evaluating severe anemia, it is essential that easily remediable causes such as nutritional deficiencies, hemolysis, and anemia of renal insufficiency are identified early and treated appropriately.
- In general, the differential diagnosis of severe anemia can be substantially narrowed by subcategorization into "microcytic," "normocytic," and "macrocytic" subtypes on the basis of mean corpuscular volume.
- However, such classification is a starting point and not infallible. Each category then can be deciphered using a stepwise approach that utilizes readily accessible laboratory tests.
- Hematology referral is always appropriate for complicated or less defined cases.

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CHAPTER 14

Nutritional Anemia in Infancy, Childhood and Adolescents

MR Lokeshwar, Nitin K Shah

Anemia is diagnosed when Hb concentration and HCT (number and size of RBCs) is lower than the level considered normal for the person's age/sex group.

Nutritional anemia (NA) is the most common cause of anemia in childhood. Nutritional anemia is a pathological condition in which hemoglobin and hematocrit levels becomes abnormally low, because of deficiency of, one or more essential nutrients needed for Hb formation, and for hemopoiesis, regardless of the cause of these deficiencies. When anemia is due to nutritional deficiency, increasing the person's intake of deficient nutrient will raise the Hb and HCT.

World Health Organization (WHO) criteria for the diagnosis of anemia (Table 1) are hemoglobin levels less than 11 gm% in children between 6 months and 6 years and below 12 gm% in children between 6 and 14 years. In developing countries like ours, besides deficiency of food specific nutrients (like iron, folic acid, vitamin B_{12}, protein, vitamin C, vitamin E, trace elements, etc.), poor health facilities, poor socioeconomic status, faulty dietary patterns, the degree of urbanization, educational background, prevalence of hook worm and other worms infestations, repeated bacterial infections, tuberculosis, urinary tract infections (UTI), etc. vitamin A deficiency and other mineral deficiencies, etc. also influences the incidence of anemia particularly in children.

However, many individuals with seemingly normal Hb level respond to iron administration with a rise in Hb, which implies that they were actually deficient in iron. Hence assessing the frequency of iron deficiency anemia in a population by means of Hb measurement tends to underestimate true prevalence.[2]

Table 1 Criteria for diagnosis of anemia
Hemoglobin levels to diagnose anemia (g/dL)[1]

Age group	*No anemia*	*Mild*	*Moderate*	*Severe*
Children 6–59 months of age	>11	10–10.9	7–99	<7
Children 5–11 years of age	>11.5	11–11.4	8–10.9	<8
Children 12–14 years of age	> 12	11–11.9	8-10.9	<8
Non-pregnant women (15 years of age)	> 12	11–11.9	8–10.9	<8
Pregnant women	> 11	10–10.9	7–9.9	<7
Men	> 13	11–12.9	8–10.9	<8

Source: Hemoglobin concentration for the diagnosis of anemia and assessment of severity. WHO

IRON DEFICIENCY ANEMIA IN CHILDREN

Iron lacks the glitter of gold, and the sparkle of silver, but it outshines both in biologic importance. **—Nancy C Andrews.[3]**

Historical

Pallor known since *Mahabharata*. Father of *Pandavas* was known as *Pandu* as he was looking pale white. Therapeutic use of iron was known in Greek mythology—Drinking wine in which sword rusted, was line of treatment. *Loha Bhasma* and *Mandura Bhasma* being used in *Ayurveda* since 5000 years use of iron salt is the main therapy of modern medicine.[4]

Iron Deficiency Anemia

Iron deficiency anemia (IDA) is most widespread micronutrient deficiency and affects infants, young children

(6–24 months), preschool children, adolescents and particularly women of childbearing age, and pregnant and lactating mother.

Inspite of efforts of increasing the awareness, education, food fortification and multiple control programmes nutritional anemia still remains widely prevalent with significant morbidity in developing countries and to a lesser extent even in the developed countries.[57]

Iron is important for a number of iron-dependent enzymes including the catalaze, peroxidase, cytochromes, and ribonucleotide reductase. It plays a vital role in hemoglobin which is important for oxygen carriage in multicellular organisms like man.

An Estimated Global Prevalence[1-30]

One-third (20–30%) of world's population, i.e. 2150 million people are iron deficient, out of which 1200 million are anemic, of which 90 percent live in third world countries. Recent World Health Organization (WHO)/ United Nations Children's Fund estimates suggest that the number of children with iron-deficiency anemia (IDA) is greater than 750 million.[1] Although the prevalence of anemia is estimated at 9 percent in countries with high development, in countries with low development, the prevalence is 43 percent in absolute numbers; anemia affects 1.62 billion people globally with about 293 million children of preschool age, 56 million pregnant women estimated to be anemic. With global anemia prevalence estimates of 47 percent in children younger than 5 years, 42 percent in pregnant women, and 30 percent in non-pregnant women 15–49 years. In the tropical countries, the incidence is at times almost 100 percent. IDA is an extremely serious public health problem in India, 1624 babies die everyday due to IDA (The World Health Report–Preventing Risks, Promoting Life, 2002).

Young children and pregnant women are most affected, with an estimated global prevalence of 43 percent and 51 percent respectively. IDA is most common in the age group of six months to three years.[21-30] Children aged 12 to 17 years (adolescence) and especially among pregnant women and lactating mothers.

The reported prevalence of nutritional anemia in preschool children varies from 44 to 74 percent.[22-30]

In the adolescent period (10–19 years), it has been found that the incidence of nutritional anemia is about 11 to 90 percent and increases from 10 years onwards and continues to remain high till 18 years of age.[31-40] With increasing age, the prevalence rate declines in males, and not in the females.

At least half of all married women aged 15 to 49 years and adolescent girls are believed to have some degree of IDA. Anemia is estimated to contribute to more than 115,000 maternal death and 591,000 perinatal deaths globally per year. Almost 58 percent pregnant women in India are anemic and it is estimated that anemia is the underlying cause for 20–40 percent of maternal deaths in India. Iron deficiency is by far the most common nutritional cause of anemia. The prevalence of anemia has actually increased from NFHS-2 to NFHS-3. The percentage of children with any anemia increased from 74.3 percent in NFHS-2 to 78.9 percent in NFHS-3 in the period between the two surveys, there was an increase in the prevalence of mild anemia (from 23% to 26%) and moderate anemia (from 46% to 49%) percent of pregnant women and 24 percent of men. The prevalence of anemia among ever married women increased from 52 percent in NFHS-2 to 56 percent in NFHS-3.[30,69] However, it may be associated with folic acid deficiency and other nutritional deficiencies like vitamin B_{12}, pyridoxine, copper, etc. The condition is more prevalent in developing countries (36%) than in industrialized developed countries (8%).

India falls in the category of high prevalence for nutritional anemia.[2,41]

IDA among Pregnant and Lactating Women[42-51]

The overall iron requirement increases from a pre-adolescent level of 0.7 to 0.9 mg Fe/day to as much as 2.2 mg Fe/d or perhaps more, in heavily menstruating young women. Hence, adolescent girls are unlikely to acquire substantial iron stores during this period; intake may average as little as 10 to 11 mg Fe/day. The low iron stores in these young women of reproductive age, will make them susceptible to IDA during pregnancy. Dietary intake alone is insufficient in most cases to meet the requirement of pregnancy. Adequate iron supplementation of adolescent girls is essential towards lowering the incidence of anemia during pregnancy.

Recent study showed that prevalence of IDA among pregnant and lactating women is over 75 percent and more than half of pregnant women and a third lactating women are moderately or severely anemic. In some states, an anemia prevalence as high as 87 percent has been found among pregnant women from disadvantaged groups.[47]

In pregnant women, the incidence of nutritional anemia vary from developed countries:

• Europe	18%
• South Asia	75%
• South-East Asia	63%
• India	88, 38–88%

Each year out of 500,000 maternal deaths 100,000 are due to iron deficiency. Mortality attributable to anemia was found to be 20 percent in Africa and 22.6 percent in

Asia. Iron requirements are higher during the second and the third trimester and iron balance during this period depends more on the adequate intake of bioavailable iron rather than the store at conception.

Iron Requirements in Different Age Groups

• Pregnant females	30 mg/day
• Females 11–30 years	15 mg/day
• Adult males	10 mg/day
– Up to 10 years	10 mg/day
• Full-term infants	1 mg/kg/day from 4 months of age
• Low birth weight babies	2 mg/kg/day from 2 months of age
• Babies < 1000 g	4 mg/kg/day from 2 months of age
– 1000–1500 g	3 mg/kg/day from 2 months of age

Surprisingly, IDA is not just a problem in developing countries. According to CDC, although ID is more common in developing countries, a significant prevalence was observed in US during early 1990s in toddlers and women of childbearing age. In one study, 7 percent toddlers (1–2 years), 9–16 percent of adolescent and adult females (12–49%) were found to have iron deficiency. Although incidence of iron deficiency is uncommon, they are still above the healthy people 2010 objectives of 5 percent, 1 percent and 7 percent for toddlers, preschoolers and females 12 to 49 years respectively. Anemia is a major contributory factor for increased maternal morbidity and mortality and accounts for 19.1 percent maternal death.[44,45,48,50]

REPORTED INCIDENCE OF IDA IN CHILDREN (TABLE 2)

- Nearly half of the world women with anemia live in South–Asia
- In a recent study, in an urban slum of Delhi, nearly half of the anemic young children had other nutritional deficiencies notably vitamin B_{12} and folic acid as the direct or associated cause[52]
- Gopalan[37] in 1997 reported that only 38 percent from urban Delhi, 19 percent of rural Rajasthan only had normal Hb level
- Dr Currimbhoy[17] reported anemia 70 to 80 percent in children in 60s
- Dr Mamta Manglani et al. 60 to 70 percent in 90s
- Dr MR Lokeshwar reported in office practice — 50 to 60 percent (unpublished data)
- Thirty to ninety percent of adolescent children in India are anemic depending upon socioeconomic condition and whether from rural or urban area[31-40]

Table 2 Incidence of anemia in developing countries like India[1-25,27,30-36,40]

Amongst preschool children	70% (76–90%)
1–3 years	63–74%, (ICMR 1977, NFHS1998-99)
School age children	37%, (60–80%)
Children between the age group of 6–35 months	74
3–6 years ICMR in 1977[8]	44%
Adolescent period 10–19 years, and increases from 10 years onwards and continues to remain high till 18 years of age[2]	50%
Nonpregnant women	35%, (81–84%)
Pregnant women	50% (30–50%) 80–88%
Women from South Asia	60%
South-East Asia	50%
Africa	40–50%
Adult male	18% (48–56%)

- Report of the NFHS-2[30,79] shows that the prevalence of anemia has not much changed in 1998 to 1999 and is still 74 percent among children of 6 to 35 months of age. It has now been realized that iron deficient state without anemia is even much more common. Over the last 50 years, the prevalence of iron deficiency anemia has ranged between 68 and 97 percent in children. Infants, toddlers, preschool and adolescents are at a great risk of developing IDA.[21-25,27,30-36,40]

Shahbuddin et al. from Bangladesh[35] reported anemia in 94 percent adolescent boys and 98 percent adolescent girls. Who conceive during or shortly after adolescence are likely to enter pregnancy with low or absent iron stores or IDA.

Classification of countries with respect to degree of public health significance is:[16]

High-risk	40 or > % of population anemic
Medium risk	15–40% population anemic
Low-risk	Less than 15% population anemic

SOURCES OF IRON

Major sources of food iron and type of dietary iron available:

- Ultimate absorption of iron into mucosal cells mainly depends upon bioavailability of iron in the various foodstuffs.

- Dietary iron is available as.
- Heme iron.
- Nonheme iron.

Heme Iron

- The nonvegetarian foods is available as hemoglobin and myoglobin in meat, fish and poultry. It is the richest source of iron. Heme iron is highly bioavailable, since it is absorbed intact within the porphyrin ring and the absorption of this is not affected by any another food and is better absorbed than nonheme iron. It is the richest source of iron containing 10 to 18 mg of iron per 100 gm. But in developing countries like ours, the intake of these products is generally low.
- Nonheme iron is available in the form of ferric complexes. Nonheme iron is markedly affected by promotive and inhibitory iron binding ligands.
  Foods rich in iron are cereals, pulses, legumes, *Bajra*, nuts, dates, jaggery, green leafy vegetables, and meat, fish and liver preparations.
- *Milk is a poor source of iron:* Breast milk, the primary source of infant nutrition is poor in iron, containing 0.28 to 0.73 mg/L. Whereas the bioavailability of iron in cow's milk is just 10 percent and that of breast milk is 50 percent (20–80%) making it a good source of iron. Hence, iron deficiency rarely occurs in exclusively breastfed infants till the age of 4 to 6 months. Breast-feeding does not protect against iron deficiency after the age of 6 months, unless iron containing weaning foods are introduced.
  - *Factors affecting iron absorption:* Heme iron is not affected by presence of any factors in the gut. The absorption of nonheme iron is retarded by alkaline pH, presence of phosphates, phytates, bran, starch, tannins, calcium, antacids, other metals (Co, Pb), etc. Phytates, which constitute 1 to 2 percent of many cereals, nuts and legumes, play a major role in the causation of nutritional anemia in the developing world.

It is enhanced by ascorbic acid, free hydrochloric acid, presence of sugars and amino acids in the diet, presence of heme iron (nonvegetarian source of iron) and EDTA.[53-56] The bioavailability of iron from a particular dietary source affects the amount absorbed. Ferrous iron is better absorbed compared to ferric iron. It is estimated that in wheat based diet, iron absorption is around 2 percent and in rice based diet, it is 5 to 13 percent.[53,54] Poor bioavailability of iron in largely cereal-based diet is major cause of IDA in most developing countries. Fish, meat and poultry are good sources of iron and bioavailability is around 20 to 30 percent. Increasing the dietary intake to meet the caloric needs will also increase the dietary intake of iron by one- third. Calcium in the form of milk, cheese or (calcium added to the bread), depresses iron absorption.

Administration of 50 mg of vitamin C increases iron absorption by two fold.

An average adult has about 3 to 5 g of iron and children have 55 mg/kg/body weight. It is more in males as compared to females. Seventy percent of iron in the body is in the form of Hb. 3.9 percent is incorporated in myoglobin and various other iron containing enzymes. Plasma iron forms only 0.1 percent of the body iron. Iron balance in the body is achieved mainly by control of absorption of iron rather than its excretion, most of the iron is recirculated in the body. Only 1.15 mg of irons is excreted daily. Thus a daily requirement is minimum. Absorption of iron mainly depends upon dietary content of iron.

Mucosal Cell Control

Site of absorption is the 1st and 2nd part of the duodenum, and at times jejunum. Maximum absorption of iron occurs from the duodenum.

Two steps are involved in the absorption of iron:

1. Entry of iron from the intestinal lumen into the mucosal cell.
2. Its passage from the mucosal cell into the plasma.

Appropriate iron balance in the body is achieved by mucosal cell control through transferrin and apoferritin receptors. The iron molecule that is taken into the mucosal cell across the brush border, can bind either to the apoferritin molecule or the ferroportin molecule in the mucosa. Iron status of the body at the time of the formation of the mucosal lining cells determines the amount of iron that is absorbed.

If iron in the plasma is adequate, or increased iron stores, there is increased messenger iron in the mucosal cell. This messenger iron stimulates the production of apoferritin. Iron binds to apoferritin, which remains in the mucosal cell. There is increased transferrin saturation.

Thus, whenever there is increased transferrin saturation or, serum iron is normal and adequate, a larger fraction of the iron entering the mucosal cell is held back as ferritin and discarded, as the cell is desquamated and ultimately excreted after 3 to 4 days, and gets denuded with the cell within 3 to 4 days. If iron is required in the body, it is bound to the ferroportin, which is then transferred to the transferrin (produced in the liver), which carries it across the mucosa. It is then utilized in the bone marrow for hemoglobin production, in the muscle tissue for myoglobin and in the body for various other enzymes. Any excess iron is stored in the form of ferritin in the liver. The RBCs circulate for their life span of approximately 120 days and are then destroyed in the spleen, liberating the free iron,

which is then retransported to the bone marrow and other tissues for its reutilization. Thus, most of the iron is cycled continuously in the body, with only 1 to 1.5 mg/day of iron being excreted through the intestinal epithelial cells after completion of their life span. Since 10 percent of ingested iron is absorbed and the daily loss is only 1 to 1.5 mg, one needs to ingest about 10 mg of iron daily, except during periods of extra needs. Iron is stored in the body in the form of ferritin and hemosiderin.

In iron deficiency state, the mucosal cell transports the iron rapidly to the circulation, where it combines with transferrin and is transported to the site of utilization and storage. Only 1/10th of the dietary iron is absorbed by the GI mucosa.

Hepicidin and its Role in Iron Metabolism[57,58]

Hepicidin plays a key role in the regulation of iron metabolism and iron deficiency. Hepatocytes produce hormone peptide (cysteine rich) hepicidin. Hepicidin controls the release of iron from variety of cell such as macrophages, hepatocytes enterocytes, etc. to plasma. It primarily controls iron absorption. The recycling of the iron from red cell lysis and release of iron from tissue iron stores is carried by interaction of hepicidin with ferroprotein which is a cellular iron exporter. Release of ferroprotein is controlled by hepicidin.

Iron Transport and Storage

Transferrin helps in transport of iron from the intestines to the site of its utilization.

Transport of Iron Across the Placenta

The transport of iron across placenta occurs against a gradient, thereby protecting fetus against iron deficiency. Babies with low iron stores may be born, to mothers who are severely iron deficient during pregnancy. Most of the placental transfer of the iron occurs during the 3rd trimester of pregnancy. As a consequence of this, all preterm babies invariably develop anemia unless supplemented by iron and iron deficiency in the mother may cause preterm labor.

Normal infants at birth have about 75 mg of iron per kg body weight, two-third of which is present in red blood cells. Once the iron is assimilated in the body it is not excreted. Normal body losses of iron are about 20 ug/kg/day. Most of these loss occur by shedding of cells from intestinal mucosa. Average loss of iron per day in children is 0.9 mg/day or 0.5 mg/m^2/day. 0.6 mg/day is lost in the GIT in the form of RBCs, bile or exfoliated mucosal cells. The rest is lost from the sweat, desquamated cells of the skin and the urinary tract.

Causes of Iron Deficiency

- *Decreased iron stores:* Preterms, small for date babies
- Decreased intake/assimilation and reduced absorption
  - Prolonged breastfeeding and delayed introduction of iron rich, complementary feeds after the age of 6 months adds fuel to the fire
  - *Diet containing low iron or nonbioavailable dietary iron:* It is estimated that in the wheat millet based diet, iron absorption is around 2 percent and in rice based diet, iron absorption is around 5 to 8 percent.
  - Low over all dietary intake
  - Nonbreastfed infants on cow's milk
  - *Malabsorption of iron:* Chronic diarrhea, celiac disease, cow's milk allergy, GI surgery, giardiasis, etc.[22]
  - Rarely genetically determined absorptive defect specific for iron.
- *Increased requirement:*
  - During periods of growth—preterm infants, toddlers, puberty
  - During reproductive age in females
  - Pregnancy
  - Lactation.
- *Increased losses:* Blood loss due to any cause/chronic blood loss. In gastrointestinal bleeding the chronic loss of few milliliters of blood daily is sufficient to deplete iron stores and lead to iron deficiency.
  - Gastrointestinal bleeding
  - Diverticulitis
  - Polyps
  - Fetomaternal hemorrhage
  - Repeated blood sampling
  - Blood losses in the menstrual cycle (Menorrhagia). Females loose 40 mL blood per month in the menstrual cycle, this increases the daily iron losses to 1.5 mg/day.
  - *Intestinal-parasites–hookworm-infestation giardiasis:*[59-65] Infants are exposed to intestinal helminths from the time they crawl. Such infants suffer repeatedly from gastroenteritis, and other infections further depleting their iron stores.[51,52] Particularly for those from rural areas. Four hundred fifty million people all over the world harbor this parasite and about 0.2 mL of blood/worm of ankylostoma per day may be lost and with nector infestation each worm accounts for loss about 0.1 to 0.5 mL/day. Female subjects harboring more than 100 worms (5 mL/day blood loss) and male subjects harboring more than 250 worms (12.5 mL/day blood loss) tend to become anemic. Chakma T, et al.[62] from Madhya Pradesh reported intestinal parasite in 50 percent of adolescents (Hook worm 16.3% lumbricoid 18.5%).

- *Adolescents:* Adolescence, is a period of rapid growth, weight gain and blood volume expansion. Iron requirement increases from preadolescent level of 0.7 mg to 0.9 mg iron per day to as much as 2.2 mg iron per day or more particularly heavily menstruating women
- In pregnancy, 4 mg of iron is lost per day in the last two trimesters. During lactation 0.5 to 1 mg of iron is lost per day
- *False concern about the body figures, food fads:* During adolescence false concern about the body figures, food fads, ignorance, particularly in girls lead to iron deficiency. Lack of knowledge of nutritional factors further adds to the problem particularly in adolescent girls
- *Poor iron absorption:*
  - Malnutrition/iron poor diet/malabsorption syndromes chronic infection/chronic diarrhea celiac disease/giardiasis/helminthiasis, sprue, hypoproteinemia, cow's milk allergy also contribute to a high prevalence of anemia
  - *Gastrointestinal surgery:* Polyps/Meckel's diverticulum/hemorrhagic telangiectasia/peptic ulcer, diverticulitis are other causes of bleeding diathesis and iron loss
  - Milk-induced enteropathy is the most common cause of occult GI bleeding seen in approximately more than 50 percent of infants with IDA seen in the western world
  - Rarely genetically determined absorptive defect specific for iron.
- *Fetomaternal hemorrhage:* Among the other causes of blood loss leading to anemia in newborn. In about 50 percent of all pregnancies there is some degree of fetomaternal hemorrhage. Eight percent are significant (0.5 – 40 mL fetal blood loss)
  - *Repeated venipunctures* for investigations, hemodialysis, and regular blood donations are important iatrogenic causes of iron deficiency due to chronic blood loss
- *Inadequate transport:*
  - Atransferrinemia
  - Antitransferrin receptor antibodies.

PATHOGENESIS

Stages of Iron Deficiency

Iron deficiency anemia (IDA) is the end stage of a relatively long drawn process of deterioration in the iron status of an individual.

The spectrum of iron nutrition status can be divided into three stages:

Iron deficiency stage: This refers to lack of iron of sufficient severity to restrict production of hemoglobin.

1. *First stage—storage iron depletion:* Iron reserve is decreased or absent. It is characterized by reduced serum ferritin, reduced iron concentration in marrow and liver tissue. Hb, serum iron, transferrin levels and saturation are within normal limits
2. *Second stage—iron limited erythropoiesis (Iron deficiency without anemia):* Refers to a milder form of iron deficiency where Hb has not fallen enough to meet the criteria of anemia. It may be transient and consists of a decrease in the transportation of iron Hb level may still be normal or in the low normal range. Serum iron is low and total iron binding capacity (TIBC) increased with low transferrin saturation and low serum ferretin levels
3. *Third stage—iron deficiency anemia:* It represents the more severe form of iron deficiency. The supply of iron to erythroid cells in marrow is impaired, causing a reduction in Hb concentration, with progressive microcytic hypochromic anemia. Hb concentration has fallen and decreased serum iron, transferrin saturation and serum ferritin levels. There is an increase in the FEP (free erythrocyte protoporphyrin) detectable anemia, microcytosis, hypochromia on the peripheral smear with low MCV and MCH and high RDW.

Clinical Features of IDA

Iron deficiency an isolated hematological condition associated with anemia. It is a systemic disease involving multiple systems. The appearance of symptoms depends upon:

- Hemoglobin level
- The rate of fall of hemoglobin
- Hemostatic adjustment of various organs systems
- Age of the child
- Maturity of various organs
- Underlying cause.

If the fall of hemoglobin is gradual as in iron deficient anemia, then the onset of symptoms is insidious and may go unnoticed till Hb drops to as low as 4 to 5 gm/dL. Child may not have any obvious symptoms. Gradual onset of pallor may escape notice even when the hemoglobin falls to 4 to 5 gm.

However, if the drop of Hb is rapid, child may be brought in serious conditions—tachycardia, signs of congestive cardiac failure and even gasping conditions as it some times occur in G6PD deficiency or autoimmune hemolytic anemia. Initial symptoms include pallor, tiredness, lassitude, easy fatigability, anorexia, weakness, lack of concentration, breathlessness, irritability, puffiness, edema feet, etc.

However, all cases of anemia may not have pallor, especially in mild cases of anemia, and associated with icterus, cyanosis, dark pigmentation, may mask pallor.

Pallor can also be seen in nonanemic conditions as color of the skin not only depends on the Hb content, but on the state of blood vessels of the skin (Vasoconstriction as seen in vasovagal syncope), presence of edema as seen in nephrotic syndrome, myxedema, congestive cardiac failure, even in absence of anemia. Hence, it is always prudent to rely on Hb or HCT estimation to detect anemia.

- Later hyperdynamic circulation leads to palpitation, shortness of breath, easy fatigability, reduce exercise tolerance and heart failure
- *Changes in epithelial cells:* These include koilonychias, platonychia, atrophic glossitis, angular cheilosis are uncommon in children, however may be observed, especially with long standing anemia and in adolescent children (Fig. 2). The triad of dysphagia due to esophagal webs, koilonychias (Fig. 1) and splenomegaly in a child with IDA is known as Plummer–Vinson or Paterson–Kelly syndrome and are not common in children
- Mild hepatosplenomegaly is also not uncommon in children with iron deficiency anemia
- *Pica:* Pica usually is the manifestation of iron deficiency and is relieved when condition is treated. Pica is a habitual ingestion of unusual substances like:
  - *Geophagia:*[83] Mud or clay and can decrease absorption of iron and aggravate IDA
  - *Amylophagia:* Eating laundry starch—uncooked rice
  - *Pagophagia-ice:* It is unexplainable symptom. Often seen in pregnant women.
- Other common causes of pica are lead poisoning and psychological disturbances
- Clay[84] can behave in the gut as an exchange resin and can interfere with iron absorption.

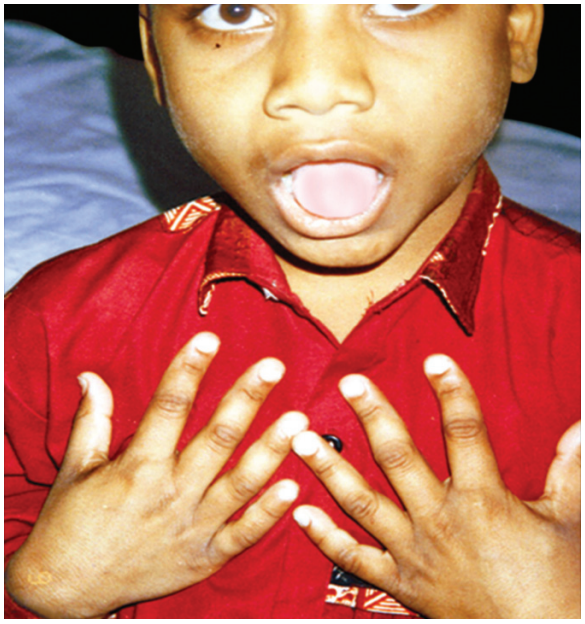


Fig. 1 Koilonychia and puffiness of face and eyes and severe pallor in a case of IDA (Rare in children)

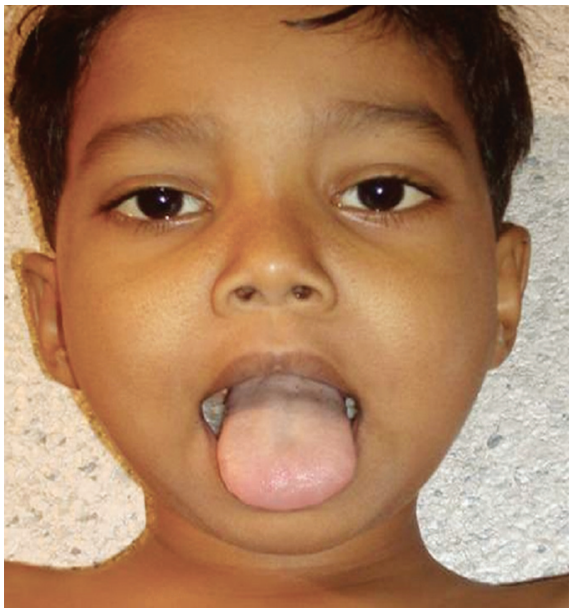


Fig. 2 Adolescent child with IDA

Pica though may be a manifestation of iron deficiency, is also considered to be a predisposing factor. It is both effect and cause of IDA.

Clinical features may be due to anemia or in addition due to underlying etiology of anemia[28,29,66-70]

In mild anemia: There may be no signs and symptoms but a definite sense of well being and better exercise tolerance is observed following treatment. All the symptoms of the anemia like fatigue, breathlessness, irritability, anorexia, etc. may be seen. Spleen is often enlarged slightly in children, but is of normal consistency.

Patients with Mild to Moderate Anemia (Hb 6–10 gm%)[1,71]

- May or may not have any symptoms
- They are usually diagnosed during routine laboratory testing
- This is because of compensatory mechanisms like increase in 2,3 DGP levels and a shift of O_2 dissociation curve.

Severe IDA < 5 gm%

- May present with fatigue, breathlessness, tachycardia, systolic murmur and later on, signs of CCF may develop
- Anorexia, loss of appetite
- Increased infections
- Irritability
- *Other features:* Depletion of nonheme iron containing tissue proteins is responsible for various other manifestations.

Diet History

A detailed diet history is very important, especially in infant with anemia.

- Exclusive breastfeeding for 4 to 6 months
- Introduction of good home made weaning food containing iron thereafter
- Iron deficiency develops where there is poor breastfeeding, prolonged breastfeeding beyond 1 to 2 years especially with introduction of improper weaning food is also a cause of nutritional anemia

Iron deficiency has a wide range of clinical and functional consequences:

- *Behavioral changes:* These changes occur due to diminished activity of aldehyde oxidase, required for serotonin catabolism, thus leading to increased levels of serotonin and 6-hydroxy nidole compounds. MAO which is also required for catabolism of catecholamine is also reduced.
- Short attention span, irritability, stubbornness, decreased cognition and scholastic performance and conduct disorders are common in children with iron deficiency, leads to learning disabilities and scholastic backwardness. These neurological changes that occur due to iron deficiency may be long term or even irreversible. Anemic children have poorer endurance capacity and lack physical fitness.[72-81]
  - Breath holding spasms in less than 3 years child.
  - *Altered immune response:* It is believed that IDA children have increased susceptibility to infections due to immunosuppression. Humoral, cell-mediated and nonspecific immunity and the activity of cytokines which have an important role in various steps of immunogenic mechanisms are influenced by iron deficiency anemia.[82] Iron deficiency affects both cell mediated as well as humoral immunity, though phagocytic activity may be normal.
- Some studies state that immunity is enhanced in iron deficient state. This is due to increased unsaturated transferrin which inhibits bacterial growth and hence high dose IV iron therapy could be harmful in such cases. However, oral iron therapy only minimally changes saturation of transferrin and hence practically it does not have any adverse effect on incidence of infection. In several studies, results show that infants who receive iron supplementary formulae have fewer episodes of respiratory and gastrointestinal infections than those who receive unsupplemented formula.
- Reduced myeloperoxidase can also lead to altered white cell function.

Growth Retardation

There is marked reduction in weight of iron deficient children, though height seems to be unaffected.

Exercises Intolerance[80,81]

Maximum work capacity, work output and endurance are impaired in iron deficiency state. This is due to reduction in the mitochondrial enzyme α-glycophosphatase dehydrogenase besides due to anemia.

Effects on Pregnancy Outcome[42-46,48,50,51]

Estimates from the World Health Organization report that from 35 to 75 percent (56% on average) of pregnant women in developing countries, and 18 percent of women from industrialized countries are anemic. However, many of these women were already anemic at the time of conception, with an estimated prevalence of anemia of 43 percent in nonpregnant women in developing countries and of 12 percent in women in wealthier regions. Even for women who enter pregnancy with reasonable iron stores, iron supplements improve iron status during pregnancy and for a considerable length of time postpartum, thus providing some protection against iron deficiency in the subsequent pregnancy.

Maternal iron deficiency in pregnancy reduces fetal iron stores, perhaps well into the first year of life. Iron deficiency anemia in pregnancy is a risk factor for preterm delivery and subsequent low birth weight and possibly for inferior neonatal health.

Regulation of Iron Transfer to the Fetus

Most iron transfer to the fetus occurs after week 30 of gestation. Serum ferritin usually falls markedly between 12 and 25 weeks of gestation, probably as a result of iron utilization for expansion of the maternal red blood cell mass. Serum transferrin carries iron from the maternal circulation to transferrin receptors located on the apical surface of the placental syncytiotrophoblast, holotransferrin is endocytosed, iron is released, and apo-transferrin is returned to the maternal circulation. The free iron then binds to ferritin in placental cells where it is transferred

to apotransferrin, which enters from the fetal side of the placenta and exits as holotransferrin into the fetal circulation. This placental iron transfer system regulates iron transport to the fetus.

When maternal iron status is poor, the number of placental transferrin receptors increases so that more iron is taken up by the placenta. Excessive iron transport to the fetus may be prevented by the placental synthesis of ferritin. There is a substantial amount of evidence showing that maternal iron deficiency anemia early in pregnancy can result in low birth weight subsequent to preterm delivery.

Diagnosis of Iron Deficiency Anemia (Flow Chart 1)[84-90]

Laboratory tests in iron deficiency anemia are required to diagnose IDA and to establish its cause. Iron deficiency anemia, should initially be suspected clinically by taking proper detailed history, dietic history, socioeconomic status, and proper clinical detailed examination—looking at tongue, mucosa of inner side of lip and palate, conjunctiva or nails, paleness around the crease of the palm and thorough systemic examination to detect underlying cause.

It is desirable that anemia and its severity are then quantified through the accurate laboratory tests.

Screening Tests[85-91]

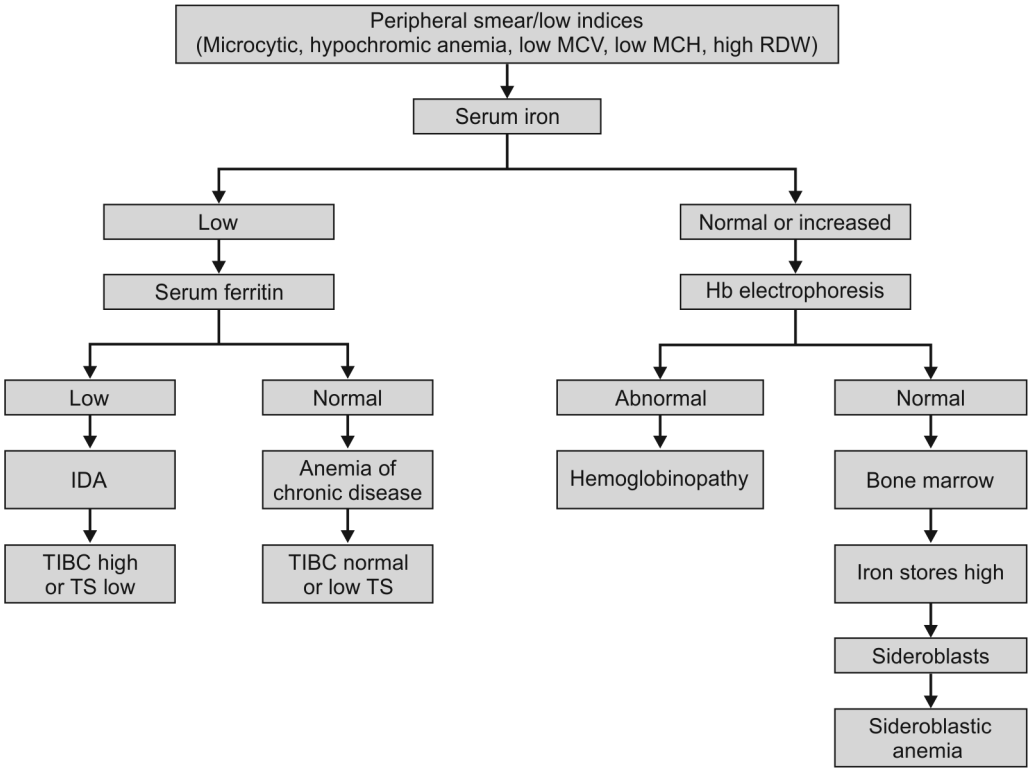
Manual determination of these red cell indices are time consuming and not very reliable and not reproducible. With the availability of electronic particle counters estimation of RBC parameters—Hb, PCV, MCV, MCHC, RBC count, RDW, has become easy to perform, accurate, reproducible and practical.

Evaluation of peripheral smear examination is must.

The changes in these parameters include:

- Red cell count, hemoglobin and hematocrit are all decreased in IDA. MCV, MCH and MCHC are also decreased.
- The peripheral blood film shows hypochromic, microcytic red cells (Fig. 3).
- If anemia is severe, morphological abnormalities such as poikilocytosis and target cells. Pencil cells may be seen.
- When iron deficiency is associated with deficiency of other hematinics like vitamin B_{12} or folate, there may be a dimorphic picture with hypochromic, microcytic red cells along with macrocytic red cells. These routine

Flow chart 1 Approach to IDA-hypochromic microcytic anemia



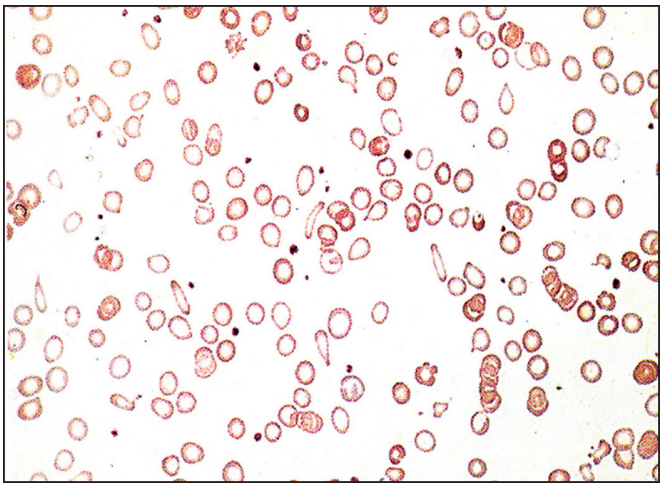


Fig. 3 Peripheral smear examination showing microcytic hypochromic anemia

investigations may not be useful to diagnose early iron deficiency state.

- Reticulocyte count is normal unless the patient has had a recent acute blood loss or the patient has received. Hematinics, in which case it may be increased. Following iron therapy there is reticulocytosis which peaks at 1 to 2 weeks.
- In severe IDA, reticulocyte count may be decreased. Low levels of Hb, MCV< 80 ug/dL, MCH < 27 Pg and high levels of RDW with low SI, TS, TIBC and FEP indicate IDA.
- Klee[92] G in his study showed that more than half of the 62 patients with IDA had a MCH value clearly within a normal range and nearly 70 percent of cases exhibited distinct microcytosis, suggesting that MCV is much more sensitive than MCH in determining changes of iron deficiency. MCHC < 33 percent and is the last of the indices to be affected and is the least important in diagnosis of IDA. MCV is more sensitive than MCH in diagnosis of IDA. RDW is the quantitative measure of anisocytosis. It is increased in IDA and normal or low RDW values are unlikely to be present with IDA.
- Microcytosis also may be seen in other conditions.

Differential Diagnosis of Microcytic Hypochromic Anemia (Table 4)

- Iron deficiency anemia
- Anemia of chronic infection and inflammation
- Thalassemias and abnormal hemoglobinopathies traits
- Lead poisoning
- Sideroblastic anemia.

Child is said to be anemic when the hemoglobin and/or hematocrit is two standard deviation (SD) below the mean for that particular age and sex.

Reticulocyte count is usually normal unless the child has an acute or recent blood loss or has received hematinics. However in mild iron deficiency, RBC morphology and indices are not altered.

Confirmatory Tests (Table 3)

- Serum iron is reduced (N = 50–180 ug/dL)
- TIBC is increased, more than 470 mcg/dL (N = 250–450 ug/dL)
- Transferrin saturation is low—less than 16 percent suggestive and less than 7 percent diagnostic of severe IDA.
- Serum ferritin is less than 10 to 12 ng/mL. However, when infectious or inflammatory diseases like rheumatoid arthritis, collagen disorders, liver disorders, chronic renal disease or malignancy are also present, the serum ferritin level is usually higher, but less than 50 to 60 ng/mL as ferritin is an acute phase reactant. The test still lacks sensitivity and normal value does not reliably exclude iron deficiency.

Free Erythrocyte Protoporphyrin

Free erythrocyte protoporphyrin (FEP) and protoporphyrin—heme (PH) ratio: Erythrocyte protoporphyrin, the precursor of heme accumulates in red blood cells when it has insufficient iron to combine with the form of heme. The blood can be conveniently tested by putting drop of blood on a cover slip and reading the result directly. The FEP can be measured by a simple fluorescence assay performed directly on the thin film of blood. Both FEP and PH ratio is elevated in iron deficiency. Normal values of FEP are 30 to 40 ucg/dL RBC and PH ratio 16 (+5.3). FEP values above 70 mcg/dL RBC and of PH ratio above 32 is considered to represent iron deficiency.

- FEP/Hb ratio increases when iron reserve is exhausted, even before anemia becomes apparent

Table 3 Confirmatory tests

Age in years	*Serum ferritin ng/dL*	*Transferrin saturation percent*	*RBC/FEP ug/dL*
0.5–4	< 10	<12	>80
5–10	< 10	<14	>70
11–14	< 10	<16	>70
>14	< 12	<16	>70

Table 4 Differential diagnosis of microcytic/hypochromic anemia

	Iron deficiency	*Thalassemia minor*	*Chronic infection*
Clinical			
Age	6–24 months	Any	Any
Community	Any	High risk	Any
Pica	++++	—	—
Diet	Milk/bottle feeding	—	—
Behavior	Irritable/listless	—	—
Breath holding	+++	—	—
Epithelial/nails	Koilo/ Platonychia	—	—
Laboratory			
RBC count	Low	High	Low
MCV	Low	Low	N-Low
RDW	High	N	High
Hemolysis	N	++	N
RBC count	Low	High	Low
TIBC/FEP	High	N	High
Ferritin	Low	N	High
sTfR/serum ferritin	High	N	Low
ESR	N	N	High
HbA2	N	High	N
Iron trial response	Good	Poor	Poor

- The ratio is normal in thalassemia trait and renal anemia
- FEP/Hb ratio remains elevated during iron therapy and returns to normal only after majority of cells containing FEP formed during iron deficiency are replaced
- FEP/Hb ratio is not subject to daily fluctuations and sudden changes as in transferrin saturation
- The highest value of FEP is seen in lead intoxication—a level of FEP greater than 160 ug/dL of RBC is taken as cut-off value for detection of lead intoxications.
- FEP/Hb ratio in the range of 5.5 to 17.5 ug of Hb may be attributed to either IDA or lead intoxication with or without associated iron deficiency. This is not used regularly due to the cost of the machine and problems of standardization. Advantage of FEP is that unlike serum iron studies, FEP values are not altered immediately after iron therapy.

Soluble transferrin receptor (sTfR) measures the severity of IDA and values more than 9 mg/L are considered abnormal. Transferrin receptors (TfR) facilitate the entry of transferrin-bound iron into cells by a process of endocytosis. sTfR increases with enhanced red cell production but iron deficiency is the only disorder in which there is increased serum receptor combined with a low level of red cell production. Unlike the serum ferritin, which only identifies iron deficiency, sTfR measures its severity. The normal reference value is 2.8 to 8.5 mg/L. Values above 9 mg/L are considered abnormal. Unlike many other iron measurements, the level remains normal in patients with anemia of chronic inflammation or infection.

The most sensitive method available to distinguish IDA from anemia of chronic disease is a combination of plasma sTfR and the log of plasma ferritin concentration, i.e. sTfR-ferritin complex. If it is high (>4) it indicates IDA; if <1 it indicates chronic disease.

Molecular genetics of iron deficiency: Human transfer in gene has been reported that human transferrin G2775 mutation is a risk factor for iron deficiency.

Depletion of stainable iron from bone marrow: (Routinely bone marrow examination not required) Bone marrow aspirates can be stained for hemosiderin by Perl's reaction and iron content is graded from 0 to 4. Although is the most accurate technique to evaluate iron status, it is an invasive procedure and therefore impractical.

Response to Therapy

- In uncomplicated IDA, administration of iron shows a predictable reticulocytosis and a rise in Hb. A positive response to therapy can be defined as a daily increase in Hb concentration of 0.1g/dL (0.3 or 1% rise in HCT) from the fourth day onwards.
- If the serum ferritin is low but the hemoglobin is normal, the individual is at risk of iron deficiency, while if the hemoglobin is low but the serum ferritin is normal further hematological assessment is required to identify the cause of anemia.

Laboratory Tests in Iron Deficiency Anemia

• Low serum iron	Less than 75 mg/dL
• Increased total iron binding capacity	More than 470 mcg/dL
• Low transferrin saturation	Less than 12% and 14% for infant and children
• Serum ferritin	< 10 µg/L in children and 12 µg/L in adults
• Increased red cell protoporphyrin concentration	
• Depletion of stainable iron from bone marrow	Graded from 0 to 4

TESTS FOR THE STORAGE COMPARTMENT

- *Serum ferritin:* A level of < 10 μg/L in children and 12 μg/L in adults is suggestive of iron depletion. Serum ferritin is increased in infections, inflammation, liver disease, parasitic infestations, enteric infections and even upper respiratory tract infections
- *Bone marrow evaluation:* Morphologically bone marrow shows erythroid normoblastic hyperplasia though the normoblasts may be smaller than normal (micronormoblasts)

  Bone marrow aspiration is not routinely indicated in the diagnosis of IDA. The degree of cellularity and the proportion of myeloid to erythroid cells on bone marrow examination vary depending on the severity as well as the duration of IDA
- Prussian blue staining of the bone marrow is also used for diagnosing IDA, though it is rarely indicated. The iron content is graded from 0 to 4.

TESTS FOR PLASMA IRON COMPARTMENT

It includes:

- Serum iron
- Total iron binding capacity (TIBC), transferrin saturation (SI).
  It is a major drawback is diurnal variation after 3 years of age. Morning samples show higher levels. Hence if morning sample shows a serum iron < 30 ug/dL, it is suggestive of IDA.
- *TIBC:* It is less subject to biological variations. But normal range is 250 to 400 ug/dL. An increase in TIBC indicative of IDA.
- *Transferrin saturation:* It is the ratio of above two values and is consistent and hence is a very useful test for IDA.

$$TS = \frac{\text{Serum iron}}{\text{TIBC}} \times 100$$

In adults TS < 16 percent is suggestive of IDA. In infants and children the diagnostic value is < 12 to 16 percent. TS < 6 percent is diagnostic of IDA at any age.

It is important to realize that one should collect fasting sample (nonlipemic) and all forms of iron supplements should be stopped for 48 to 72 hours before collection while performing iron studies.

When to Suspect IDA?

- Anemia during age of rapid growth, i.e. first 6 months to 3 years of life and adolescence.
- Child not breastfed but fed with faulty diet, diluted artificial powder milk especially improper weaning, prolonged breastfeeding, bottle feeding.
- Irritable, cranky child, breath holding spasm, history of pica, worms' infestation, chronic bleeding.
- Microcytic, hypochromic anemia on PS, relative decrease in RBC count and increased RDW and low MCV
- Leukocyte count is usually normal.

Hypersegmented neutrophils may be seen due to concomitant B_{12} or folate deficiency or due to iron deficiency-induced interference with folate utilization, or due to IDA-induced impairment of jejunal function leading to poor absorption of B_{12} or folate. Thrombocytosis may occur in patients with IDA, as a result of iron therapy *per se* or due to underlying condition such as malignancy or bleeding:

- In infants and children the TS < 12 to 16 percent is diagnostic. Ferritin value is < 12 to 16 ng/dL
- Increase in FEP
- *Therapeutic test:* Treat the child with 3 to 5 mg/kg of elemental iron.

There is increase in retic count at 1 to 2 weeks and Hb level reaches to normal by 2 to 3 months.

When to Suspect β-thalassemia Trait?

- *Community:* Kutchi, Lohana, Punjabi, Sindhi, Neo-buddhist, Mahars and other high-risk communities
- Microcytic/hypochromic anemia with target cells, polychromasia, relative increase in RBC count and normal RDW. Normal iron study including ferritin level
- Poor and inadequate response to iron therapy.

Screening Tests

- NESTROFT,[93,94] discriminant functions.[93,94] PS examination
- *Confirmatory tests:* Increase in HbA2 more than 3.5 percent
- β-chain synthesis (silent carrier).
- *Free erythrocyte protoporphyrin (FEP) and protoporphyrin—heme (PH) ratio:* Erythrocyte protoporphyrin, the precursor of heme, accumulates in red blood cells when it has insufficient iron to combine with to form heme. Elevation of FEP mainly EZP level is an early and sensitive indicator of iron deficiency. FEP can be measured by a simple fluorescence assay performed directly on a thin film of blood on a glass slide. Both FEP and PH ratio are elevated in iron deficiency. Normal values of FEP are 30 to 40 mcg/dL RBC and PH ratio 16 (+5.3). FEP values above 70 mcg/dL RBC and of PH ratio above 32 is thought to represent iron deficiency. In uncomplicated iron deficiency anemia, red cell FEP levels may range

from 100 to1000 μg/dL. EZP is also elevated in chronic lead poisoning and sideroblastic anemia

- *Serum iron:* Normal serum iron level varies considerably. It has a diurnal variation with a peak in the morning and trough in the evening. Serum iron concentration may also be affected by chronic infection, malignancies and chemotherapy as well as iron medication. Values below 40 mcg/dL (<12 mcg/dL in young children) are considered diagnostic of iron deficiency (in absence of infection or other disorders which affect iron metabolism)
- *Total iron binding capacity (TIBC) and transferrin saturation (TS):* TIBC is the measure of plasma transferrin, which is free, not bound to iron. The normal value of TIBC is 250 to 350 mcg/dL. Since serum iron is about 100 mcg/dL, normally only one-third of transferrin is utilized to bind iron, giving a normal transferrin saturation of 33 percent. In iron deficiency states, TIBC is increased (> 350 mcg/dL) and transferrin saturation is reduced to below 16 percent (<12% for children)
- TIBC below 200 mcg/dL is characteristic of inflammatory disease
- Factors that affect serum iron concentration do not alter values of TIBC
- *Serum ferritin:* The serum ferritin is a sensitive laboratory index of iron status. It is the best non-invasive test (gold standard with a high specificity and adequate sensitivity) for evaluating iron status in the body. It is estimated that each ng/mL of serum ferritin is equivalent to 8 to 10 mg of storage iron. A serum ferritin value of less than 12 ng/mL is highly specific for iron deficiency but gives no information about its magnitude.[91] Ferritin levels are estimated by radioimmunoassay (RIA), or ELISA techniques. Serum ferritin is increased in chronic disorders, e.g. chronic infection and inflammation, malignancies, chronic liver disorders. In presence of any of these, a coexisting iron deficiency anemia can be missed.
- *Soluble plasma transferrin receptor (sTfR):* Transferrin receptors (TfR) facilitate the entry of transferrin bound iron into cells by a process of endocytosis in iron deficiency anemia, transferrin receptors are increased probably due to an increased turnover associated with ineffective erythropoiesis and, an increase in cellular transferrin receptor expression produced by iron starvation. Unlike the serum ferritin, which only identifies iron deficiency, the serum transferrin receptors measure its severity. Value above 9 mg/L are considered abnormal health level in healthy male and female are 56 mg/L and is normal in anemia of chronic infection and inflammation.[95]

Bone Marrow Examination

Bone marrow aspiration is not recommended for the diagnosis of IDA, as there are simpler, noninvasive and relatively inexpensive tests, which diagnose IDA reasonably well. In contrast, bone marrow iron staining, though a gold standard, is very painful, expensive and cumbersome to perform. However, bone marrow when done shows increased cellularity with micronormoblastic erythroid hyperplasia. On staining with prussian blue (Perl's reaction), there is little or no stainable iron seen.

Response to Therapy

In uncomplicated IDA, administration of iron shows a predictable reticulocytosis and a rise in hemoglobin. Hb concentration remains the most dominant predictor of response to therapy in uncomplicated iron deficiency. A positive response to therapy can be defined as a daily increase in Hb concentration of 0.1 g/dL (0.3 or 1% rise in HCT) from the fourth day onwards. Lower the initial Hb, greater is the response following iron therapy.

Treatment of Iron Deficiency Anemia

Basic principles of management include:

1. Correction of anemia
2. Treatment of underlying cause

- *Management of IDA:* If the patient is severely pale and sick looking, breathless, has tachycardia, raised JVP and tender hepatomegaly, it is suggestive of congestive cardiac failure. Such a patient needs immediate attention and prompt treatment including admission, diuretics, anti-CCF measures and packed cell transfusion. And other treatments depending on underlying cause
- One should not waste time in lengthy diagnostic tests and do as minimum tests as required.
- Even removing too much blood for various tests in small infants, can be hazardous as it can precipitate cardiac failure. Instead, one can arrange for packed cell transfusion and remove blood for various tests just before starting transfusion.
- Other way if the patient is pale but comfortable and not sick, there is neither need to give packed cell transfusion nor start 'gun shot' therapy without proper investigations and establishing the diagnosis.

The management of iron deficiency anemia is considered in two parts:

1. Treatment of the individual patient
2. Treatment at public health level.

The successful management requires:

- Confirmation of the diagnosis
- Thorough investigations to find out the etiology and to treat the cause
- Supplementation of iron.

It is important to find out the etiological factor for iron deficiency to prevent failure of therapy and recurrence of deficiency after treatment is stopped, especially in older children who are likely to have a secondary IDA associated with underlying cause. Treatment of worms, giardiasis, bleeding from any site, recurrent infections is must to treat the patient adequately besides iron.

Infants usually have poor dietary history. Lack of breastfeeding associated with bottle feeding or prolonged breastfeeding and improper weaning and poor intake of iron containing food as a cause of iron deficiency.

Promotion of exclusive breastfeeding for first 4 to 6 months. Thereafter introduction of proper and age appropriate food items after 6 months and continuing breastfeeding for as long as possible, along with prophylactic iron supplementation will prevent iron deficiency during infancy and early childhood. In older children diet modifications to improve total calorie intake and iron containing foods in diet will prevent iron deficiency.

- Iron therapy—including replenishment of stores.
  - Oral
  - Parenteral.
- Treatment of underlying causative factor
- To prevent recurrence of deficiency preventive measures:
  - Diet counseling
  - Iron supplementation
  - Iron fortification.

IRON THERAPY

Aim is to give iron in enough dose, for enough number of days so as to normalize the Hb levels and replenish stores, in a convenient way with least number of side effects**.**

It can be given either:

- Orally
- Parenterally.

Oral Iron Therapy

Advantages of oral iron therapy are cheap, effective, safe, convenient and well tolerated and preferred and advocated route of therapy.

- Best absorbed when given on an empty stomach or in between the meals in divided doses.
- It may be given at bed time as compliance is better in children as child goes to sleep and vomiting and pain abdomen is much less as intestinal activities are slow during sleep. Compliance in the first month of therapy is important as majority of iron absorption occurs during this period. It is continued for at least 2 to 3 months after hemoglobin becomes normal, to replenish stores.[66,85]

Dose

- *For infants and children:* Iron store present at birth and the highly bioavailable iron in breast milk protect an infant from IDA up to 6 months. Supplementation with medicinal iron has been recommended by WHO for all children beyond 4 to 6 months of age and low birth weight babies from 2 months onwards, for preventive supplementation, iron dosage is 2 mg/kg per day for children of all age groups. Children of 6 to 35 months of age should receive a daily uniform dose of iron folic acid (IFA) supplement (20 mg elemental iron + 100 mcg folic acid) in liquid from (3–6 mg/kg/day of elemental iron). Although the desired Hb level is usually reached in 2 months, iron therapy should continue for another 3 months to build up iron stores.
- *For women (15 years +) with severe anemia (Hb < 7 g/dL):* National Nutritional Anemia Control Program (NNACP)[96] recommends two tablets of iron-folate tablet per day (each tablet containing 100 mg of elemental iron and 500 mcg of folic acid) for a minimum of 100 days. Prolonged duration of treatment is required to correct the anemia and replenish iron stores.

Restoration of Hb to normal with ferrous salts requires 3 to 6 months of Rx. Replenishment of body iron stores requires further therapy for additional 2 to 4 months.

- Risk of accidental iron poisoning in small children
- In developed countries, tablet containing ferrous iron are the second most common cause (after aspirin) of accidental poisoning among small children leading to hospitalization and several death.
- Preparations of iron formulations.

All dietary iron has to be reduced to ferrous form to enter the mucosal cells. Various iron salts available include ferrous fumerate, ferrous gluconate, ferrous sulphate-hydrous anhydrous form, etc.

Bivalent iron salts like ferrous sulfate, fumarate, gluconate, succinate, glutamate and lactate have been preferred over ferric salt preparations.

- *Type of iron salt:*
  - Ferrous sulfate (20% elemental iron) is commonly used for tablet preparations.

- Ferrous fumarate (33% elemental iron) is environmentally more stable.
- When ferrous preparation is taken on empty stomach absorption increases, however side effect also increases.

Many new iron preparations are not affected by food or milk enabling administrations without consideration of the timing of feed. These are iron polymaltose complex and carbonyl iron (highly purified metallic iron with a particle size less that 5 mm). However, most of these preparations have not been found to be effective in clinical setting.

Ferrous salt is the drug of choice as it is absorbed best and is cheapest. Iron salts of carbonate, citrate, choline citrate and pyrophosphate are not absorbed efficiently (Table 5).

Other salts available are:

- Ferrous fructose
- Ferrous succinate
- Ferrous lactate
- Ferrous carbonate
- Ferrous glycine citrate
- Ferric ammonium citrate
- Colloidal iron.

Various Forms of Iron Preparation

- *Uncoated tablets:* Uncoated tablets are cheaper but have more side effects. Enteric coated tablets have better availability with less side effects but are expensive. Flavored chewable tablets are available which have better compliance but again are costly. And overdose is more common.
- Syrups and drops can lead to staining of tongue and teeth and are costly, may not be stable.
- Iron in hemoglobin form is also available but has little advantage. Content of elemental iron is low and is expensive and often fails to provide the required amount of iron.
- *Side effects of oral iron therapy:* Nausea, vomiting, abdominal cramps, diarrhea, constipation, staining of tongue and teeth, blackish discoloration of stools, etc. are common side effects. By and large the side effects are less and well tolerable if proper counseling is done and before starting therapy.
- Rarely case of acute iron poisoning occurs by taking accidental or suicidal overdose.
- Once or twice a week oral iron therapy has been found to be equally effective if not better with less side effects.

Table 5 Percentage and amount of iron in some commonly used iron tablets preparation of iron compounds[97]

	Per tab (mg)	*Per tab iron*	*Elemental % iron (mg)*
Ferrous fumarate	200	66	33
Ferrous gluconate	300	36	12
Ferrous sulfate ($7H_2O$)	300	60	20
Ferrous sulfate (anhydrous)	200	74	3

Daily versus Weekly Supplementation[99-104]

In humans, the intestinal mucosal turnover time is 5 to 6 days and serves as the basis for the weekly preventive supplementation regimen:

- Iron absorption from GI tract depends upon iron content in the mucosal cells. Less the iron contents more the absorption
- One of the major problem with daily supplementation regimen is that supplements must be taken for a long period of time for achieving desired improvement in iron status
- It has been seen that iron deficient and anemic women can absorb as much as 30 to 40 mg dose of iron ingested on an empty stomach
- Iron administration every 3rd day (intestinal mucosal turn over time in rat is 3–4 days) was more efficient in iron deficient rats than when administered daily.[98] Indeed, in weekly dosing food iron absorption is also better maintained. Weekly dose is considered as cost effective with requirement of lesser number of doses, with fewer side effects and better compliance. Recent research in experimental animals and field studies among the preschool children in China and other countries have indicated that intermittent therapeutic dosage can be as effective as daily dosage in correcting the mild to moderate anemia and iron deficiency and in anemia prophylaxis.[96,97] A number of studies in Indian setting have shown weekly iron supplementation is effective
- With daily administration of iron, gastrointestinal complaints were more common in majority of the studies and were rare with weekly dose. Serum ferritin levels were nonsignificantly higher in daily as compared to weekly supplement
- In Bombay, Mehta et al.[100] in a study of 1748 adolescent girls (10-18 years) from urban slums revealed prevalence rate of anemia in 63.8 percent. Iron supplementation resulted in beneficial effect and prevalence of anemia decreased to 61.16 to 26 percent in daily group and 65 to 33.9 percent in weekly group and no changes 64.8 to 58.4 percent in control group and there was statistically significant increase in Hb level—rise of mean 0.939 g. Hb in daily group as compared to 1.54 g in weekly group. Rise in ferritin level was 5.04 ng/mL in weekly group as compared to 4.69 ng/mL in the daily group. Sheshadri et al. from Baroda[101] in her study

reported rise in Hb in daily group was 0.749 percent as compared to 0.44 gm% in the weekly group side effects of iron supplementation were almost absent in weekly regimen as compared to 15 percent in daily regimen

- A WHO report based on 11 trials published involving 14000 subjects for a period of 4 to 7 months in school children, adult, nonpregnant women and pregnant women revealed weekly supplementation was as effective as when given daily whereas in some studies slightly less therapeutic effect was seen in weekly supplementation as compared to the daily supplementation.

Based on these studies and recent multicentric study in India has now recommended that adolescent girls on attaining menarche should be given weekly dosage of the IFA tablet containing 100 mg elemental iron and 500 ug folic acid once a week accompanied by appropriate dietary supplementation.[97]

Response to Therapy

The first response is the decreased irritability and a subjective improvement

↓

This is followed by a marrow response and reticulocytosis peaking at 5 to 7 days.

↓

Hb rises at a slower rate, i.e. 0.25 to 0.4 g/dL/day. It normalizes by 6 to 8 weeks but it may take little longer.

Failure of oral iron therapy:

- Wrong diagnosis
- Inadequate dose, inadequate length of treatment
- Poor compliance, etc. This can be easily tackled by proper knowledge on part of physician and proper counseling of parents
- Having severe side effects
- Decreased absorption as seen in various GI disorders
- Gastrointestinal bleeding, which is aggravated by oral iron therapy
- Increased iron loss not met with oral therapy, e.g. ongoing GI bleeds, or true intolerance. Such cases can be treated with parenteral iron therapy
- Chronic malabsorption syndromes—chronic diarrhea, cystic fibrosis, Crohn's disease, etc.

Oral iron not sufficient to compensate the need for increasing deficit as in persistent bleeding (Epistaxis).

Parenteral Iron Therapy

Intravenous Iron

Parenteral iron therapy should usually be avoided as having severe side effects.

Indications

- Oral iron not sufficient to compensate the need for increasing deficit as in persistent /significant bleeding-like epistaxis (telangiectasia), gastrointestinal bleeding, etc.
- Decreased absorption as seen in various GI disorders–*Chronic* malabsorption syndromes: Chronic diarrhea, cystic fibrosis, Crohn's disease, surgery, gastrointestinal disease, etc.
- True intolerance for oral iron therapy.
- Having severe side effects on oral therapy.
- For receiving recombinant erythropoietin therapy, or for use in treating functional iron deficiency.
- Noncompliance may make oral iron treatment in some patients inadequate

Types of Parenteral Iron Preparations

- Intravenous (IV)
- Intramuscular (IM)
- Parenteral iron products available are:
  - Iron dextran,
  - Ferric gluconate,
  - Iron sucrose.

Following parenteral administration of iron, the iron carbohydrate complex is separated by the reticuloendothelial system. Iron is gradually released into the circulation.

Iron Dextran Complex

Iron dextran is a colloidal solution of ferric oxyhydroxide complexed with polymerized dextran.

Total dose to be given as, divided intramuscular injections or as full dose IV therapy.

The iron requirement can determine from the equation:

Iron (mg) = weight (kg) × Hb deficit (g/dL) × 80/100 × 3.4 × 1.5 or

weight (kg) × Hb deficit (g/dL) × 4.

Dose: Dose of iron (mg) = weight (lbs) × Desired increment of Hb (g/dL) × 3.

a. *Intravenous route (IV):* There are two methods:
   - Infusion of iron dextran diluted in ratio of 5 mL of iron dextran complex in 100 mL of normal saline. Initially flow rate should be kept at 20 drops/min for 5 to 10 minutes and there are no reactions, then rate can be increased to 40 to 60 drops/min.
   - Bolus injection of iron dextran: Bolus dose of iron dextran diluted in 20 mL of saline.[105-107] Both these routes are however used after a prior sensitivity testing where 1 mL of iron dextran solution is diluted in 20 cc of normal saline and injected slowly over 10 to

15 minutes following which one should observe for reactions for 1/2 to 1 hour

- Sodium ferric gluconate intravenous injection or infusion.

  Ferrlecit (sodium ferric gluconate complex in sucrose injection) is indicated for treatment of iron deficiency anemia in pediatric patients age 6 years and older. Standard dose of 125 mg in 100 mL of normal saline intravenously over 60 min. If the patient's serum ferritin is less than or equal to 100 ng/mL and the transferrin saturation is less than or equal to 20%, the dose can be repeated over 8 weeks.
- Iron sucrose injection Venofer
  - Iron sucrose cucrose injection (Venofer) is an iron hydroxide sucrose complex in water.
  - Iron sucrose is administered by intravenous injection or infusion. The recommended schedule is to administer 100 mg intravenously over 5 min, 1–3 times weekly upto 1,000 mg if required.

  The rate of administration should not exceed 20 mg per minute. Side effect includes hypotension, nausea, and lower back pain.

Intramuscular route (IM): This is very painful and may lead to serious allergic reactions and hence not used in children. Intramuscular injections are best given into the upper outer quarter of gluteal region using Z tract technique. A dose of 0.1 mL should be given as test dose intramuscularly and there are no reactions within 1 hour, full dose (to a maximum of 0.5 cc) can be given.

Though most of the reaction are mild and transient, anaphylactic reactions may be life-threatening and hence one should always keep injection adrenaline, hydrocortisone and resuscitative measures handy before injecting.

Response to Therapy

- Rapid hematologic response can be confidently predicted in iron deficiency.
- A positive response to therapy can be defined as a daily increase in hemoglobin concentration of 0.1 g/dL (0.3 or 1 percent rise in hematocrit) from the fourth day onwards.
- Approximately 2 months are required to achieve a normal Hb level.
- Reticulocytes increase within 3 to 5 days and reach a maximum at 5 to 10 days, reticulocyte counts being 8 to 10 percent in severe anemia.
- The maximum rate of recovery from severe anemia in a child may be 0.25 to 0.4 g/dL per day increase in Hb or a 1 percent per day rise in hematocrit which is more rapid than is anticipated in the adult.
- Iron absorption is maximum during the initial phase of therapy and declines from 14 percent in the 1st week to 7 percent in the 4th week to 2 percent after 4 months.

Blood transfusion may be needed in most severe cases of IDA when the hemoglobin level is below 3 to 4 g/dL or when superimposed infection may interfere with optimal therapeutic response. Packed red cells may be slowly given preferably 2 to 3 mL/kg at one time.

Side Effects

Reactions can occur with both IM and IV therapy and can be either immediate or delayed.

- Immediate reactions
  - Pain at the injection site
  - Vomiting, nausea, headache, malaise, flushing, metallic taste, such reactions are brief in duration and often are relieved by slowing the rate of infusion.
  - Severe reactions like anaphylaxis, hypotension, cardiac arrest, etc. should be contraindicated to further doses.
- Delayed reactions
  - Arthralgia, fever, myalgia, regional lymphadenitis

Prevention of IDA

The basic approaches to the prevention of IDA are:

- Supplementation with medicinal iron
- Dietary modification (Table 6)
- Fortification of foods with iron
- Other measures, which could play an indirect role in improving the iron status are control of viral, bacterial and parasitic infections (hookworm infestation-corrected by regular deworming measures), malaria. Improvement in poor health facilities, poor socioeconomic status, faulty dietary patterns, the degree of urbanization, educational background, provision of safe water, environmental sanitation, health education, vitamin A deficiency and immunization, etc.
- *Protection and promotion of breastfeeding:* Exclusive breastfeeding till the age of 4 to 6 months and promoting breastfeeding for as long as possible, even up to 2 years. Human breast milk is low in iron about 0.5 mg/L. An infant taking 600 to 650 mL of breast milk daily ingests approximately 0.3 mg of iron/day. However, the bioavailability of this iron is quite high, (50%) as much as 0.15 mg of iron per day is absorbed which is sufficient for an exclusively breastfed baby. Breast milk appears to be adequate to cover the dietary

Table 6: Iron content of food articles[108-110]

Food	*Iron content (mg/100 g)*	*Articles rich in iron (>10 mg/100 g)*
Cereals	2.5–14.0	*Bajra*, wild barley, *kangri ragi*, rice flakes, whole wheat flour, *kodra (Harik)*
Pulses and legumes	2.7–11.0	Bengal gram, cowgram, soyabean
Leafy vegetables	0.9–40.0	*Amaranth*, beet greens, Bengal gram leaves, coriander, *alu* leaves, pudina, neem, radish top *rajgira* leaves, turnip greens, all types of green *bhajis* (spinach, methi, lettuce, etc.)
Roots and tubers	0.4–13.9	
Other vegetables	0.2–22.2	Amaranth seeds, dhaincha seeds
Nuts and oil seeds	2.5–10.0	Garden cress, gingelly, mustard,
Fruits	0.1–10.0	Dates, karvands, raisins
Seafood	1.0–11.5	Most Indian fish, crab
Meat	2.0–18.8	Beef
Milk	0.2–0.8	
Miscellaneous		Jaggery, yeast

iron requirements of normal birth weight full term and infants up to the age of 6 months. From 6 months of age the iron requirement increases markedly and hence the iron from breast milk alone is no longer sufficient.

- Encouraging the timely introduction of iron containing weaning food is an important step in prevention of anemia in early infancy and childhood. As maternal transplacental transfer of iron from mother to child takes place during the last trimester of pregnancy, iron storage in premature and low birth weight infant is affected and low. Hence, low birth weight infant require iron supplementation from the age of two months.
- *Nutrition education and dietary modification: Food based intervention:* Anemia (IDA) is hardly ever observed in exclusive breastfed infants till the age of 4 to 6 months even though mother is severely anemic. Breast milk has high bioavailability for iron absorption. Breastfeeding along with appropriate complimentary feed, including iron rich or iron fortified foods where possible through the second year of life is important modality to prevent IDA.
  - Introduction of iron containing food after the age of 4 to 6 months is the most important step in prevention of anemia of infancy.
  - Commercially prepared iron rich weaning foods though available in developed countries, are very expensive and beyond the reach of the majority of the families and so not recommended.
  - Encourage natural homemade food items, initially semisolids and then solid items so that child should consume normal family diet by 9 to 12 months of age.
  - Home made weaning foods rich in iron and vitamin C are cooked vegetables, raw fruits, parents should be taught how to prepare mashed vegetables? Thick palak soup with boiled dal and vegetable, citrous fruit juices, egg preparation, minced mutton, etc. and motivated to introduce these to the infants in early life after 4 to 6 months of age.
  - Foods rich in vitamin C like orange, citrous food, guava, tamarind are seasonably available and are however expensive particularly in developing countries. In developing countries where meat intake is low, vitamin C (ascorbic acid) is the single most important enhancer of iron absorption. Adding as little as 50 mg of ascorbic acid to a meal will double the iron absorption (an orange or lemon, or cabbage 100 g or 200 g of amaranth will sufficient amount of vitamin C). The presence of vitamin C 25, 50, 100, 250, 500 mg in given meal is associated with 2, 3, 4, 5, 6, fold enhancement of iron absorption respectively.[5] Approximately 50 to 80 percent of vitamin C originally present in food can be lost during cooking. And hence should consumed a raw form. Inhibitors of iron absorption such as phytate and tannin. (Phytates are present in wheat and other cereals). Tannin present in tea and to a lesser extent in coffee, nuts and legumens). hence should not be consumed with meals or shortly after meals.

Approximately 50 to 80 percent of vitamin C originally present in food can be lost during cooking. Vitamin C content of food cooked and left standing decreases, reheating reduce still further. In poor households food is cooked once and reheated 12 hours apart, in which case difficult to ensure that enough vitamin C is retained. Best would be to consume raw fruits but caution for hygienic care for GI infections. Common household strategy, processing methods, germination, malting and fermentation, increases vitamin C content and lower

tannin and phytic acid content or both, e.g. germination of some cereals and legumes for 24 to 48 hours is associated with appearance of 10 to 70 mg ascorbic acid/100 gm and reduction of 8 to 25 percent in tannin, 25 to 35 percent in phytic acid and bioavailability increases 2 folds.

- Heme containing food like red-meat, fish and other sea-food have highly absorbable iron and promotes the absorption of the iron more than other less bioavailable food sources. However, it is a very small fraction of the total iron intake particularly in persons from developing poor countries. Heme iron is directly absorbed (20–30% absorption). Its bioavailability is little affected by the nature and composition of the meal, like other associated foods such as phytate, etc.

Approximate ascorbic acid content: Fruits and vegetables vitamin C mg/100 g of food[109,110]

Fruits	Guava	326
	Lemon fresh (juice)	37–50
	Orange fresh	46
	Pineapple fresh	37
	Mango fresh	42
Vegetables		
	Cabbage raw	54–60
	Cabbage boiled	15
	Cauliflower raw	60 –96
	Cauliflower boiled	20
	Potato raw	21
	Potato boiled	12–18
	Sweet potato raw	25–37
	Sweet potato boiled	15
	Spinach boiled	7–25
	Tomato raw	20–26
	Turnip boiled	17

- Nonvegetarian foods not only have a rich amount of heme iron, but they enhance the absorption of the non-heme iron contained in the rest of the meal. However, enhancing meat consumption is not practical by the poor rural people from developing and religious objections to the consumption of meat also pose a problem**.**
- Some breast milk substitutes particularly cow's milk is prone to cause gastrointestinal bleeding in infants leading to IDA.
- Increasing total consumption of habitual food in young children (Mothers should include to feed 4–5 meals per day for young children.) so that their energy needs are fully met and ensure that total iron intake is high even though the percentage of iron absorbed from each meal remains low.
- Dietary modification process like germination (sprouting of green grams) and intake of green leafy vegetables are also helpful in prevention of IDA. Promotion of low viscocity, nutrient dense food for infants is useful. Germinating, malting, fermenting also increases iron absorption by increasing the vitamin C content and lowering tannin and phytic acid which inhibits iron absorption from the gut. Germination increases the bioavailability by almost 2 folds. Just inclusion of guava fruit/citrous food with lunch and dinner have shown to raise Hb level by 2.2 g/dL.[110]
- For older children and adult, iron supplementation can be done by cooking of the food in iron pots may increase the iron content of a meal several fold. This is especially true for soups containing vegetables. Frying in iron pans does not increase the food's iron content.
- Health program and nutrition education on dietary diversification are useful strategies to improve iron intake in intervention area.

Strategies for iron fortification: Basic approach to prevention of IDA:

- *Food based strategies:* The problem of IDA in children largely disappeared in North America when foods fortified with iron and other micronutrients became available for children. In this group, the prevalence of IDA has fallen from 21 percent in 1974 to 13 percent in 1994.
- *Nonfood based strategies:* Food based strategies are important for raising the iron status of populations. They will not be enough to improve the iron status rapidly.

Current Approaches to Food Based Fortification with Iron

- Exclusive breastfeeding
- Avoid bottle-feeding
- Introducing of iron containing weaning foods from 6 months of age
- *Older children:* Encourage diet containing iron—cereals wheat, *ragi*, *jowar* sprouted cereals, green and leafy vegetables, jaggery, nonvegetarian food like mutton, chicken, fish, egg and liver preparations.
- Commercially prepared iron rich weaning foods is not affordable to our poor patients
- Increasing utilization of iron in poor communities
- Increasing total consumption of habitual food so that their energy needs are fully met (Increased by 25–30 percent when the energy shortage was corrected)
- Enhancing the bioavailability of iron by germinating, malting, fermenting by almost 2 folds
- Enhancer heme iron, vitamin C, meat, fish
- Avoiding inhibitors of iron absorption like phytic acid, phytates in plant based diets, tannin in tea and coffee.

Supplementation of Medicinal Iron

Supplementation with oral iron tablet (syrup) is the most widely used approach to control global problem of iron deficiency anemia. For the past 150 years or more, oral ferrous sulphate syrups have been the primary strategy to control IDA in infants and young children.[3]

1. Although pockets of infants and children remain at risk, generally, the eradication of iron deficiency in developed countries is recognized as a successful public health accomplishment. This solution has not worked in developing countries where commercially purchased fortified foods are not affordable or are not used.
2. However, adherence to the syrups is often limited owing to a combination of their unpleasant metallic after taste, the dark stain they leave on the child's teeth, and abdominal discomfort.

In the developing world, there are three major approaches available to address iron deficiency:

1. Dietary diversification so as to include foods rich in absorbable iron.
2. Fortification of staple food items (such as wheat flour),
3. The provision of iron supplements.

Dietary or fortification strategies are not logistically or economically feasible, supplementation of individuals and groups at risk is an alternative strategy.

For the past 150 years or more, oral ferrous sulphate syrups have been the primary strategy to control IDA in infants and young children.

The earth's crust contains 4.5% iron whereas the human body contains a mere 0.005% of iron. Accordingly iron overload, should be more of a problem rather than iron deficiency. However, this is not so, because the form of iron in the environment as well as food is insoluble and difficult to assimilate. In early times the earth had a reducing atmosphere and an abundance of ferrous iron was available for incorporation into biological molecules. Later with an increase in the atmospheric oxygen, iron existed largely in its less available ferric form and special devices had to be developed by life-forms, for the acquisition of iron. Bacteria for example synthesize and excrete high affinity chelating agents that extract otherwise unavailable iron from the surroundings environments. Roots of the plants also secrete substances that augment iron absorption. In the mammalian species, mucosal transferrin appears to perform this function.

The body had been genetically designed to absorb hemoglobin iron, as then human beings were hunters and carnivorous. The progressive change in diet that began 10,000 years ago, with the cultivation of grain and vegetables led to replacement of highly available iron in meat, by the small amounts of iron from cereal diet. This explains the high prevalence of iron deficiency.

Despite the fact that iron is one of the most abundant metals on the earth crust, iron deficiency is the most prevalent deficiency in the world. **PARADOX IS "POVERTY IN THE MIDST OF PLENTY".**

It is useful for rapid treatment as well as for prophylaxis and prevention of anemia can be targeted at high-risk group of becoming iron deficient, such as pregnant women adolescent girls. Preschool and school and children, infants and 'captive audience' such as school children or plantation workers who can receive the supplements at school or work. Problems encountered include side effect of the drug and lack of motivation to continue the drug for a minimum 3 to 4 months in patients who perceive themselves not to be ill.

Pregnant Women

Pregnancy creates a larger demand for iron which is needed for the development of the fetus and placenta and to expand the woman's blood volume. Iron also is lost with blood lost during delivery. About 100 mg of iron are needed to cover the iron requirement of the mother and the fetus during pregnancy. Dietary absorption of iron is reduced during the first trimester and morning sickness, nausea, vomiting adds to the problem. In most of the developing countries 25 to 30 percent of women have little or no iron stores even before conception. Particularly in pregnant teen age mothers the situation is farther critical. Supplementation should be done primarily during the second half of pregnancy. Pregnant women are a priority group for iron supplementation. A number of programs—National Nutritional Anemia Program 1970, National Nutritional Anemia Control Program (NNACP)[111] 1989 have been implemented and dosage was revised from 60 mg elemental iron to 100 mg and 500 mg folic acid per tablet. Poor compliance due to side effects like nausea, vomiting, pain in abdomen, constipation, loose motions, etc. and lack of awareness regarding the real need for iron during pregnancy and the importance of iron for their health, for the unborn fetus and the newborn.

Adolescent Girls[31-40]

Why Concentrate on Adolescents Girls?

The greatly elevated iron requirement of pregnant woman indicates need for prepregnancy reserve. Daily requirement of iron of pregnant women are three times as compared to the need of nonpregnant women and total requirement of iron during pregnancy is about 1000 mg. Though food based strategies are important for raising the

iron status of population they will not be enough for many iron deficient women who become pregnant. Hence there is a higher requirement of iron during adolescent period and reproductive age and during lactation. In developing countries 24 to 30 percent women have no iron reserve. As poor iron store before conception is one of the cause of IDA during pregnancy, there is need to raise iron stores of women (adolescents) before they become pregnant. Iron loss during menstruation is estimated to be 16 mg/kg/day and basal requirement during this period in both male and females estimated to be 44 mg/kg/day or total of 3 mg/kg/day in female[97] and hence need for supplementation of iron in adolescent girls (10–18 years) 100 mg of elemental iron daily to all adolescent girls every year for 100 days. Addition of 25 mg of vitamin C to iron folate tablet has shown a higher Hb response as compared to iron folate tablet alone in adolescent girls.[34] The most common form of iron in iron tablet used are ferrous sulfate (20% iron) fumerate (33% iron) and gluconate (12% iron).

Iron Supplementation

- In breastfed term infants 1 mg/kg/ day of oral iron in single dose starting from 5th month of life will prevent IDA at later age.
- In preterm and LBW babies one may give 2 to 3 mg/kg/day of oral iron and start it early, i.e. by 2nd and 3rd months of age.
- Iron 'tonic' to infants is more important than multi-vitamin drops!
- Iron supplementation can also be given to pregnant women, school children, and other high-risk groups.
- *Community level:* Fortification of staple foods like cereals, grains, sugar or salt will be effective.Vitamin C or meat increase the iron absorption. Salt fortification gives an iron content of 1 mg/g of salt in preparation. Common[112] salt fortified with iron orthophosphate and sodium hydrogen sulphate with ascorbic acid has been found stable and effective in field trials in India.
- Similarly for infants fortification of formula feeds and cereals have been successful in developed country.
- Fortification of foods with iron constitute the most desirable, cost effective and sustainable methods of preventing iron deficiency and is a long-term measure for improving the iron status of the entire population.
- Fortify a staple food that is consumed in significant quantity regularly by most people. Widely consumed condiment—salt, sugar, fish sauce, curry powder[113] and have all been successfully fortified with iron. In South America both dried and liquid milk and milk products like yogurt, fortified infant food have been fortified with iron. Ferrous fumerate, ferrous gluconate, lactate and ferrous sulfate, ferric orthophosphate, sodium acid sulfate, orthophosphoric acid have been extensively used for the fortification of wheat flour, bread and other bakery products, corn-soya-milk preparation (CSM) salt, sugar, fish sauce, rice, etc. Iron salt EDTA (ethylene diamine tetra-acetate)[112-117] has been successfully used to fortify sugar/wheat flour in Guatemala[115] (13 mg of iron/100 mg sugar). A formula for double fortified salt, salt fortified with iodine and iron has been developed and has been found effective.[112]

Home Fortification with Sprinkles[112-123]

The idea of sprinkles was formulated in 1996, when a group of consultants determined that the prevention of childhood. IDA was a United Nations Children's Fund priority, yet available interventions (syrups and drops) were not effective—single-dose sachets containing micronutrients in a powdered form, which are easily sprinkled onto any foods prepared in the household. This would be a successful method to deliver iron and other micronutrients to children at risk .

In sprinkles, the iron (ferrous fumarate) is encapsulated within a thin lipid layer (soya-based hydrogenated lipid layer) to prevent the iron from interacting with food. There are minimal changes to the taste, color, or texture of the food upon adding sprinkles. Other micronutrients including zinc, iodine, vitamins C, D, and A, and folic acid may be added to sprinkles sachets. Any homemade food can be fortified with the single-dose sachets, hence the term "home fortification". Two formulations have been developed, a nutritional anemia formulation and a complete micronutrient formulation.

Encapsulation prevents the micronutrients from oxidizing the food. No change in the color and taste, fixed dose sachet (15–45 mg).

Home fortification can be done by, parents, health care giver and can be given as Intermittent Fe therapy under supervision or can be integrated into existing health program. Sprinkles is a novel approach and this can be sprinkled on any complementary food at containing micronutrients in a powder form, which are easily sprinkled onto any foods prepared in the household. Sprinkles have been shown to be efficacious in the treatment of anemia in many developing countries.

- Periodic de worming should be considered in endemic areas.
- Administration of one pediatric (small) tablet containing 20 mg of iron and 100 ug of folic acid daily for 100 days every year has been recommended by National Nutritional Anemia Control Program.Various contact points like measles (9 months) and DPT booster (16–18 months) in ICDS scheme should be utilized for the distribution of iron folate.

Control of Viral, Bacterial and Parasitic Infections

Effective, timely curative care could diminish the adverse nutritional consequences of viral and bacterial disease. Although the number of infective episodes is unlikely to be reduced, the curative services reduce the duration and severity of infections, thereby improving iron status even if there is no increase in dietary iron consumption. Preschool children who have such would benefit from such improvements in health care. It is vital to educate families frequent infections about proper feeding practices during and after periods of infective illness, as the young children are often semistarved when ill , either because of their poor appetite because of illness and myth of parents about restrictions of diet during illness. Breastfeeding must be confined as it helps preventing infections apart from its direct effect on iron status. Along with encouragement for taking timely immunizations, primary health care measures like improvement in personal hygiene, environmental sanitation, provision of safe water go a long way in improving the nutritional status and indirectly iron status of the children. The main culprits in causing anemia due to chronic blood loss are parasites hookworm ankylostoma and necator and schitosoma. Heavy giardial infestation can reduce iron absorption. Although routine deworming is recommended in actual practice, it becomes costly, when advised to entire community and reinfections are common. As it does not improve hemoglobin [Hb] value significantly, addition of supplemental iron or food fortification with iron has better results for rise in (Hb) status even when deworming is not done. However, in individual cases with heavy infestation deworming and use of cheap footwear advised to prevent reinfestation.

CONCLUSION

Iron deficiency anemia is a public health problem of high magnitude. Iron deficiency is the most common malady known to mankind since ages, though iron is available in plenty in environment. In early fetal life and young children, iron deficiency could impair mental (intellectual potential) and motor development irreversibly. Low intake of iron rich food is a very important cause for developing anemia. Hemoglobin, MCV, RDW and serum ferritin estimation can identify most IDA cases correctly. The reasons for IDA are mainly faulty dietary habits especially during growth period. Exclusive breastfeeding and proper weaning thereafter 6 months age, and measures taken during last few decades like iron supplementation food fortification. Education have reduced IDA, atleast its severe forms. Iron supplementation in high risk group is another alternative. All it needs realization of magnitude of problem of IDA will and open eyes to prevent, detect and treat IDA in time!

Did you Know!

- Thirty percent of world population suffers from IDA. Out of which > 80 to 90 percent in developing countries.
- < 70 to 90 percent Indian children at 1 to 2 years of age, adolescents, pregnant women, lactating mothers suffer from IDA.
- Pica is a common symptom of IDA but can also be seen in lead poisoning.
- Always suspect IDA in patient with breath holding spasm.
- Exclusive breastfeeding protects a full term infant from IDA till 6 months of age.
- Preterms can develop IDA as early as 2 to 3 months.
- IDA early in life can reduce the intellectual potential and cognitive functions of the child permanently.
- Adding vitamin C (fruit juices) or meat can increase iron absorption by 3 to 4 folds.
- β-thalassemia is a common differential diagnosis of IDA. RDW helps in differentiation as it is normal in thalassemia minor and increased in IDA.
- Simple way to prove IDA is by iron therapeutic test.
- HbA2 levels can be low in patients with thalassemia minor if they have coexisting IDA. Repeat HbA2 after iron therapy and will show increased values.
- Cheapest and best form of iron is ferrous sulfate tablets.

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CHAPTER 15

Megaloblastic Anemia

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INTRODUCTION

Nutritional anemia is one problem that has haunted the developing world for ages now and still continues to do so. Nevertheless the modern "zero figure trends" and the resultant increase in the followers of vegan diets has lead to a surge in the number of anemia cases in the western world also. As science progressed, a better insight was gained into the causes, pathogenesis and molecular details of the biology of the various kinds of anemias consequent to which tremendous advances in the diagnostics and treatment of the same have been made. Much has been discussed and spoken about the iron deficiency anemia which was so far considered to be synonymous to nutritional anemia but as has been realized over the last few decades cobalamin and folic acid deficiency is an equally big problem challenging the practicing pediatricians and hematologist, if not more. This chapter intends to provide an overview of megaloblastic anemia in the pediatric population, both of the nutritional and other varieties with emphasis on clinical approach, laboratory diagnosis as well as treatment and preventive aspects of the disorder.

HISTORY[1-3]

In 1855, Thomas Addison at Guy's Hospital described a lethal, idiopathic anemia that in 1872 was given the name pernicious anemia by Biemer. For years it was believed that pernicious anemia was a result of the positive acting deleterious influence of an unknown infectious agent or biological product which caused increased destruction of the red blood cells. Ehrlich was amongst the first to describe megaloblastic bone marrow in 1891. In the 1920s two milestones discoveries were made that changed the way in which the medical fraternity treats anemia today. While Dr George Whipple (1), a pathologist along with his colleagues at the Rochester university firmly established the fact that properties of food affected blood formation, a concepted not previously accepted, George Minot and William Murphy (2) at Harvard Medical School developed a special diet that could reverse the pathology of pernicious anemia and cure patients. These milestone discoveries lead another prominent investigator of the era WB Castle (3) to put in decades of investigation into uncovering the complex pathophysiological mechanisms underlying pernicious anemia.

Pernicious anemia is another example where an effective treatment was found before an understanding of the underlying pathogenic processes was found. Even before the therapy for megaloblastic anemia was described in 1926, it was known that marrow morphology could return to normal with reticulocytosis and correction of anemia even in a previously documented megaloblastoid marrow. The observation that despite feeding the same liver extracts to various subjects the response to the pernicious anemia was variable lead Castle to think that some "intrinsic factor" must also influence the absorption of the yet not discovered deficient nutrient and that it could be corrected by feeding beef stimulated gastric juice from healthy patients along with the liver extracts. Later on the fact that pernicious anemia was caused by inability of the stomach to absorb vitamin B_{12} because of atrophy of its mucosal lining was realized. A major clinical leap was when it was acknowledged that cobalamin and folic acid deficiencies could manifest as neurological symptoms like peripheral neuropathies, SCDSC and NTDs in the unborn child of a folic acid mother. With advances in the science, details of the absorption and transport of these compounds in the body, their role in the DNA synthesis

and other single carbon transfer reactions in the body have been elucidated and this has also allowed clinicians to arrive at diagnoses in conditions with megaloblastosis where the nutrition seems to be adequate. Elevated levels of methyl malonic acid and homocysteine levels could be used as a means of diagnosing subclinical deficiencies of vitamin B_{12} and folic acid, that these two correlated with the incidence arteriosclerotic diseases, bowel and other cancers and that correcting these levels could decrease the incidence of such events was also an outcome of elaborate insights into the metabolism and physiological role of the concerned nutrients.

DEFINITIONS

Megaloblastic anemia is used to describe a group of disorders characterized by a distinct morphological pattern in the hematopoietic cells most often macrocytosis of the red blood cells often accompanied by leukopenia and thrombocytopenia. The prominent feature is a defective DNA synthesis with minimally altered RNA synthesis and protein synthesis leading to a state of unbalanced cell growth and impaired cell division. These cells in a vain attempt to divide increase their DNA contents by two to four times and often get arrested in the S phase of the cell cycle. A larger than normal immature nuclei with finely particulate chromatin afloat in the mature cytoplasm of a large cells having unimpaired RNA and protein synthesis is the hallmark of megaloblastosis. Thus in a nutshell, megaloblastosis results in a cell whose nuclear maturation is arrested while its cytoplasmic maturation proceeds normally independent of the nuclear events. Although megaloblastic hematopoiesis commonly manifests as anemia, it reflects a global defect in DNA synthesis which affects all the proliferating cells of the body.

CAUSES OF MEGALOBLASTIC ANEMIA (TABLE 1)

Although cobalamin and folate deficiency is amongst the major causes of megaloblastic anemia, it is not the sole

Table 1 Causes of megaloblastic anemia*[4]

Cobalamin deficiency	*Folate inhibitors: Antifolates (methotrexate, pyrimethamine, sulfones, trimethoprime)*
Nutritional cobalamin deficiency: Vegetarians, breastfed infants of mothers with pernicious anemia.	*Hereditary folate malabsorption* (PCFT mutations)
Abnormal intragastric events: Atrophic gastritis, hypochlorhydria, PPI, H_2 blockers	*Damage to the ileal mucosa*: Tropical and nontropical sprue, regional enteritis, infiltrative disorders of the small bowel (lymphoma)
Loss/atrophy of gastric oxyntic cells: Total or partial gastrectomy, pernicious anemia, caustic damage	*Defective CSF transport*
Insufficient pancreatic secretions: ZE syndrome	*Inherited disorders of folate utilization*: Methylenetetrahydrofolate reductase deficiency, methionine synthase deficiency[18,19]
Usurping of luminal cobalamin: By bacterial overgrowth in cases of blind loop syndrome, diverticulosis; infection by *D latum*	**Other causes**
Disorders of ileal mucosa/Cobalamin-IF receptors: Ileal bypass, nontropical sprue, Crohn's disease *CUBAM receptor defects: Imerslund-Gräsbeck* syndrome *Drug effects:* Metformin, neomycin	*Defects in purine and pyrimidine synthesis* *Orotic aciduria* *Myelodysplasia and leukemia, HIV* *Drug-induced*
Congenital TCII deficiency	*Thiamine responsive megaloblast anemia*
Metabolic disorders (cells not able to use vit B_{12}): CblA to CblG disorders, nitrous oxide intoxication	*Scurvy*
	Pyridoxine responsive anemia
Folate deficiency	
Decreased dietary intake: Poverty, psychiatric illnesses, maternal deficiency affecting fetus or infant, prolonged feeding of goat's milk	
Increased requirements: Pregnancy, lactation, hemolysis, hyperthyroidism, anticonvulsant therapy, Lesch-Nyhan syndrome, prematurity, homocystinuria, psoriasis	

*modified from ref no. 4

reason for a megaloblastic picture. Other conditions are also enumerated in the Table 1.

COBALAMIN (VITAMIN B_{12})

Chemistry:[5] Cobalamin, a member of the corrin family has a structure described below in the Figure 1. A planar corrin ring similar to heme, coordinates with a central cobalt atom, with a 5, 6 dimethylbenzimidazole and an upper axial ligand which varies in different biologic and pharmacological cobalamins like cyano-, methyl-, hydroxyl-, 5'deoxyadenosylcobalamines. These axial ligands are attached when the central Co is in its most oxidized state, the cob(III) state. It can also exist in the cob(II) and cob(I) states. These various forms confer a distinct identity to the cobalamin while it participates in the various one carbon metabolism reactions.

Nutrition:[6-8] Cobalamin in the nature is produced only by microorganisms and humans receive it solely from diet. Certain bacteria and fungi produce this vitamin in excess and form the major resources of cobalamin for commercial purposes and therapy. Herbivores (and humans) obtain their cobalamin from plants contaminated with cobalamin-producing soil bacteria found in the roots of legumes. For all practical purposes, there is no uncontaminated plant source that could be a source of vitamin B_{12}. Nevertheless the colon of some individuals contains cobalamin producing bacteria like Klebsiella pneumoniae, which can be absorbed.

Animal protein especially parenchymal meat is a major dietary source of vitamin B_{12} for non-vegetarians (mcg/100 gm dry wt). Milk and milk products and eggs contain 1 to 10 mcg/100 gm dry weight. While an average non-vegetarian diet contains 5–7 mcg/day of cobalamin, the average vegetarian consumes only 0.25–0.5 mcg/day (Table 2).

Although heat does not influence the stability of cobalamin, ascorbic acid readily changes the active forms of cobalamin into inactive analogs. With the liver storing 1 mg of the total 4–5 mg of the adult stores and an obligatory loss of 0.1 percent/day, it takes about 3–4 years to deplete the stores even if the dietary cobalamin is abruptly withdrawn. However a deficiency would take longer to set in due to an efficient enterohepatic circulation which accounts for a turn over of 5–10 mcg/day of cobalamin.

Table 2 Recommended daily allowance of vit B_{12}

	Recommended daily allowance (mcg)
Men	2.4
Nonpregnant women	2.4
Pregnant and lactating women	2.6
Children 9–18 years	1.5–2

ABSORPTION AND TRANSPORT OF VITAMIN B_{12}

Cobalamin in the food is in the coenzyme form and nonspecifically bound to protein. On reaching the stomach, the low pH causes proteolysis and releases the cobalamin which now preferentially binds to a high affinity (>intrinsic factor) 150 kda cobalamin-binding protein, R protein (a haptocorrin) from gastric juice and saliva. The cobalamin-R protein (holo R protein) complex along with IF.

Passes into the duodenum where the pancreatic juices cleave the cobalamin from the complex. However the IF does not undergo proteolysis and the cobalamin is now transferred to this 45kDa glycoprotein secreted by the oxyntic cells in the fundus and cardia of the stomach in response to food ingestion by membrane associated vesicular transport stimulated by vagal and hormonal signals. IF is produced in an amount far access of that required for absorption and only 2–4 mL of normal gastric juice can correct cobalamin deficiency in adults lacking IF. While R binder binds both active cobalamin and its inactive analogs IF binds only the active forms. This property is used in order to excrete the inactive analogs secreted in the biliary secretions which are excreted along with R protein while the active form attaches to IF and is reabsorbed thus providing an efficient system of enterohepatic circulation of cobalamin.

This stable cobalamin-IF complex now passes into the jejunum and into the ileum where the IF through its receptor binding site attaches to the receptors present on the microvilli of the ileum. The functional IL cobalamin receptors are composed of two proteins collectively known as CUBAM-cubulin and amnionless.[8] The cubulin is the larger extracellular portion of this complex which is anchored to the membrane by the smaller amnionless.[10] These are very specific for the IF-cobalamin complex and do not bind any of the components when presented singly or in combination with the R protein. The human ileum contains cubam receptors to bind 1 mg of IF bound cobalamin. Once internalized into the enterocyte the cubam is recycled back to the surface. The cobalamin is now released from the IF in the lysosome and is attached to the transcobalamin(II) either within the eneterocyte itself or at the basal surface of the ileal enterocyte, while the IF is degraded. Holo-TC appears in the portal circulation in about 3–5 hours and reaches peak levels in about 8 hours.

Cobalamin when given in large doses can diffuse passively through buccal, gastric and jejunal mucosa and less than 1 percent of such orally administered drug

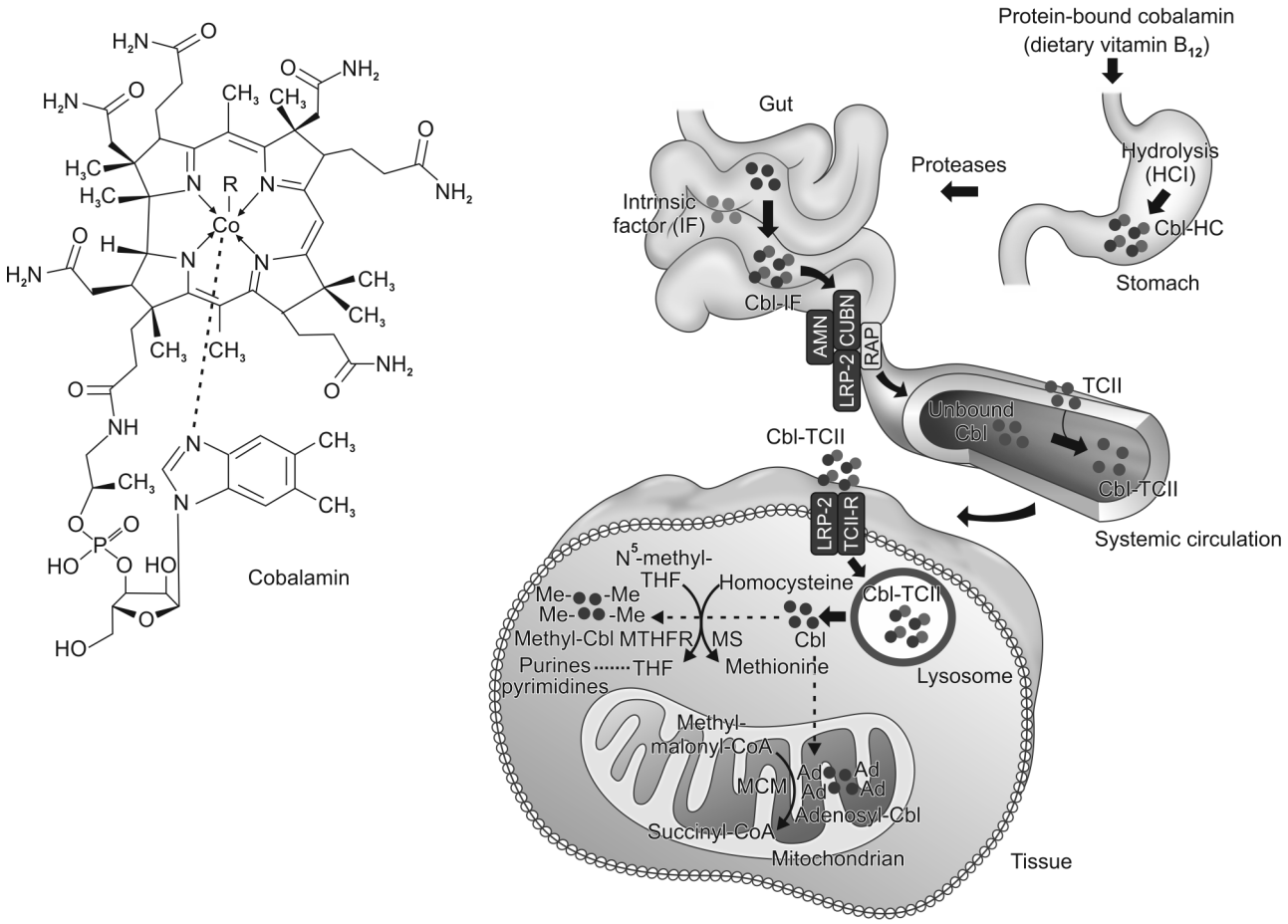


Fig. 1 Absorption and transport of cobalamin (Image[9] Adopted from: Dr Joseph Mercola, Alexa Natural Health Website)

then appears in the circulation within few minutes. This property has been utilized to treat patients with impaired absorption with oral supplements instead of parenteral therapy (Fig. 1).

In the blood the cobalamin is bound to three types of proteins. Transcobalamin (TC) I, II and III.[11,12] >90 percent of recently absorbed cobalamin is bound to TCII which is the specific transport proteins that delivers this important nutrient to the tissues. The TCII-cobalamin complex is rapidly cleared from the circulation in less than an hour as it binds to the various cells with receptors for this complex and is internalized. Major circulating form which is the methyl form is never found free to in the plasma. The major (70%) circulating form found in the plasma is that bound to TCI which binds both active and inactive forms and is largely considered to be a plasma-storage protein for cobalamin. TCIII which is a asialoglycoprotein binds all forms of cobalamin analogs with high affinity and within minutes delivers them to the liver through the asialoglycoprotein receptors present on the surface of hepatic cells and from there into the bile for fecal excretion.

CELLULAR PROCESSING

The TCII-cobalamain complex is internalized[13,14] via conventional receptor mediated endocytosis and within the lysosome, at the low pH the TC is cleaved off the cobalamin and the cobalamin is transported into the cytosol. Here it can have two fates. It either goes to the mitochondria to participate in reactions involving methyl malonyl-CoA or stays in the cytosol to be a part of the methionine synthase complex (Fig. 2).

In the mitochondria, cob(I) alamin is converted to its coenzyme form adenosyl cobalamin which along with methylmalonyl-CoA mutase mediates transfer of a -CH moiety to convert methylmalonyl CoA to succinyl-CoA which can now take part in the Kreb's TCA cycle and help generate ATP.[14-17]

In the cytosol, cobalamin in its methylcobalamin form acts as a coenzyme along with methionine synthase, a complex enzyme requiring both folates and cobalamin for carrying out one carbon metabolism reactions. First a methyl group is transferred from 5-methyl-tetra-

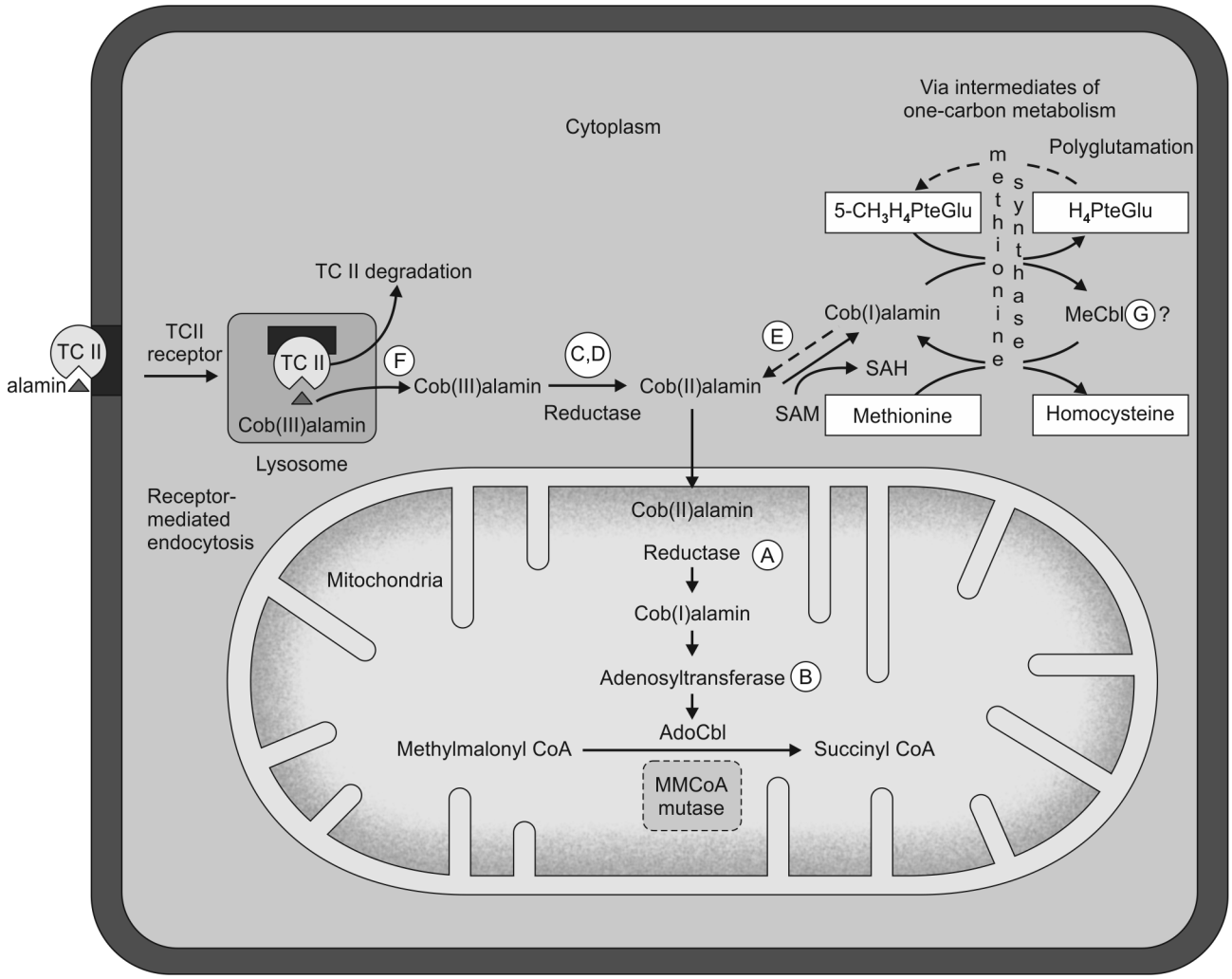


Fig. 2 Intracellular metabolism of cobalamin

hydrofolate to methionine synthase–bound cob(I) alamin to form methylcobalamin, followed by transfer of this methyl group to homocysteine to form methionine and regeneration of cob(I) alamin.[14-17]

During these reactions sometimes the cob(I) alamin is spontaneously converted to the inactive form cob(II) alamine which needs to be reduced back to the active form cob(I) alamin before it can accept and transfer a methyl group. This is carried out by the enzyme methionine synthase reductase with the help of NADPH and SAM. This enzyme is defective in people with cbIE mutations.

Intracellular reactions involving cobalamin: In vivo substitutions include the replacement of hydroxocobalamin or cyanocobalamin by a 5′-deoxyadenosyl group attached by a covalent bond, giving rise to adenosylcobalamin (AdoCbl). Methylcobalamin (MeCbl) is the main form in plasma. In vivo, 5-methyl-tetrahydrofolate readily donates its methyl group to cob(I) alamin in a reaction involving methionine synthase to form methylcobalamin. The approximate loci for defects in cobalamin mutants, cblA to cblG, are shown. *MMCoA mutase,* methylmalonyl-CoA mutase; *SAH, S*-adenosylhomocysteine; *SAM, S*- adenosylmethionine (Fig. 2).[15]

DEVELOPMENT OF COBALAMIN DEFICIENCY

Nutritional deficiency: People following vegetarian diets can be either pure vegans who exclude all animal products from their diets and need to be routinely supplemented with cobalamin or they could be those following lactovegetarian diets or lacto-ovo vegetarian diets which incorporates milk and egg products into their food also need external supplementation, even if they are asymptomatic as they are too likely to suffer from subclinical deficiencies.

Although severe cobalamin deficiency can often lead to sterility, adverse pregnancy outcomes like preterm labor, intrauterine growth retardation, neural tube defects and recurrent miscarriages are also frequent manifestations of undetected cobalamin deficiency in the potential young mother.

There is a critical period in prenatal and early postnatal neurodevelopment when sufficient folate and cobalamin is required for the proper formation of neurologic circuits. Any perturbation of neurodevelopment during this period can give rise to subtle changes that can manifest in behavioral abnormalities long after the folate and cobalamin deficiency is reversed.

Cobalamin deficient mothers with low serum cobalamin levels are not able to provide enough vit B_{12} stores to their fetuses at birth neither are they able to compensate for it when they breastfeed their child exclusively since their breast milk cobalamin levels are also found to be equally low, often below 362 pmol/L. In India the duration of exclusive breastfeeding is longer than the western world and hence the relevance of supplementing the mother with B_{12} and folic acid supplements in order to prevent deficiency in the infant cannot be emphasized less. The maternal stores can exert a strong effect on the vit B_{12} stores of the infant for around 12 months.

Children of mothers who have been on macrobiotic diets (nearly vegan with occasional serving of animal protein in form of fish) are also at risk not only because the cobalamin deficient mother is not able to provide the child with enough cobalamin stores during her pregnancy and lactation but she also tends to feed her child according to the principles of macrobiotic diet. Elevated urinary methylmalonic levels were found in 15 to 16 percent of breastfed infants of vegetarian mothers who consumed macrobiotic diets.[18,19]

Cobalamin deficient infants have also been found to be born to mothers who are on apparently balanced nonvegetarian diets highlighting the fact that pregnancy places an additional stress on the mothers cobalamin stores which needs to be corrected. This can negatively affect their breastfed infants' cobalamin status at 6 weeks; indeed, over two-thirds of Norwegian infants of otherwise healthy mothers had a metabolic profile consistent with cobalamin deficiency, which reverted to normal after cobalamin replenishment. This emphasizes that many more breastfed infants may need cobalamin supplements early in life than previously realized. Such studies raise new questions as to whether the optimal intake of cobalamin in women should be much higher than 2.4 mcg/day, and be raised to 4–7 mcg/day.

The prevalence of cobalamin deficiency among older children and adolescents is also high, ranging from 40 percent to 80 percent in various communities, because of consuming monotonous diets low in animal-source foods prepared by unaware parents or self-imposed by the adolescent to keep up with the so called "healthy vegan diet" trends and food fads. Low serum cobalamin levels have also been found in adolescents infected with HIV without having the frank immunodeficiency syndrome. Treatment with HAART resulted in improved status in these candidates.

Pernicious anemia:[20-23] This lymphocyte mediate destruction of the oxyntic cells of the stomach gives rise to deficiency of the IF and constitutes one of the most important causes of cobalamin deficiency in the adult population. Autoantibodies are directed against the H^+, K^+ ATPase of the parietal cells. Patients who lack IF after gastric resection and those with genetic mutations yielding defective or undetectable IF are not considered to have pernicious anemia.

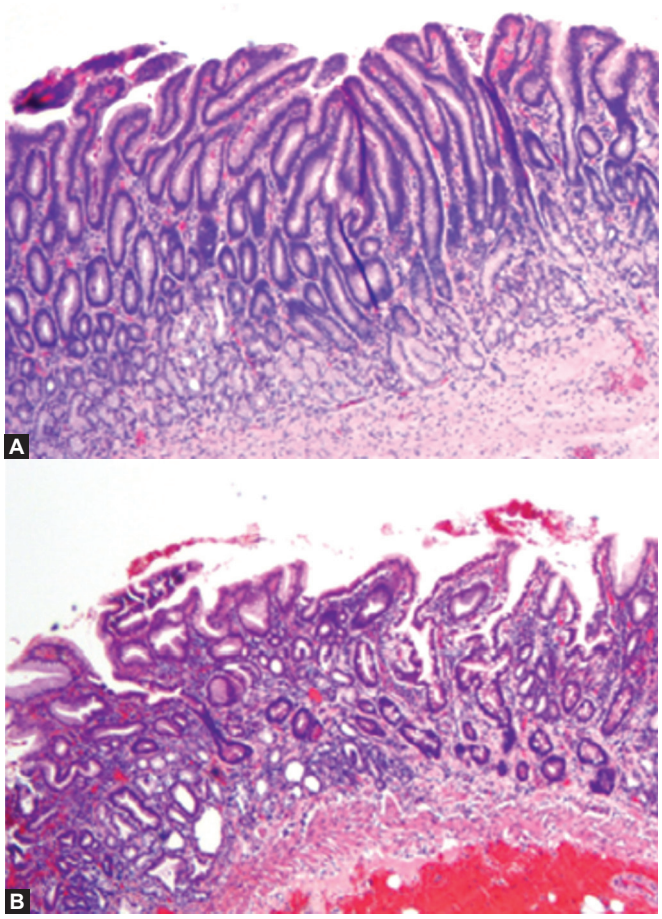
The annual incidence of pernicious anemia is approximately 25 new cases per 100,000 persons older than 40 years. Although the average age of onset is about 60 years, pernicious anemia dose not avidly comply with the boundaries of age, race, or ethnic origin. Although a genetic basis that predisposes one to develop pernicious anemia has long been suspected, but neither the mode of inheritance nor the initiating events or primary mechanism is precisely understood. There is a positive family history for about 30 percent of patients, among whom the risk for familial pernicious anemia is 20 times as high as in the general population.

Other autoimmune diseases, including Grave's disease (30%), Hashimoto thyroiditis (11%), vitiligo (8%), Addison disease, idiopathic hypoparathyroidism, primary ovarian failure, myasthenia gravis, type 1 diabetes mellitus, and adult hypogammaglobulinemia have been found to have significant associations with pernicious anemia.

Histologic features of stomach in pernicious anemia compared to normal: The normal gastric mucosa (A) is contrasted to that seen in pernicious anemia (B), in which there is atrophy of gastric glands, intestinal metaplasia with goblet cells, and loss of parietal cells (not visible at this magnification) (Figs 3A and B).

Abnormal events in the small bowel: Insufficient or inactivated pancreatic proteases as occurs in the Zollinger-Ellison syndrome, fail to cleave the cobalamin from the R binder to allow it to bind to IF in order to get absorbed through the ileal receptors which are highly specific for the cobalamin-IF complex.

Bacterial overgrowth in the intestine as occurs during bowel stasis in conditions like blind loop syndrome, leads to usurpation of the cobalamin from the intestinal lumen making less it available for absorption. This type of deficiency has been corrected with a 7–10 days antibiotic course which sterilizes the gut.



Figs 3A and B (A) Histologic features of stomach in normal mucosa; (B) Pernicious anemia[24]

Infestation by the fish tapeworm, *Diphyllobothrium latum,* which avidly usurps cobalamin for growth, affects around 3 percent of the population and such people can develop frank cobalamin deficiency. Plerocercoids of this parasite reach the human intestine when they consume partially cooked or raw fish containing, where they develop into adult worms in the jejunum in about 6 weeks, growing to a length of 10 m, with up to 4000 proglottids; when these worms lay eggs, the life cycle is repeated. Stool examination showing the ova can give a diagnosis. This followed by praziquantel (10–20 mg/kg as a single dose taken orally) which leads to expulsion of the worms and cobalamin replenishment is curative.

Disorders of ileal IF-cobalamin receptors or mucosa: Resection of only 1–2 feet of the distal ileum which has the maximum density of the concerned receptors can cause clinically significant cobalamin deficiency by reducing the number of interactions between the IF-cobalamin complex and its respective receptor.

Imerslund-Gräsbeck syndrome[25-28] is an autosomal recessive disorder in children arising from biallelic mutations (in 80% of cases) involving either the cubulin (CUBN) or amnionless (AMN) genes that constitute the functional IF-cobalamin receptor (i.e., cubam) resulting in selective cobalamin malabsorption. Presentation is commonly between ages of 3–10 years with hematological and neurological manifestations with low serum cobalamin. Consequent to the role of cubam in the renal tubular absorption of several other proteins, a persistent but benign proteinuria is also found in Imerslund-Gräsbeck syndrome. Mutational analysis of gastric IF, CUBN and AMN genes can give the diagnoses.

Nitrous oxide exposure: Nitrous oxide (N_2O) by inactivating coenzyme forms of cobalamin by oxidizing the fully reduced cob(I) alamin to cob(III) alamin causes a state of functional intracellular cobalamin deficiency. First identified in patients with tetanus who were given nitrous oxide for up to 6 days, similar manifestations were also found in persons exposed to nitrous oxide for open heart surgery and through chronic exposure either accidental, or occupational. These groups are considered to be at high risk for developing megaloblastosis and cobalamin-deficient neuromyelopathy. Megaloblastosis develops within 24 hours and lasts less than 1 week after a single exposure.

Inborn Errors of Cobalamin Metabolism[20,21]

Inborn errors of cellular cobalamin metabolism can affect synthesis of AdoCbl, synthesis of MeCbl, or synthesis of both cobalamin coenzymes, depending on which step in metabolism is affected. Essentially, defects that affect AdoCbl synthesis or directly affect methylmalonyl-CoA mutase result in isolated methylmalonic acidemia and aciduria; defects affecting synthesis of MeCbl result in hyperhomocysteinemia and homocystinuria; and defects resulting in deficiency of both cobalamin coenzymes results in combined methylmalonic aciduria and homocysteine.

cblA: The cblA disorder is caused by mutations in the MMAA gene on chromosome 4q31.1–q31.2. It plays a role in transfer of AdoCbl from cobalamin adenosyltransferase to methylmalonylCoA mutase and in stabilization of mutase-bound AdoCbl.

cblB: Caused by mutations in the MMAB gene on chromosome 12q24 encoding cobalamin adenosyltransferase, it result in decreased synthesis of AdoCbl and therefore decreased activity of the AdoCbl dependent enzyme methylmalonyl CoA mutase.

Patients with the MUT disorder, caused by mutations in the gene encoding methylmalonyl-CoA mutase itself also have a similar clinical presentation although the synthesis of AdoCbl is normal.

When compared to serum and urine MMA levels in dietary cobalamin deficiency the levels here are very high.

Patients usually present in the first year of life, but late and benign presentations along with silent carrier states of these mutations are also known. Lethargy, failure to thrive, hypotonia, recurrent vomiting and dehydration constitute common presenting symptoms. Any stress including infections and even dietary changes can precipitate fatal metabolic acidotic crises.

cblC: This disorder with more than 500 documented cases is the most common inborn error of cobalamin metabolism is the most common inborn error of cobalamin metabolism. Around 70 different types of mutations affecting the MMACHC gene, which encodes a protein that apparently plays a role as a cobalamin chaperone and in removal of the upper axial ligand of exogenous cobalamins are likely to be the cause of this disorder. It is found that there is a problem not with the uptake but with the retention of cobalamin in the fibroblasts from cblC patients, perhaps because it does not become associated with cobalamin-binding enzymes.

There is decreased synthesis of both AdoCbl and MeCbl, and decreased activity of both cobalamin-dependent enzymes.

cblD: This disorder is caused by mutations in the MMADHC gene, which encodes a gene of unknown function. Affection of the N-terminal domain of the MMADHC protein resulted in variant 2, while mutations affecting its C terminal domain caused variant 1. Although the exact defect is still to be found, it is suggested that the MMADHC protein plays a role in partitioning of cobalamin between the mitochondrial (methylmalonylCoA mutase) and cytoplasmic (methionine synthase) compartments.

cblE: Mutations in the MTRR gene have been identified in cblE patient's locus on chromosome 5p15.3–p15.2, which encodes methionine synthase. Both the cblE and cblG disorders show rapid improvement in biochemical and neurological parameters when treated with intramuscular OHCbl while such is not the case with most cblB patients which respond poorly.

cblF: This disorder is the result of mutations in the LMBRD1 gene on chromosome 6q13, which encodes a lysosomal membrane protein containing 9 transmembrane domains which leads to inability to transfer cobalamin freed from transcobalamin in the lysosome across the lysosomal membrane into the cytoplasm. Cells from cblF patients accumulate large amounts of free cobalamin within lysosomes, but there is a deficiency of both cobalamin coenzyme derivatives and decreased activity of methylmalonylCoA mutase and methionine synthase.

cblG: Mutations at the MTR locus have been identified in cblG patients.

All the above disorders can present in various combinations as well.

Treatment of these disorders involves protein restriction, or feeding with formula deficient in valine, isoleucine, methionine and threonine, to limit the levels of the amino acids that are the major source of methylmalonylCoA within cells. Supplementation with OHCbl or CNCbl may be useful in cblA patients as well as some MUT and cblB patients. Therapy with carnitine has been advocated, as has treatment with lincomycin and metronidazole to reduce generation of propionate (the precursor to methylmalonylCoA) by gut bacteria. Even with treatment, outcomes may be poor.

FOLATES

Chemistry:[30,31] More than 100 compounds are known which are together known as folates. Folic acid (pteroylmonoglutamate [PteGlu]) is the commercially available parent compound. PteGlu consists of three basic components: a pteridine derivative, a *p*-aminobenzoic acid residue, and an L-glutamic acid residue. This must be first reduced at positions 7 and 8 to dihydrofolic acid (H2PteGlu) and then to 5, 6, 7, 8-tetrahydrofolic acid (THF; H4PteGlu), and one to six additional glutamic acid residues must then be added by means of γ-peptide bonds to the l-glutamate moiety (subscripted *n* in PteGlun denotes polyglutamation) before it can play its part as a coenzyme (Fig. 4). The major role of folate coenzymes is in donation or acceptance of one-carbon units in numerous reactions in amino acid and nucleotide metabolism.

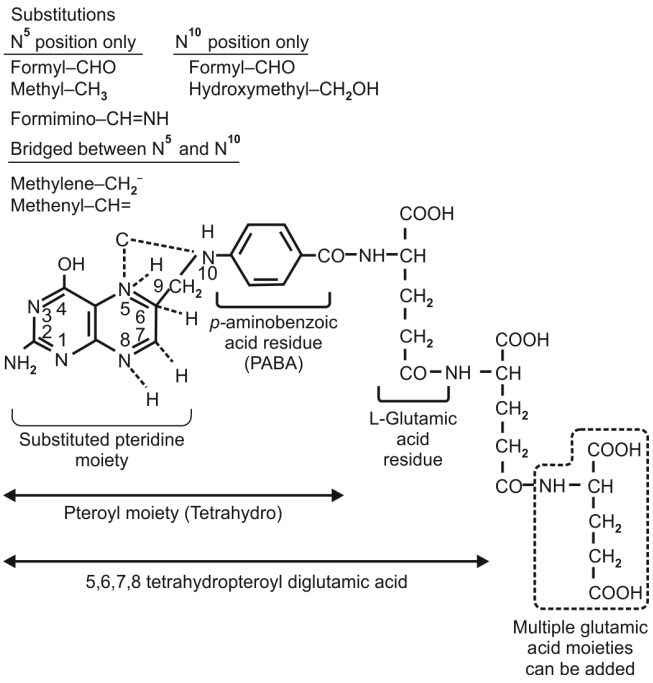


Fig. 4 Folate structure[29]

Various substitutions in H4PteGlun occur at positions 5 or 10, or both; position 5 can be substituted by methyl (CH_3), formyl (CHO), or formimino (CHNH), and position 10 can be substituted by formyl or hydroxymethyl (CH_2OH). Positions 5 and 10 can be bridged by methylene (–CH_2–) or methenyl (–CH=).

Nutrition: Vitamin B_9 or folates are synthesized by microorganisms and plants, including leafy vegetables (spinach, lettuce, broccoli), beans, fruits (bananas, melons, lemons), yeast, and mushrooms, and are also found in animal meats.

Bioavailability of folates from various sources varies greatly mostly as result of the following factors.

1. *Food stability:* The labile and susceptible reduced natural folate is damaged by oxidative cleavage by nitrates or light exposure and prolonged cooking/boiling for over 30 minutes which can reduce the bioavailabilty by 50–80 percent. However folic acid is much more stable. Similarly refrigeration of leafy foods exposed to artificial fluorescent light in supermarkets doubles the folate content and ascorbate when consumed along with folates increases the bioavailability.
2. Pureed foods allow easier access to the glutamate carboxypeptidase II (also known as folate-polyglutamate hydrolase), which converts folate polyglutamates to simpler folate monoglutamates before absorption; any perturbation of this enzyme by organic acids (orange juice), sulfasalazine, or ethanol can preclude absorption; conversely, folate-binding proteins in human or cow's milk can increase folate absorption for infants and women.
3. Interference with jejunal absorption of folaes due to intestinal disease.
4. Drugs that interfere with the proton-coupled folate transporter (PCFT) can also compromise folate absorption.

The recommended daily allowances of folate are as follows (Table 3):

Table 3 Recommended daily allowance of folate

	Recommended daily allowance
Adult men and nonpregnant women	400 mcg
Pregnant women	600 mcg
Lactating women	500 mcg
Children 9-18 years	400 mcg
1-6 years	3.3 mcg/kg/day
Infants	3.6 mcg/kg/day

ABSORPTION AND TRANSPORT

First the dietary folate which is mainly in the polyglutamate form is converted to folate monoglutamate form by γ-glutamyl hydrolase at the enterocyte brush border.[31,32] This is followed by their transport through the duodenal and jejunal brush border by high-affinity membrane-associated, luminal surface–facing proton-coupled folate transporters (PCFT). At pH 5.5, they act most efficiently and have equivalent affinity for transport of both physiologic reduced folates and folic acid, but at pH 6.5, reduced 5-methyl-tetrahydrofolate is transported more efficiently. PCFT being a folate-hydrogen symporter results in a net translocation of positive charge along with every folate molecule transported.

Within the enterocyte, after reduction to tetrahydrofolate and methylated before release into plasma as 5-methyl-tetrahydrofolate the efflux from the basolateral membarane into the portal blood being aided by the multidrug resistance–associated protein 3 (MRP3). These proteins with a low affinity but high capacity can be best described to function as cellular "sump pumps" that eject excess folates as well as antifolates out of cells. With the help of MRP2, which mediates folate transport into the bile, an efficient enterohepatic circulation is maintained thereby allowing the body to retain folates.

Less than 5 percent of average folate requirement can be derived from that produced by the intestinal bacteria by absorption across the large intestine. However, this fraction is largely used up by the colonocytes themselves for purpose of nutrition.

At high pharmacological concentrations passive diffusion of folic acid is probably the primary mechanism of intestinal mucosal folate. Peak folate levels in plasma are achieved 1 to 2 hours after oral administration.

Unlike cobalamin, folates are not privileged with a specific serum transport protein which enhances their cellular uptake. In the plasma, one-third of the folate is free, two-thirds is nonspecifically bound to serum proteins, and a very small fraction binds high-affinity, hydrophilic 40-kDa folate-binding proteins, which are structurally related to hydrophobic (native) folate receptors. Specialized, high-affinity, glycosyl-phosphatidylinositol-anchored (membrane) folate receptor-α, which takes up these folates at physiologic concentrations found in serum and transfer into the intracellular compartment of proliferating cells. The folate–folate receptor complex is then endocytosed. Acidification of the perinuclear endosomal compartment to pH 6 cleaves the folate from folate receptor, thereby releasing the folate to pass across the acidified endosome into the cytoplasm by a trans-endosomal pH gradient, mediated most often by the PCFT.[33,34]

Folate receptor-α mediates the cellular uptake of folates in proliferating normal and malignant cells besides their transport across the placenta to the fetus, into the brain, and in renal conservation of folates. While β receptors are expressed mostly in monocytes and macrophages, α receptors are found in abundance in many kinds of malignant cells and these are now being explored as potential diagnostic and therapeutic targets for certain malignancies.

Placental Transport[35]

Fetal and newborn blood folate is invariably more elevated than maternal blood folate which is proof enough for existence of a placental mechanism for preferential maternal-to-fetal folate transport. Transfer receptors are abundant and polarized to the maternal-facing microvillous membrane of the syncytiotrophoblast wherein they become the first to bind maternal folate at physiologic concentrations and pH. For physiologic transplacental folate transport a continued provision of adequate dietary folate intake by the mother followed by capture of maternal folate by placental folate receptors is essential. This results in an intervillous blood concentration that is three times that of maternal blood and subsequent concentration gradient based transfer of the folate into the fetal circulation. Inadequate intake of folate by the mother thereby leads to reduction in maternal-to-fetal folate transfer which in turn predisposes the embryo/fetus to very serious developmental defects.

Folate Receptors in Embryonic and Fetal Development[36]

Folate receptors-α are among the earliest genes activated in embryonic stem cells coinciding with the period when there is the need for increased folate requirements to support DNA synthesis during bursts of intense cell proliferation of initial phases of organogenesis. They are abundantly expressed in early stage neural tube cells and neural crest cells and their experimental perturbation can lead to profound abnormalities in neural tube closure and in heart, facial, and eye development. A striking human correlate of such experimental studies is the significant increase in blocking autoantibodies against placental folate receptor-α seen in women with pregnancy complicated by neural tube defects.

Cerebral Folate Transport Across the Choroid Plexus

As has already been mentioned folate receptor-α and PCFT are found in the basolateral membranes of the choroid plexus. To maintain the normal cerebrospinal fluid–to blood-folate ratio in healthy humans of 3:1 the folate first binds to the folate receptor-α in the choroid plexus, and is transported into the CSF with the assistance of the PCFT.

A syndrome of severe developmental regression in early childhood associated with movement disturbances, epilepsy, and leukodystrophy that is reversed by folinic acid occurs due to cerebral folate deficiency consequent to a mutation in folate receptor-α, which perturbs folate transport into the cerebrospinal fluid.

Similarly antifolate receptor-α antibodies that can prevent uptake of folate into the cerebrospinal fluid, lead to either infantile acute cerebral folate deficiency or one of two autism spectrum disorders (Rett syndrome and infantile low-functioning autism with neurologic abnormalities). High doses of oral folinic acid can lead to partial or complete clinical recovery in 12 months by normalizing CSF folate levels.

The role of PCFT in transport of folates across the choroid plexus is supported by the fact that mutations in PCFT result in hereditary folate malabsorption with low to undetectable cerebrospinal fluid folate values.

Renal Retention of Folates (Cobalamin)

Once the folate reaches proximal tubule after filtration it is bound to the folate receptor-α in the brush border membranes of these absorptive cells and is internalized rapidly by folate receptor-α–mediated endocytosis followed by its dissociation in the acidic environment of the lysosome. It is then transported across basolateral membranes into the blood, with recycling of apofolate receptor-α back to the luminal brush border membrane. Megalin, a large 550-kDa membrane protein interacts found in renal proximal epithelial cells interacts with cubulin and functions as a multiligand receptor for a variety of macromolecules. Beside it also specifically binds to and mediates endocytosis of TCII-cobalamin complexes as well as filtered folate bound to soluble folate-binding proteins in kidney proximal tubules.

Regulation of Folate Homeostasis[37-39]

Upregulation of cell surface folate receptor-α in response to low extracellular and intracellular folate concentrations through transcriptional, translational, and post-translational mechanisms allows it to bind all available folate and thereby restore cellular folate homeostasis.

The basic molecular mechanism has now been deciphered. Intracellular deficiency of folates leads to accumulation of homocysteine which covalently binds to a protein known as heterogeneous nuclear ribonucleoprotein-E1 (hnRNP-E1), which is already known to mediate the translational upregulation of folate receptor-α. Homo-

cysteinylation of hnRNP-E1 at specific cysteine–cysteine disulfide bonds leads to the unmasking of an underlying messenger RNA (mRNA)-binding pocket for which folate receptor-α mRNA has a high affinity thereby triggering the biosynthesis of folate receptors which ultimately results in a net increase of cell surface folate receptors to bind more available folate and thereby normalize cellular folate levels.

Intracellular One-Carbon Metabolism

Polyglutamylation is important not only because it is the folate form that can be retained within the cell but also because, polyglutamylated folates are more efficient substrates for folate-dependent enzymes. In human erythrocytes, folate is accumulated at earlier stages within the marrow by folate receptors;[8] on maturation, more than 90 percent of H4PteGlu(n) molecules interact with hemoglobin, which, because of its high capacity, assists in intracellular folate retention (Fig. 5).

Compartmentalization and Channeling of Folate Metabolism

Folate metabolism and folate-dependent enzymes are very strategically compartmentalized with a distribution such that approximately 40 percent are in the mitochondrial matrix, 50 percent in the cytoplasm, and 10 percent in the nucleus.

After cellular uptake, 5-methyl-THF (which is the major form that is transported intracellularly) it must first be converted to THF via methionine synthase (in the methylation cycle). This is because THF is the preferred physiologic substrate for polylpolyglutamate synthase, which adds multiple glutamate moieties to THF. Only once this is accomplished the polyglutamylated form of THF participate in one-carbon metabolism where it can be converted to either 10-formyl-THF—used in *de novo* biosynthesis of purines, or to 5,10-methylene-THF—used for synthesis of thymidylate. Also 5, 10 -methylene-THF and 10-formyl-THF can be interconverted by intermediates.

The mitochondrial compartment contains its complement of folate cofactors, and homologues of the major cytosolic enzymes. Other one-carbon donors like serine, glycine, dimethylglycine, and sarcosine also enter mitochondria and ultimately generate formate that crosses back into the cytoplasm. In the cytoplasm, C1-THF synthase uses this mitochondria-derived formate with THF to form 10-formyl-THF, which is required for the *de novo* synthesis of purines; this enzyme can also catalyze the interconversion of THF, 10-formyl-THF, 5, 10-methenyl-

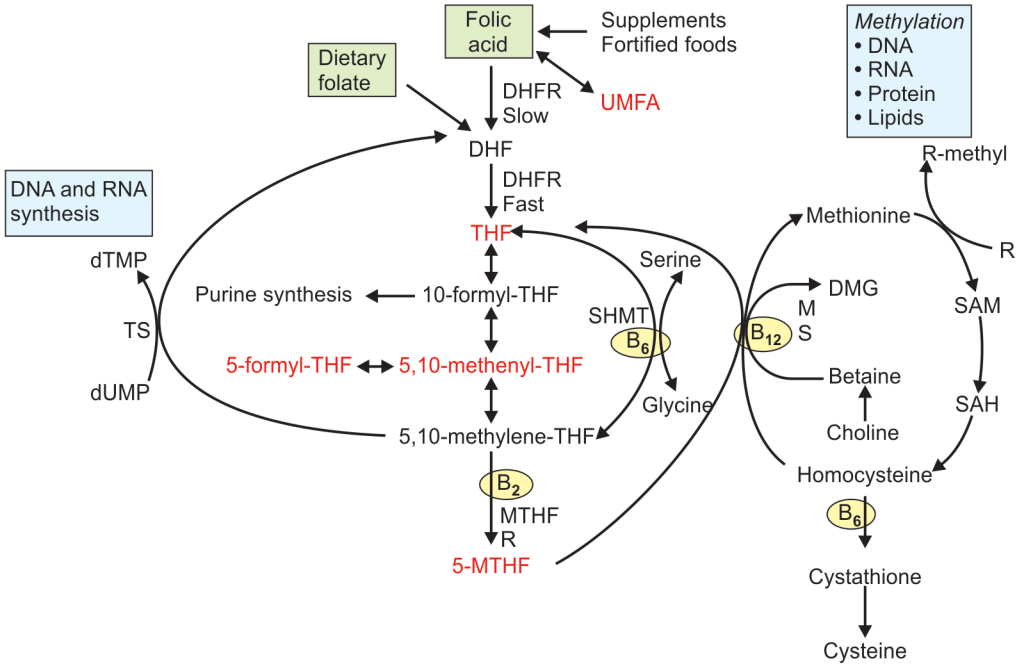


Fig. 5 One carbon metabolism[40] (*Abbreviations:* B2: Riboflavin; B6: Pyridoxal phosphate; B12: Cobalamin; DHF: Dihydrofolate; DHFR: dihydrofolate reductase; DMG: Dimethylglycine; dTMP: Deoxythymidine 5′-phosphate; dUMP: 2′-deoxyuridin-5′-phosphate; MS: Methionine synthase; 5-MTHF: 5-methyltetrahydrofolate; MTHFR: Methylene tetrahydrofolate reductase; SAM: S-adenosylmethionine; SAH: S-adenosylhomocysteine; SHMT: Serine hydroxymethyltransferase; THF: Tetrahydrofolate; TS: Thymidylate synthetase; UMFA: Unmetabolized folic acid. Adopted from ref no. 40)

THF, and 5, 10-methylene-THF. Thus a continued delivery of mitochondrial formate helps perpetuate cytoplasmic one-carbon metabolism. Formation of 5, 10-methylene-THF from serine (which is derived from glycolytic intermediates) forms another major entry point of one-carbon units into cytoplasmic folate metabolism. After 5, 10-methylene-THF is converted to 5-methyl-THF by the enzyme methylenetetrahydrofolate reductase, it can be used in the methylation cycle that involves methylation of homocysteine via methionine synthase to form methionine and tetrahydrofolate.

The methionine that is generated can be converted to a methyl donor through its adenosylation to SAM which in turn is a universal donor of methyl groups for critically important biologic methylation reactions involving over 80 proteins, membrane phospholipids, the synthesis of neurotransmitters.

5, 10-methylene-THF can be converted to 10-formyl-THF, it can be used for *de novo* synthesis of purine nucleotides for DNA and RNA or can also be used in the thymidylate cycle via the enzyme thymidylate synthase to generate thymidylate for DNA synthesis.

Methylfolate Trap[41-45]

The methylfolate trap is a normal physiological response to impending methyl group deficiency resulting from a very low supply of methionine which decreases cellular S-adenosyl-methionine (SAM) thereby endangering important methylation reactions, including those required to maintain myelin. To protect against such catastrophy and considering availability of SAM as its utmost priority the cell behaves as explained as:

- Decreased SAM causes the folate co-factors to be directed through the cycle involving 5-methyl-tetra hydrofolate (5-methyl-THF) and methionine synthetase and away from the cycles that produce purines and pyrimidines for DNA synthesis. This not only enhances the remethylation of homocysteine to methionine and SAM but by restricting DNA biosynthesis decrease the requirement of methionine for protein synthesis and with it cell, division thereby allowing the limited methionine to be conserved for the vital methylation reactions in the nerves, brain, and elsewhere.
- Since in the absence of methionine homocysteine cannot be formed which as discussed earlier is essential to allow folate to be retained intracellularly, there sets in a state of intracellular folate deficiency which restricts the rate of mitosis in the cells and hence decreases requirements of methionine further.

Vitamin B_{12} deficiency is mistakenly perceived as methione deficiency by the cells, thus resulting in an inappropriate response of downregulating the multiplication of hematopoietic precursors and causing lethal anemia. In these circumstances the methylation reactions are also partly protected by the reduced rate of cell division. Hence when only folic acid is administered in cobalamin deficiency without simultaneous supplementation of B_{12}, cell division is induced and the subsequent consumption of methionine in protein synthesis, impairs methylation of myelin and precipitates or exacerbates subacute combined degeneration (SCD).

The selective use of available folate to conserve methionine, together with the ability of nerve tissue to concentrate folate from the plasma, explains the absence of SCD in folate deficiency.

Development of Folate Deficiency

Nutritional: The body stores of folate are adequate for only about 4 months any additional stress that increase folate requirements like illness, hemolysis, anorexia will tip an individual who was chronically in a negative folate balance to develop frank folate deficiency.

Socioeconomic status has a major impact as folate deficiency often coexists with poverty, malnutrition, and chronic bacterial, viral and parasitic infections. ignorance about cooking practices like overheating food also contributes to the nutritional losses of folates.

In the economically well off countries food faddism, alcoholism, or unbalanced slimming diets usually lead to decreased folate intake in adolescents.

Pregnancy and infancy: Folate requirements (over 400 mcg/day) during pregnancy and lactation are increased tremendously for growth of the fetus, placenta, breast, and other maternal tissues. There is also increased urinary loss of folate in pregnancy (about 14 mcg/day versus approximately 4.2 mcg/day in nonpregnant women) because of a lower renal threshold. Additional folate during pregnancy is required to prevent both pregnancy complications (pre-eclampsia, placental abruption or infarctions, recurrent miscarriage) and poor pregnancy outcomes (preterm delivery, NTDs, congenital heart defects, and intrauterine growth retardation).

The rapidly proliferating tissues in children also have an absolute requirement for exogenously supplied folate. Although human milk can maintain folate balance in breastfed infants, the breast milk content of folate is low when the mother's folate status is poor.

Folates and neurodevelopment: All inborn errors of folate metabolism, which result in reduced folate availability to the developing brain, give rise to mental retardation and related mental health problems. Since the fetal brain is dependent on sufficient provision of maternal folate during embryogenesis maternal folate deficiency can compromise the delivery of folate to the developing

fetal brain, and depending on the degree of deficiency, there could be a spectrum of neurologic abnormalities ranging from full-blown NTDs to more subtle changes that manifest in childhood as behavioral abnormalities. Many studies have confirmed this theory and marked improvement in pregnancy outcomes in form of reduced incidences of NTDs as well as better neurocognitive status in the offsprings of mothers who were adequately supplemented with folate perinatally.

Folates and intrinsic hematologic disease: Because folate is necessary for hematopoiesis, folate requirements are increased when there is significant compensatory erythropoiesis in response in hemolytic disorders, abnormal hematopoiesis, or infiltration by abnormal cells in marrow. In fact folate deficiency developing in hemolytic disorders can lead to an acute aplastic crisis and hence the recommendation of routine prophylactic administration of folate in all follow up cases of hemolytic anemias has been laid down. An unexpected increase in transfusional requirement or a fall in platelets can also suggest folate deficiency.

Drugs and folates: Although *trimethoprim* and *pyrimethamine* bind to bacterial and parasitic dihydrofolate reductase with much greater affinity than to human dihydrofolate reductase, but patients with underlying folate deficiency appear to be more susceptible to the effects of these drugs. The megaloblastosis can be reversed by folinic acid (5-formyl-tetrahydrofolate [5-formyl-THF]; leukovorin). *Methotrexate* binds with high affinity to human dihydrofolate reductase and leads to trapping of folate as a metabolically inert form (dihydrofolate). This leads to a true depletion of THF within hours and consequently to functional deficiency of 5, 10-methylene-THF and reduced thymidylate synthesis. Although megaloblastosis can develop rapidly, the toxic effects of methotrexate can be avoided by rescue with 5-formyl-THF (leukovorin). *Sulfasalazine* produces megaloblastosis in up to two-thirds of patients taking full doses (over 2 g/day) by decreasing absorption of folates and induction hemolytic anemia (i.e. increased requirements). *Anticonvulsants* can induce NTD, and guidelines have stressed the importance of ensuring that pregnant women and children with epilepsy be prescribed folates together with anticonvulsants. The only caveat is to correct B_{12} deficiency before prescribing long-term folates.

PATHOLOGY OF MEGALOBLASTIC ANEMIA

Hematological Manifestations[46-48]

Peripheral smear and bone marrow examination (Table 4): Widening disparity in nuclear-cytoplasmic asynchrony as a cobalamin- or folate-deficient cell divides, until the more mature generations of daughter cells die in the marrow or are arrested (as megaloblastic cells) at various stages of the cell cycle is the hall mark. Although megaloblastosis affects all proliferating cells including those of the intestinal lumen, cervix, uterus, changes are most striking in the blood and the bone marrow.

Table 4 Morphology[49]

Morphology in megaloblastosis from cobalamin and folate deficiency
Peripheral smear
• Increased mean corpuscular volume (MCV) with macro-ovalocytes (up to 14 μm), which is variously associated with anisocytosis and poikilocytosis
• Nuclear hypersegmentation of polymorphonuclear neutrophils (PMNs) (one PMN with six lobes or 5% with five lobes)
• Thrombocytopenia (mild to moderate)
• Leukoerythroblastic morphology (from extramedullary hematopoiesis)
Bone marrow aspirate
• General increase in cellutarity of all three major hematopoietic elements
• Abnormal erythropoiesis—orthochromatic megaloblasts
• Abnormal leukopoiesis—giant metamyelocytes and "band" forms (pathognomonic), hypersegmented PMNs
• Abnormal megakaryocytopoiesis—pseudohyperdiploidy

As the megaloblastic erythroid cells are prone to programmed cell death, ineffective hematopoiesis extends into long bones, and the bone marrow aspirate exhibits trilineal hypercellularity, especially of the erythroid series. This apparent exuberant cell proliferation seen within the marrow with numerous mitotic figures is misleading because these cells are actually proliferating very slowly. Elevated LDH, increased bile pigments and iron are outcomes of this ineffective erythropoiesis.

The mature erythrocytes are of various sizes with higher mean corpuscular volumes (MCV). In fact increase in (MCV) with macro-ovalocytes (up to 14 μm) is one of the earliest manifestations of megaloblastosis. Because these cells have adequate hemoglobin, the central pallor, which normally occupies about one-third of the cell, is decreased. Poikilocytosis and anisocytosis are seen when severe anemia is present. Cells containing remnants of DNA (Howell-Jolly bodies), arginine-rich histone, and nonhemoglobin iron (Cabot rings) may be observed. Extramedullary megaloblastic hematopoiesis may give rise to a leukoerythroblastic picture.

Nuclear hypersegmentation of DNA in PMNs is found to be a strong and pretty consistent indicator of megaloblastosis when associated with macro-ovalocytes. In megaloblastosis greater than 5 percent PMNs with more

than five lobes or a single PMN with more than six lobes supports the diagnosis and a formal lobe count/PMN (i.e. lobe index) above 3.5 may be obtained.

Thrombocytopenia and neutropenia often accompany severe anemia although they may be seen even in the absence of anemia.

Erythroid hyperplasia reduces the myeloid-to-erythroid ratio from 3:1 to 1:1. Proerythroblasts often do not exhibit much abnormality except for a larger size but the later precursors show many abnormalities. These megaloblasts are not only larger but instead of having a densely packed chromatin they have an open, finely stippled, reticular, sieve-like pattern. The orthochromatic megaloblast, with its hemoglobinized cytoplasm, continues to retain its large sieve-like immature nucleus, in sharp contrast to the clumped chromatin of orthochromatic normoblasts.[80-90] percent of the potential progeny of proerythroblasts that develop into later megaloblastic die in the bone marrow. Effective scavenging dead or partially disintegrated megaloblasts by the marrow macrophages forms the basis for ineffective erythropoiesis (intramedullary hemolysis).

Leukopoiesis is hit as well. There is an absolute increase in the myeloid precursors, which are large and have similar sieve-like chromatin. Spectacular giant (20–30 μm) metamyelocytes and "band" forms are often seen and are pathognomonic for megaloblastosis. Their clinical relevance lies in the fact that such giant metamyelocytes and band forms are not seen in the megaloblastoid bone marrow of leukemia and MDS. They may persist in the marrow for 10–14 days after the initiation of treatment for megaloblastosis (Figs 6A to E).

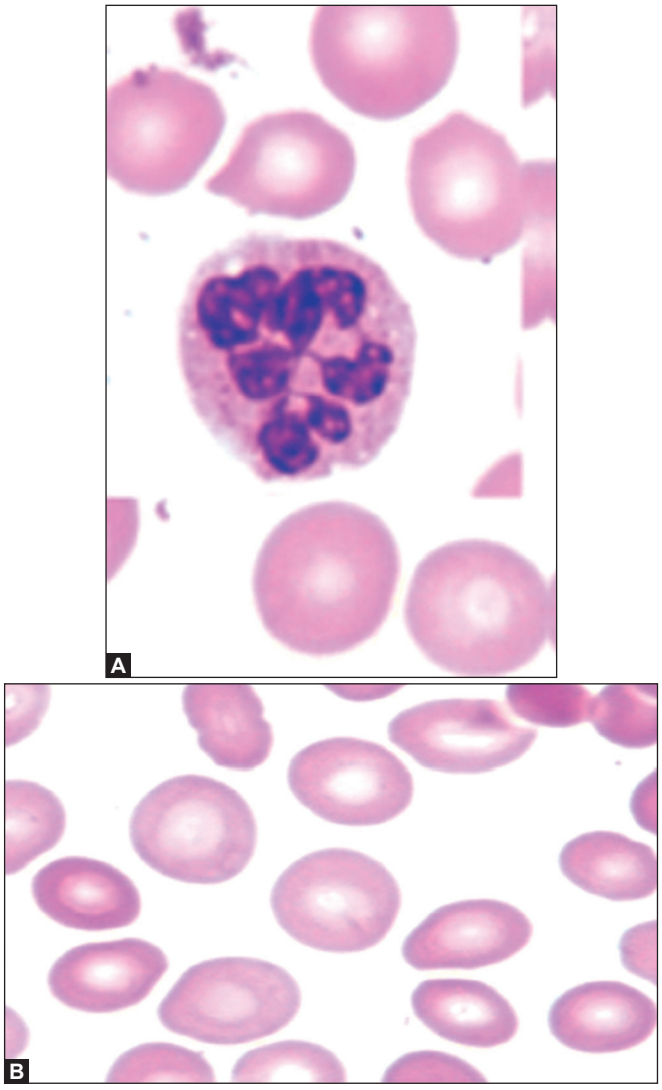
Megaloblastosis[50,51] when affects the rapidly proliferating cells of the gastrointestinal tract leads to their atrophy which in turn cause further malabsorption of cobalamin and folate thereby fueling a vicious cycle wherein megaloblastosis begets more megaloblastosis which can be adequately interrupted by specific therapy with folates and cobalamin.

Megaloblastosis versus Macrocytosis

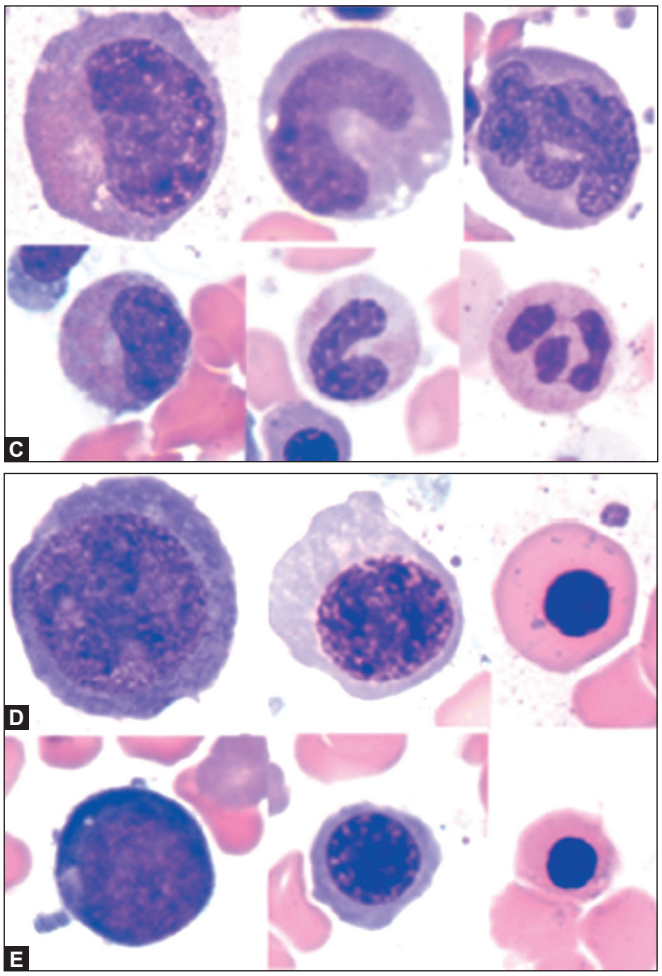
The central pallor that normally occupies about one-third of the normal red blood cell is decreased in macro-ovalocytes. This contrasts with the finding of thin macrocytes, in which the central pallor is increased. This is because the hemoglobinization in cobalamin and folate deficiency is only increased as the cell now takes more time to mature and hence the decrease in central pallor (Table 5).

Neurological Manifestations

Cobalamin deficiency can manifest as a myriad of neurological defects. The basic underlying pathogenic process is a patchy demyelination affecting both the brain and the spinal cord. It begins as a swelling of the myelin sheath followed by its breakdown and eventual axonal degeneration which is often followed by secondary Wallerian degeneration of the long tracts. Lesions first involve the dorsal columns in the thoracic segments spreading contiguously to engulf the corticospinal tracts and ultimately affecting the spinothalamic and spinocerebellar tracts as well. Degeneration of the dorsal root ganglia, celiac ganglia, the Meissner plexus, and the Auerbach plexus also occurs. Demyelination may also extend to the white matter of the brain. Whether the peripheral neuropathy is caused by a distinct lesion or results from spinal cord disease is still to be found.



Figs 6A and B The peripheral smear (A) Hypersegmented polys; (B) Macro-ovalocytes



Figs 6C to E Microscopic picture of megaloblastosis[49] (C). Giant metamyelocyte (Band form cell) Details from the cells in the aspirate (D) compared with normal hematopoiesis at same magnification (E)

Table 5 Macrocytosis versus megaloblastosis[51]

Macrocytosis without megaloblastosis

- Reticulocytosis
- Liver disease
- Aplastic anemia
- Myelodysplastic syndromes (especially 5q-)
- Multiple myeloma
- Hypoxemia
- Smokers

Spurious increases in MCV without macro-ovalocytosis[51]

- Cold agglutinin disease
- Marked hyperglycemia
- Leukocytosis
- Older individuals

CLINICAL FEATURES

History

History often reveals an exclusively breast fed infant born to an apparently anemic mother who is either vegetarian by choice or forced due to socioeconomic factors. The infant would have gradually curtailed his activities and the mother is unable to notice the gradually developing pallor until it becomes severe enough that the child needs to be taken to a doctor. Occasionally children may present with shortness of breath and impending failure. Since this is a chronically developing anemia such manifestations are seen only when the hemoglobin falls below 5 gm/dL.

Gastrointestinal symptoms may predominate in some including loss of appetite, weight loss, diarrhea, nausea, vomiting, and glossitis aggravated by spicy foods.

The neurological symptomatology may vary from vague complaints like decreased memory, lethargy, irritability, mild degree of cognitive impairment to severe peripheral neuropathies a subacute combined degeneration of the spinal cord which is the most feared complication of this nutritional deficiency. SCDSC may manifest as loss of vibration sense, parestherias and weakness, all affecting the lower limbs much more than the upper.

Further enquiry regarding parity of the mother, birth history and dietary details as well as past illnesses and surgeries, drug ingestions (antiepileptics, pyrimethamine), worm infections and affection of other family members may aid in coming to an etiology of the present condition.

Physical Examination

Physical examination reveals different features in well-nourished patients and poorly nourished individuals. The latter show evidence of significant weight loss or other stigmata of multiple deficiencies due to "broadspectrum" malabsorption. Angular cheilosis, bleeding mucous membranes, dermatitis, and chronic infections hint to associated vitamin A, D, E, K deficiency with PEM. Various degrees of pallor with lemon-tint icterus (i.e. a combination of pallor and icterus best observed in fair-skinned individuals) are common features of megaloblastosis.

The skin may be diffusely pigmented or have abnormal blotchy tanning. A macular hyperpigmentation[41] with follicular accentuation may be observed in the axilla and groin; hyperpigmentation can also involve the dorsal acral distal interphalangeal joints with special emphasis on pigmentation of the nail beds and skin creases. Unlike Addison's disease there is no staining of the mucous membranes. Premature graying, observed in light-and dark-haired individuals, is reversible within 6 months of

cobalamin therapy. These pigmentation changes often resolve within 2 months of cobalamin replacement.

Glossitis with a smooth (depapillated), beefy red tongue with occasional ulceration of the lateral surface or gingival hyperplasia are found on oral examination. Increased jugular venous distention along with gallop, cardiomegaly (with or without pericardial effusions), pulmonary basal crepitations, pleural effusion, tender hepatomegaly, and pedal edema should alert the clinician towards a cardiac failure due to severe anemia. Very rarely nontender hepatomegaly, but more often splenomegaly may hint towards extrameduallary hematopoiesis (Table 6).

Also look for signs of associated hypo-or hyperthyroidism like neck swellings and loss of eyebrows, etc.

Anemia and neurological signs[52-56] are found to be inversely related. SCDSC can have all or few of the features which include decreased vibration sense below the iliac crests (mobile phone sign), loss of position sense in feet and ataxia all due to affection of posterior spinal columns. Weakness and progressive spasticity with increased muscle tone, exaggerated deep tendon reflexes with clonus, extensor plantar response, and in-coordinate or scissor gait, which may progress to spastic paraplegia indicate UMN type of lesion because of pyramidal tract involvement. The involvement of peripheral nerves may markedly modify these signs to include flaccidity and the absence of deep tendon reflexes. A positive Romberg sign as well a positive Lhermitte sign may be elicited. Loss of sphincter and bowel control, altered cranial nerve dysfunction with altered taste, smell, and visual acuity or color perception, and optic neuritis are other rare but proven manifestations of cobalamin deficiency.[56]

Data suggests that changes of SCDSC is a result of decreased remethylation of homocystiene as a result of changes in the activity of AdoMet-dependent methyltransferase.

Table 6 Clinical features of B_{12} and folate deficiency[57]

System	*Manifestations*
Hematologic	Pancytopenia with megaloblastic marrow
Cardiopulmonary	Congestive heart failure
Gastrointestinal	Beefy-red tongue and added stigmata of broadspectrum malabsorption in folate deficiency
Dermatologic	Melanin pigmentation and premature graying
Genital	Cervical or uterine dysplasia
Reproductive	Infertility or sterility
Psychiatric	Depressed affect and cognitive dysfunction
Neuropsychiatric	Unique to cobalamin deficiency with cerebral, myelopathic, or peripheral neuropathic disturbances, including optic and autonomic nerve dysfunction

Subclinical Cobalamin Deficiency[57-59]

The issue of subclinical cobalamin deficiency has been a semantic dilemma. Many persons present with subtle symptoms including fatigue, cognitive changes, lower quality-of-life measures, and subtle symptoms of neuropathy that cannot be directly attributed to cobalamin deficiency, despite the fact that these very symptoms are often seen in symptomatic cobalamin deficiency; often this triggers testing with a serum cobalamin test, and a borderline result generates a new set of problems, including the need to label this entity and thereby make clinical decisions. The incidence of subclinical cobalamin deficiency is found to be 10 times higher in the US population than the classical overt type of megaloblastic anemia. Demonstration of an increase in metabolites (i.e. serum homocysteine and MMA test results), helps pick-up many individuals having subclinical cobalamin deficiency, provided they had no subtle cognitive abnormalities.

A cut-off value of 148 pmol/L (less than 200 pg/mL) is consistent with 3 standard deviations (SDs), and will miss about 3 to 5 percent of patients with clinical cobalamin deficiency.

However, the literature does not provide the clinician with set guidelines about how to manage the entity of subclinical cobalamin deficiency, which is defined as biochemical evidence for cobalamin deficiency—reflected by a low cobalamin value (and increased MMA and homocysteine) but without overt clinical manifestations. Although some experts do not feel obliged to treat, preferring to wait until there are overt symptoms, there is another school that feels ethically bound to treat even without overt clinical manifestations rather than allow them to develop and make the patient suffer.

DIAGNOSTIC ISSUES AND INVESTIGATIONS

Peripheral Smear and Bone Marrow Aspirate

Macro-ovalocytes, although not specific for megaloblastic anemia are the hallmark of megaloblastosis. Similarly, though the importance of MCV values in suspecting megaloblastic anemia is immense only half of the patients with MCVs greater than 105 fL may have vitamin deficiency. Macrocytosis per se is not associated with megaloblastosis in around 50 percent of the cases and a complete diagnosis may be reached only after carrying out several other additional tests.

The frequency of hypersegmented neutrophils (5 percent with five lobes or 1 percent with six-lobed PMNs) in patients with megaloblastic hematopoiesis is 98 percent

with the specificity of this finding being approximately 95 percent. Hypersegmented PMNs and macro-ovalocytosis, together give results that are much more accurate with a specificity of 96 percent to 98 percent, and the positive predictive value of folate or cobalamin deficiency of about 94 percent.

Diagnostic features of a megaloblastoid marrow have been mentioned in previous sections. A question now being frequently raised is that whether a bone marrow examination always necessary to make a diagnosis of folate and cobalamin deficient megaloblastic anemia. There are many schools of thought. With highly sensitive serum tests for the specific diagnosis of cobalamin and folate deficiency now available, it would be reasonable to say that the urgency of the diagnosis should dictate the need for a bone marrow. For example, an urgent bone marrow aspirate examination showing megaloblastosis for immediate diagnosis is indispensible in a case of florid hematologic disease with or without neurologic disease suggestive of cobalamin or folate deficiency. However, in stable and non-urgent cases with characteristic peripheral smear, or for a patient with a primary neuropsychiatric presentation, proceeding with measurement of serum levels of vitamins or metabolites without a bone marrow aspiration is a reasonable option.

Masked Megaloblastosis[60]

Conditions wherein true cobalamin or folate deficiency with anemia is not accompanied by classic findings of megaloblastosis in the peripheral blood and bone marrow constitute the phenomenon of masked megaloblastosis any condition that compromises a cells capacity to carry out hemoglobinization such as iron deficiency anemia and thalassemia will simultaneously decrease the tendency to form megaloblastic cells. However certain points can help unveil this occult megalblastosis. A wide RBC distribution width (RDW) with a normal MCH and/or MCV on the Coulter counter readout may reflect megaloblastic anemia 9 or dimorphic anemia (macro-ovalocytes plus microcytic hypochromic RBCs). Since hemoglobinization has got no business with the white blood cells and their pecursors, these pathognomonic findings (giant myelocytes and metamyelocytes, and hypersegmented PMNs) remain unaltered and can be of great help in suspecting an underlying folate or cobalamin deficiency. The latter may persist for up to 2 weeks after replacement with cobalamin or folate. Once masked megaloblastosis has been recognized investigations to rule out iron deficiency, anemia of chronic disease, or hemoglobinopathies is indicated. Without correction of the iron deficiency, cobalamin or folate will not elicit a maximal therapeutic benefit. Conversely, treating with iron alone would unmask the megaloblastosis.

Biochemical Evidence[56,61]

Serum levels of cobalamin: The sensitivity of cobalamin concentration less than 200 pg/mL (or less than 148 pmol/L) exceeds 95 percent when the clinical spectrum suggests and smear examination reveals megaloblastosis. A serum cobalamin level of more than 300 pg/mL predicts folate deficiency or another hematologic or neurologic disease while 99 percent of patients with occult deficiencies will have levels less than 300 pg/mL. In view of lack of transparency related to these tests, poor validation, and poor tracking of assay performance, if the clinical picture is consistent with cobalamin deficiency, and the serum cobalamin level is normal or borderline low, it is entirely appropriate to treat as for a cobalamin deficiency. Cobalamin deficiency can falsely raise serum folate by 20 to 30 percent via methyl-folate trapping. In patients with megaloblastic anemia, the finding of a normal to increased level of serum folate, along with a reduced ratio of RBC to serum folate provides strong although an indirect evidence of cobalamin deficiency (Table 7).

Serum folate levels: When negative folate balance continues, hepatic folate stores are depleted in about 4 months. This leads to tissue folate deficiency, which clinically correlates with a decrease in RBC folate (less than 150 ng/mL) by the microbiologic assay.

Serum MMA and Homocysteine Level

Basis: Perturbation of methionine synthase activity by cobalamin deficiency results in substrate (homocysteine) buildup and elevated serum levels of homocysteine, which can be measured by a sensitive assay. Additionally cobalamin deficiency also affects the activity of methyl-malonyl-CoA mutase negatively, which leads to elevated

Table 7 Cobalamin levels and B_{12} deficiency[56]

Falsely low serum cobalamin in the absence of true[56] cobalamin deficiency

- Folate deficiency (one-third of patients)
- Multiple myeloma
- TCI deficiency
- Megadose vitamin C therapy

Falsely raised cobalamin levels in the presence of a true deficiency[62]

- Cobalamin binders (TCI and II) increased (e.g. myeloproliferative states, hepatomas, and fibrolamellar hepatic tumors)
- TCII-producing macrophages are activated (e.g. autoimmune diseases, monoblastic leukemias and lymphomas)
- Release of cobalamin from hepatocytes (e.g. active liver disease)
- High serum anti-IF antibody titer

serum MMA levels. Thus homocysteine and MMA are sensitive tests for cobalamin deficiency. Early manifestations of negative cobalamin balance are increased serum methylmalonic acid (MMA) and total homocysteine levels. This occurs when the total cobalamin in serum is still in the low-normal range. Normal levels of MMA and homocysteine rule out clinically significant cobalamin deficiency with virtually 100 percent certainty.

Values: The normal value for serum homocysteine is 5.1 to 13.9 μM and serum MMA is 73 to 271 nM, and in general the higher the values, the more severe the clinical abnormalities.[9] However age-, creatinine-, gender-, diet-, and race-dependent can cause the values to fluctuate over a wide range.

Application: When cobalamin and/or folate deficiency is suspected strongly and the cobalamin levels are suggestive but not definitive, then the MMA and homocysteine tests are an excellent means to confirm a clinical diagnosis. Patients with clinical cobalamin deficiency usually have MMA values over 1000 nM and homocysteine values that are over 25 μM. The MMA and homocysteine test results exceed cobalamin levels by quite an amount when sensitivities are compared as they increase much earlier and more consistently than the drop in cobalamin levels.

Since running serum cobalamin and folate levels compared with serum MMA and homocysteine levels is more cost effective it is recommended to first use the cheaper tests that can assist in the diagnosis of cobalamin and folate deficiency (Table 8).

Tests to Assess Absorption and Transport

Schilling test: This test is rarely used these days since less time consuming and less tedious ways of diagnosing pernicious anemia are now available. However for historical purposes the principle of this test is mentioned in brief. It is important to remember that this test intends to define an etiology of cobalamin deficiency already established by other tests and not to diagnose cobalamin deficiency per se.

In the first part of the test, the patient is given radiolabeled vitamin B_{12} orally followed by an intramuscular injection of unlabeled vitamin B_{12} given an hour later which is only enough to temporarily saturate B_{12} receptors in the liver with enough normal vitamin B_{12} to prevent radioactive vitamin B_{12} binding in body tissues (especially in the liver). Normally, the ingested radiolabeled vitamin B_{12} will be absorbed into the body. Since the body already has liver receptors for vitamin B_{12} saturated by the injection, much of the ingested vitamin B_{12} will be excreted in the urine.

- A normal result shows *at least 10 percent* of the radiolabeled vitamin B_{12} in the urine over the first 24 hours.

Table 8 Interpretation of serum levels[62]

Cobalamin (pg/mL)*	*Folate (ng/mL)*	*Provisional diagnosis*	*Proceed with metabolites?*
>300	>4	Cobalamin or folate deficiency is unlikely	No
<200	>4	Consistent with cobalamin deficiency	No
200–300	>4	Rule out cobalamin deficiency	Yes
>300	<2	Consistent with folate deficiency	No
<200	<2	Consistent with (1) combined cobalamin plus folate deficiency or (2) isolated folate deficiency	Yes
>300	2–4	Consistent with (1) folate deficiency or (2) an anemia unrelated to vitamin deficiency	Yes
Test results on metabolites: serum methylmalonic acid and total homocysteine			
Methylmalonic acid (Normal, 70–270 nM)	*Total homocysteine (Normal, 5–14 μM)*	*Diagnosis*	
Increased	Increased	Cobalamin deficiency confirmed; folate deficiency still possible (i.e. combined cobalamin plus folate deficiency possible)	
Normal	Increased	Folate deficiency is likely	
Normal	Normal	Cobalamin and folate deficiency is excluded	

*Table adopted from ref no. 62

- In patients with pernicious anemia or with deficiency due to impaired absorption, *less than 10 percent* of the radiolabeled vitamin B_{12} is detected.
  If an abnormality is found, i.e. the B_{12} in the urine is only present in low levels, then in the second part the test is repeated, with additional oral intrinsic factor.
- A normal urine collection shows a lack of intrinsic factor production, i.e. pernicious anemia.
- A low result on the second test also implies other causes of malabsorption, including infestation with D latum or giardia, celiac disease, Whipple's disease, etc.

Single–sample stool excretion test: Labeled cobalamin was fed with a nonabsorbable dye together with nonabsorbable chromium chloride (Cr51). A sample of such stained stool was counted for Cr51 and Co57 and the ratio of the counts compared with that of the sample that was fed. Normally 36–88 percent of the dose should be absorbed. This test is not readily available.

Miscellaneous

- Increase LDH, serum bilirubin and serum iron levels reflect ineffective erythropoiesis-non-specific.
- Serum lipid, cholesterol and immunoglobins may be decreased-non-specific.
- Increased serum gastrin and pepsinogen levels.
- Antibodies to IF in the serum is highly specific and indicates either present or imminent cobalamin deficiency.
- *Serum transcobalamin II levels:* As an early marker of cobalamin homeostasis, as a surrogate for the Schilling test, or to diagnose cobalamin deficiency in lieu of serum cobalamin values is still in the process of validation.

Positive Therapeutic Response[63-66]

Clinical, hematological and biochemical response to therapy with cobalamin and folic acid is another way to diagnose the deficiency in retrospect. A single injection of cyanocobalamin is given and disappearance of megaloblastoid changes in the bone marrow is looked for in the next 48 hours besides two of the following, in order to call the test positive (Fig. 7).

- 50 percent decrease in serum iron or LDH within 48 hours.
- Increase in retic count 5 to 10 days after treatment.
- Correction of neutropenia and thrombocytopenia over a period of 2 weeks.
- Once reticulocytosis subsides MCV is decreased by 5 fL or more.
- Plasma MMA and homocysteine in 2 weeks.
- Correction of anemia of 2 to 4 weeks.

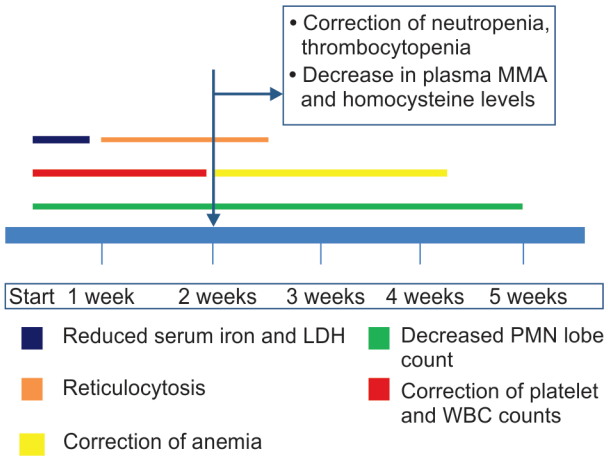


Fig. 7 Therapeutic response

- Decrease in the neutrophil lobe count to normal over a 4 weeks period.

Accelerated turnover of normal DNA in erythroid precursors also increases serum rate level, peaking by the fourth day along with increased cellular phosphate uptake for nucleotide synthesis. These together may precipitate an attack of gout if the patient has a "gouty predisposition".

If by the end of the third week, the RBC count is not above $3 \times 10^6/mm^3$ additional causes of underlying iron deficiency, hemoglobinopathy, chronic disease, or hypothyroidism should be considered.

TREATMENT OF MEGALOBLASTOSIS[67-74]

Principles

- Routinely, treatment with full doses of parenteral cobalamin (1 mg/day) and oral folate (1–5 mg) before knowledge of the type of vitamin deficiency is established should be reserved for the severely ill patient.
- Patients with vitamin B_{12} deficiency despite a normal absorption, such as vegetarians and vegans, only need a daily supplement in the form of a vitamin pill containing at least 6 µg of vitamin B_{12}.
- Patients with an irreversible cause of vitamin B_{12} deficiency are destined to lifelong treatment with a pharmacological dose of vitamin B_{12}.

Severe deficiency: When a patient of suspected megaloblastic anemia presents in failure either due to anemia itself, due to sodium retention or due to myocardial hypoxia the treatment includes oxygen administration with slow transfusion of packed cells under cover of diuretics to avoid disastrous conditions of fluid overload. Giving high initial doses of vitamin B_{12} can cause severe metabolic disturbances like hypokalemia by shifting

extracellular potassium intracellularly in response to the sudden increase in the cell replication. This is also exaggerated further by the delay in renal potassium retention. A clinician should anticipate such complications and keep a watch on the potassium levels besides starting prophylactic potassium supplements in such cases. Hence it is recommended to give small doses of 10 μg (0.2 μg/kg for severely deficient children) CNbl subcutaneously for first 2 days which should suffice to normalize serum LDH as well as iron levels besides inducing reticulocytosis within approximately a week. However, more is needed before serum MMA and homocysteine levels are normalized and body stores are replenished.

Conventional therapy:[75-78] In a stable child it is advisable to collect samples of bone marrow aspirate, those for serum folate and cobalamin levels. Conventional therapy includes once daily injection 1000 μg for a week which is succeeded by 100 μg of cyanocobalamin injection for a month and then monthly injections.

Different forms of vitamin B_{12} can be used, including cyano-, hydroxy-, and methylcobalamin.

Hydroxycobalamin may have advantages due to a slower metabolism however, the depot preparation of cyanocobalamin (cyanocobalamin-tannin complex suspended in a sesame oil-aluminium monostearate gel) is metabolized even slower than hydroxycobalamin.

All inborn errors of cobalamin metabolism adequately justify use of parenteral therapy with cobalamin. There is no major advantage of other preparations over generic cyanocobalamin. Oral 2 mg cobalamin tablets consumed daily is found to be equivalent to traditional monthly parenteral treatment with 1 mg of intramuscular/subcutaneous cobalamin among those requiring long-term cobalamin as at such high doses cobalamin is absorbed passively across the mucous membranes of oral cavity and stomach. This option is especially helpful in patients in whom parenteral therapy becomes less feasible due to refusal for daily injection, those with coagulation disorders and in them cobalamin (1–2 mg/day as tablets) can be recommended despite cobalamin malabsorption. Meals can decrease the bioavailability of cobalamin by 40 percent and taking the same doses empty stomach decreases the losses in stool.

Certain points should be emphasized when initiating treatment with vitamin B_{12}. Once vitamin B_{12} has been administered, the increase in red cell production will increase the demand on iron stores and, therefore, it is important to monitor–and correct–any signs of iron deficiency. A drop in plasma folate after initiation of vitamin B_{12} treatment is a sign of unmasking of hitherto occult folate deficiency.

Even when malabsorption is a problem oral folate (folic acid) at doses of 1 to 5 mg/day results in adequate absorption. Therapy should be continued until complete hematologic recovery is documented although if the underlying etiology for the folate deficiency is not corrected a lifelong folic acid supplemented is also warranted for. Folinic acid (i.e. 5-formyl-THF [leukovorin]) should be reserved only for rescue protocols involving antifolates (methotrexate or trimethoprim-sulfamethoxazole), for 5-fluorouracil modulation protocols, after nitrous oxide toxicity, or in pediatric cases involving cerebral folate deficiency or inborn errors of folate metabolism.

Prophylaxis

Prophylaxis with Cobalamin

- 5 to 10 mcg for nutritional causes and 1000 mcg/day for problems of malabsorption
- Infants on specialized diets
- Premature infants
- Infants of mothers with pernicious anemia
- Infants and children of mothers with nutritional cobalamin deficiency
- Vegetarianism and poverty-imposed near-vegetarianism
- Total gastrectomy.

Prophylaxis with Folic Acid

- 4 g/day periconceptionally
- All women contemplating pregnancy (at least 400 mcg/day)
- Pregnancy and lactation, premature infants
- Mothers at risk for delivery of infants with neural tube defects
- Hemolytic anemias/hyperproliferative hematologic states
- Patients with rheumatoid arthritis or psoriasis on therapy with methotrexate
- Patients on antiepileptic drugs
- Patients with ulcerative colitis.

Thiamine-responsive Megaloblastic Anemia Syndrome[79]

It is caused by mutations in SLC19A2, encoding a thiamine transporter protein. It is usually associated with diabetes mellitus, anemia and deafness. With an onset generally seen during infancy or at early childhood and most of the thiamine-responsive megaloblastic anemia (TRMA) patients are originated from consanguineous families and is thus an autosomal recessive disease whereby active thiamine uptake into cells is disturbed. Thus, at physiological concentrations (food as the only source), thiamine is not transported normally and intracellular thiamine deficiency leads to decreased activity of enzymes

associated with thiamine pyrophosphate. The role of thiamine in DNA metabolism and heme synthesis explains the megaloblastic anemia. Thrombocytopenia has been less commonly reported in TRMA patients. The rarity of leukopenia is in these patients is probably accounted for by the different needs of the hematopoietic progenitor cells to the intracellular thiamine. Diabetes mellitus in TRMA patients is a consistent finding and is most likely secondary to impairment of islet cell function by the intracellular thiamine deficiency.

While the costs for health care delivery move farther and farther out of the reach of the common man, and the ongoing debate on ways to reduce these costs, few instances in internal medicine and hematology yield more satisfying dividends than diagnosing and treating cobalamin and folate deficiency using generic vitamins that are "dirt-cheap"—costing only one or two cents a day. These conditions are devastating when undiagnosed or misdiagnosed or when cobalamin deficiency is treated with folate alone. Recognition of various populations at risk and the clinical scenarios in which folate and cobalamin deficiency are likely to be present, and the availability of sensitive and specific tests, should reduce uncertainty in diagnosis.

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CHAPTER 16

Anemia of Chronic Disease

Dilraj Kaur Kahlon, Satya P Yadav, Anupam Sachdeva

Anemia of chronic disease (ACD), the second most prevalent anemia after anemia caused by iron deficiency, occurs in patients with acute or chronic immune activation. The condition has thus been termed "anemia of inflammation."[1] ACD is an anemia of underproduction that usually is normocytic, normochromic, and relatively mild, with a hemoglobin level greater than 10 g/L. However, the anemia can be severe, and the mean corpuscular volume may be reduced. Hypochromia (mean corpuscular hemoglobin concentration, 26 to 32 g/dL) is more common than microcytosis.

The most frequent conditions associated with anemia of chronic disease are listed in Table 1.

ANEMIA

- Microcytosis in ACD is usually not as striking as that commonly associated with iron deficiency anemia; values for MCV <72 fL are rare. Another distinction from iron deficiency is that hypochromia typically precedes microcytosis in ACD but typically follows the development of microcytosis in iron deficiency.[2] The hematocrit usually is maintained between 0.25 and 0.40, but significantly lower values are observed in 20 to 30 percent of patients.[2,3]

  The percentage of reticulocytes is normal or reduced; although on rare occasions, it may be slightly increased.
- Red cell distribution width may be normal initially but is typically elevated to a moderate degree, and generally does not help in distinguishing iron deficiency and ACD.
- The degree of anemia is proportional to the severity of the underlying disease. The severity of the anemia and the activity of rheumatoid arthritis are judged by fever, severity of joint swelling and inflammation, and the erythrocyte sedimentation rate (ESR). In patients with malignant disease, anemia is more severe when metastases are widespread than when the disease is localized. Serum iron concentration and total iron binding capacity (TIBC) is decreased. Transferrin saturation is subnormal.[2]

Table 1 Causes of anemia of chronic disease

Chronic infections
• Tuberculosis
• Pulmonary infections
• Subacute bacterial endocarditis
• Pelvic inflammatory disease
• Osteomyelitis
• Chronic urinary tract infections
• Chronic fungal disease
• Human immunodeficiency virus infection
Chronic noninfectious inflammations
• Rheumatic fever
• Severe trauma
• Thermal injury
Malignant diseases
• Lymphoma
• Leukemia
• Multiple myeloma
Autoimmune
• Rheumatoid arthritis
• Systemic lupus erythematosus
• Vasculitis
Miscellaneous
• Chronic renal disease
• Chronic liver disease
• Endocrine disorders
Graft rejection

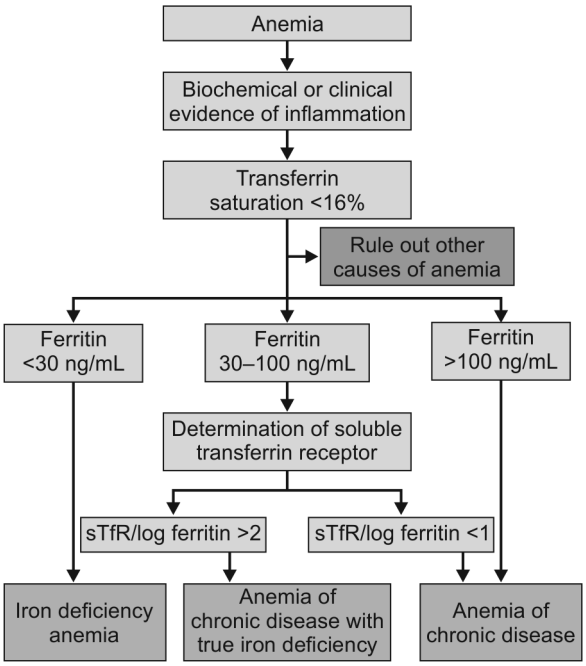
The abbreviation sTfR/log ferritin denotes the ratio of the concentration of soluble transferring receptor to the log of the serum ferritin level in conventional units.

In patients with ACD, however, the serum ferritin level indicative of adequate reticuloendothelial iron stores requires upward adjustment. Serum ferritin values usually increase in patients with inflammatory diseases and extreme elevations of serum ferritin may be a nonspecific indicator of significant underlying disease.[4] When iron deficiency coexists, the serum ferritin level falls but do not reach values as low as those found in uncomplicated iron deficiency. A patient with chronic inflammatory disease and a serum ferritin <30 μg/L is certainly iron-deficient, and a patient with a serum ferritin >200 μg/L is certainly not iron-deficient. Examination of a Prussian blue–stained marrow specimen can confirm the ferritin status. Flow chart 1 shows differences between anemia due to iron deficiency from anemia of chronic disease.

A determination of the levels of soluble transferrin receptors by means of commercially available assays can be helpful for differentiating between patients with anemia of chronic disease alone (with either normal or high ferritin levels and low levels of soluble transferring receptors) and patients with anemia of chronic disease with accompanying iron deficiency (with low ferritin levels and high levels of soluble transferrin receptors).[5]

The concentration of free protoporphyrin in the erythrocytes (FEP) tends to be elevated in patients with ACD.[6] However, FEP increases more slowly in anemia of chronic disorders than it does in iron deficiency.

Flow chart 1 Algorithm for the differential diagnosis among iron deficiency anemia, anemia of chronic disease, and anemia of chronic disease with iron deficiency



PATHOGENESIS

The pathogenesis of anemia of chronic disease has been attributed to following:

- Shortened erythrocyte survival
- Impaired marrow response
- Disturbance in iron metabolism.

The shortening of the erythrocyte survival creates an increased demand for red cell production on the marrow and the marrow is unable to respond fully because of a combination of a blunted erythropoietin response, an inadequate progenitor response to erythropoietin, and limited iron availability.

ACD is one manifestation of the systemic response to immunologic or inflammatory stress, which results in the production of various cytokines: the cytokines most often implicated in the pathogenesis of ACD are TNF[7], IL-1[8], IL-6[9], and the interferon,[10,11] concentrations of which have been reported to be increased in the serum or plasma of patients with disorders associated with ACD.[11,12]

Shortened Erythrocyte Survival

The rate of survival of cells from patients with arthritis, when transfused into normal subjects, is normal, and the survival of red cells from normal individuals in the circulation of patients with arthritis is less than the normal rate. Therefore, shortened red cell survival in patients with chronic inflammatory disorders is attributed to an extracorpuscular mechanism. IL-1 levels and shortened red cell survival are correlated in anemia patients with rheumatoid arthritis, and mice that become anemic after exposure to TNF *in vivo* also exhibit a shortened red cell survival.

Neocytolysis, a selective hemolysis of newly formed erythrocytes associated with erythropoietin deficiency, can also contribute to shortened red blood cell (RBC) survival in ACD.

IMPAIRED MARROW RESPONSE

The bone marrow which normally should compensate for decreased erythrocyte survival does not show increased marrow response.

The three proposed possible mechanisms are:

1. Inappropriately low erythropoietin secretion
2. Diminished marrow response to erythropoietin
3. Iron-limited erythropoiesis.

The erythropoietin response to anemia is blunted. This impaired erythropoietin response is cytokine-mediated. IL-1, TNF-α, and transforming growth factor-β inhibit

production of erythropoietin by various hepatoma cell lines. This reduces the tissue oxygen consumption so that normal oxygenation is maintained despite reduced hemoglobin levels. There is also increased erythrocyte 2, 3-diphosphoglycerate levels along with decreased hemoglobin oxygen affinity

TNF, IL-1, and interferon have all been reported to inhibit erythropoiesis *in vivo* and *in vitro*. TNF induces IL-1 production by macrophages, IL-1 induces interferon-γ production by T-lymphocytes, and interferon-γ can exhibit positive or negative feedback on production of IL-1 and TNF.

Treatment with recombinant human erythropoietin can correct ACD in many cases.

ABNORMAL IRON METABOLISM

A hallmark of anemia of chronic disease is the development of disturbances of iron homeostasis, with increased uptake and retention of iron within cells of the reticuloendothelial system. This leads to a diversion of iron from the circulation into storage sites of the reticuloendothelial system, subsequent on-restricted erythropoiesis. It has been proposed that lack of iron for erythropoiesis contributes to the inadequate marrow response in ACD. Evidence of a functional iron deficiency in this syndrome includes erythrocyte microcytosis, increased FEP, reduced transferrin saturation, and decreased marrow sideroblasts.

- The major contributor to hypoferremia in patients with ACD is probably a shift of iron from a transferrin-bound, available state to a ferritin-incorporated storage state. Iron absorption appears to be normal, but iron tends to remain in the mucosal cell and in hepatocytes and there is a limited availability of iron for erythroid progenitor cells. Macrophages, the major site from which iron is obtained for erythropoiesis, also exhibit increased iron storage.

Macrophage iron becomes available for erythropoiesis through two mobilization pathways:

- *Rapid pathway,* associated with almost immediate return of the iron retrieved from senescent red cells;
- *Slower pathway,* consisting of iron mobilized from storage[13]
- In ACD, the slower pathway predominates, and iron tends to accumulate. The acquisition of iron by macrophages most prominently takes place through erythrophagocytosis and the transmembrane import of ferrous iron by the protein divalent metal transporter 1 (DMT1). Recent studies of the role of the liver-produced antimicrobial peptide hepcidin strongly suggest that it is the dominant factor in this process.[14]
- Hepcidin is an acute-phase-reacting peptide, which is induced by IL-6, lipopolysaccharides and inhibited by TNF-α. Hepcidin appears to promote macrophage iron retention by causing internalization of the iron transport protein ferroportin.[15] Also causes decreased duodenal absorption of iron. Under certain conditions, hepcidin may be associated with impaired erythroid colony formation *in vitro*.[16] The mechanisms leading to anemia in anemia of chronic disease are given in Figure 1.
- *Apoferritin* is normally synthesized in response to increased intracellular iron concentration.[17] It has been suggested that excess apoferritin is made in inflammatory and malignant conditions, and the surplus binds a larger-than-usual amount of iron entering the cell.[18] In effect, such a mechanism would divert iron from the rapid to the slow pathway of iron release. Rodents injected with recombinant TNF developed a hypoferremic anemia associated with impaired storage iron release and incorporation into erythrocytes.[19]

IL-1 increases translation of ferritin messenger RNA and this additional ferritin act as a trap for iron that might otherwise be available for erythropoiesis.[20]

Nitric oxide, which is a common mediator of cytokine effects, has similar effects on ferritin expression.[21]

Lactoferrin is a transferring like protein in neutrophil-specific granules,[22] released from the neutrophil during phagocytosis or stimulation by IL-1.[23] Lactoferrin-bound iron is not immediately available for erythropoiesis as it transfers iron from its transferrin-bound, circulating state to a storage state.

In Panel A, the invasion of microorganisms, the emergence of malignant cells, or autoimmune dysregulation leads to activation of T cells (CD3+) and monocytes. These cells induce immune effector mechanisms, thereby producing cytokines such as interferon-α (from T cells) and tumor necrosis factor-α (TNF-α), interleukin-1, interleukin-6, and interleukin-10 (from monocytes or macrophages).

In Panel B, interleukin-6 and lipopolysaccharide stimulate the hepatic expression of the acute-phase protein hepcidin, which inhibits duodenal absorption of iron.

In Panel C, interferon-α, lipopolysaccharide, or both increase the expression of divalent metal transporter 1 on macrophages and stimulate the uptake of ferrous iron (Fe^{2+}). The anti-inflammatory cytokine interleukin-10 up regulates transferrin receptor expression and increases transferrin receptor–mediated uptake of transferrin bound iron into monocytes. In addition, activated macrophages phagocytose and degrade senescent erythrocytes for the recycling of iron, a process that is further induced by TNF-α through damaging of erythrocyte membranes and stimulation of phagocytosis. Interferon *a* and lipopolysaccharide down-regulate the expression

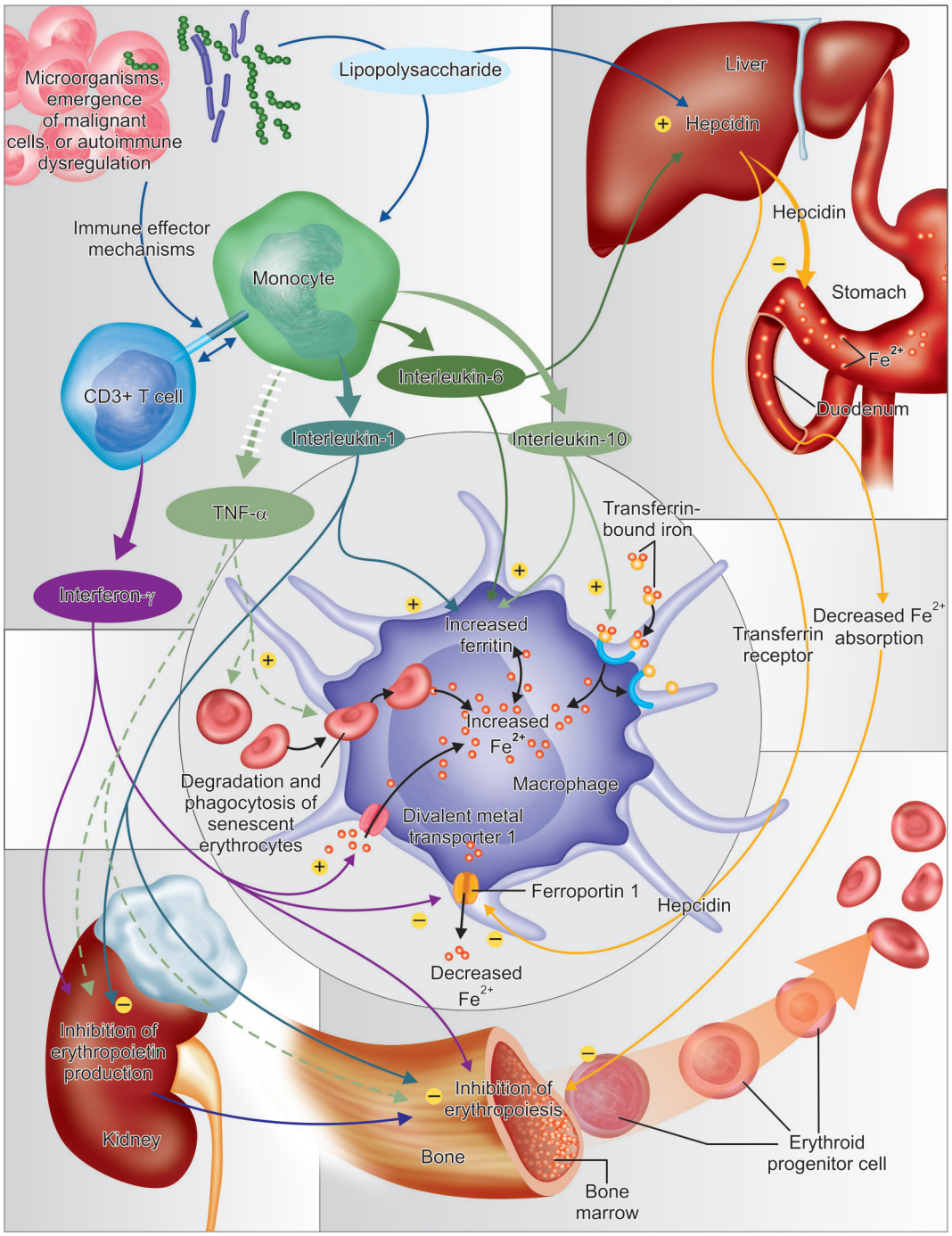


Fig. 1 Pathophysiological mechanisms underlying anemia of chronic disease

of the macrophage iron transporter ferroportin 1, thus inhibiting iron export from macrophages, a process that is also affected by hepcidin. At the same time, TNF-α, interleukin-1, interleukin-6, and interleukin-10 induce ferritin expression and stimulate the storage and retention of iron within macrophages.

In summary, these mechanisms lead to a decreased iron concentration in the circulation and thus to a limited availability of iron for erythroid cells.

In Panel D, TNF-α and interferon-γ inhibit the production of erythropoietin in the kidney.

In Panel E, TNF-α, interferon-γ and interleukin-1 directly inhibit the differentiation and proliferation of erythroid

progenitor cells. In addition, the limited availability of iron and the decreased biologic activity of erythropoietin lead to inhibition of erythropoiesis and the development of anemia. Plus signs represent stimulation, and minus signs inhibition.

Treatment Options

Moderate anemia warrants correction, especially in patients with additional risk factors (such as coronary artery disease, pulmonary disease, or chronic kidney disease), or a combination of these factors. In patients with renal failure who are receiving dialysis and in patients with cancer who are undergoing chemotherapy, correction of anemia up to hemoglobin levels of 12 g per deciliters is associated with an improvement in the quality of life.

In a retrospective review of nearly 100,000 patients undergoing hemodialysis, levels of hemoglobin of 8 g per deciliters or less were associated with a doubling of the odds of death, as compared with hemoglobin levels of 10 to 11 g per deciliter. Guidelines for the management of anemia in patients with cancer or chronic kidney disease recommend a target hemoglobin level of 11 to 12 g per deciliter.

Transfusion

Transfusions are particularly helpful in the context of either severe anemia (in which the hemoglobin is less than 8.0 g per deciliter) or life-threatening anemia (in which the hemoglobin is less than 6.5 g per deciliter), particularly when the condition is aggravated by complications that involve bleeding.

Iron Therapy

Oral iron is poorly absorbed because of the down regulation of absorption in the duodenum. Only a fraction of the absorbed iron will reach the sites of erythropoiesis, owing to iron diversion mediated by cytokines, which directs iron into the reticuloendothelial system.

In addition, iron therapy for patients with anemia of chronic disease is controversial. By inhibiting the formation of TNF-α, iron therapy may reduce disease activity in rheumatoid arthritis or end-stage renal disease.

In addition to possible absolute iron deficiency accompanying the anemia of chronic disease, functional iron deficiency develops under conditions of intense erythropoiesis[24] during therapy with erythropoietic agents, with a decrease in transferrin saturation and ferritin to levels 50 to 75 percent below baseline.[24]

Iron supplementation should also be considered for patients who are unresponsive to therapy with erythropoietic agents because of functional iron deficiency.

Erythropoietic Agents

The therapeutic effect involves counteracting the antiproliferative effects of cytokines,[25] along with the stimulation of iron uptake and heme biosynthesis in erythroid progenitor cells. Accordingly, a poor response to treatment with erythropoietic agents is associated with increased levels of proinflammatory cytokines, on the one hand, and poor iron availability.[24]

Three erythropoietic agents are currently available:

1. Epoetin alfa
2. Epoetin beta
3. Darbepoetin alfa.

These differ in terms of their pharmacologic compounding modifications, receptor-binding affinity, and serum half-life, thus allowing for alternative dosing and scheduling strategies.

The long-term administration of epoetin has been reported to decrease levels of TNF-α in patients with chronic kidney disease; reportedly, those who responded well to epoetin therapy had a significantly higher level of expression of CD28 on T cells and lower levels of interleukin-10, interleukin-12, interferon-γ, and TNF-α than did those with a poor response. Such anti-inflammatory effects might be of benefit in certain diseases such as rheumatoid arthritis, a disease in which combined treatment with epoetin and iron not only increased hemoglobin levels but also resulted in a reduction of disease activity.

The production of erythropoietin receptors by cancer cells appears to be regulated by hypoxia, and in clinical cancer specimens the highest levels of erythropoietin receptors were associated with neoangiogenesis, tumor hypoxia, and infiltrating tumors. Erythropoietin increases inflammation and ischemia-induced neovascularization by enhancing the mobilization of endothelial progenitor cells.[26, 27]

Monitoring Therapy

Before the initiation of therapy with an erythropoietic agent, iron deficiency should be ruled out. Hemoglobin levels should be determined after four weeks of therapy and at intervals of two to four weeks thereafter. If the hemoglobin level increases by less than 1 g per deciliters, the iron status should be reevaluated and iron supplementation considered.[28] If iron-restricted erythropoiesis is not present, a 50 percent escalation in the dose of the erythropoietic agent is indicated. The dose of the erythropoietic agent should be adjusted once the hemoglobin concentration reaches 12 g per deciliter.[29] If no response is achieved after eight weeks of optimal dosage in the absence of iron deficiency, a patient is considered nonresponsive to erythropoietic agents.

ANEMIA OF CHRONIC RENAL INSUFFICIENCY

The term anemia of chronic renal insufficiency refers to that anemia resulting directly from failure of the endocrine and filtering functions of the kidney. The kidney is the major source of erythropoietin and the ability to secrete this hormone is lost as the kidney fails. Renal failure is also associated with other pathologic processes that either inhibit erythropoiesis or shorten erythrocyte survival. Lack of sufficient erythropoietin is the most important factor in causing anemia; consequently, the hypoproliferative features of the anemia tend to predominate.In clinical settings, in chronic renal failure, additional factors may also contribute to the development of anemia such as :

- The presence of infection or inflammation
- Iron deficiency anemia due to blood loss from the gastrointestinal tract or hematuria or from retention of blood in the hemodialysis apparatus tubing[30]
- The megaloblastic anemia because of folate deficiency in patients on dialysis[31]
- Certain types of renal disease, including the hemolytic-uremic syndrome or thrombotic thrombocytopenic purpura, are associated with microangiopathic hemolytic anemia
- Aluminum intoxication can cause microcytic anemia in dialysis patients.[32]

Clinical Description

The nature of the underlying disease has little relation to the degree of anemia, although anemia may be less severe in patients with hypertensive renal disease and with polycystic disease.[33]

The severity of the anemia bears a rough relationship to the degree of renal insufficiency.

Anemia is not routinely observed until the creatinine clearance falls to <40 mL/minute, which corresponds roughly to a serum creatinine of 2.0 to 2.5 mg/mL in an average-sized adult. At creatinine clearance rates below that, a statistically significant correlation between creatinine clearance and hematocrit has been reported.[34]

Laboratory Findings

Hemoglobin and PCV: Anemia tends to become more severe as renal failure worsens, but in most patients the hematocrit ultimately stabilizes between 0.15 and 0.30.[2] The apparent degree of anemia may be exaggerated or minimized by alterations in plasma volume.

Peripheral blood film (PBF): The erythrocytes usually are normocytic and normochromic. The majority of red cells appear normal on blood smears. Occasionally, "burr" cells (Figs 2A and B) are observed along with some triangular, helmet-shaped, or fragmented cells.

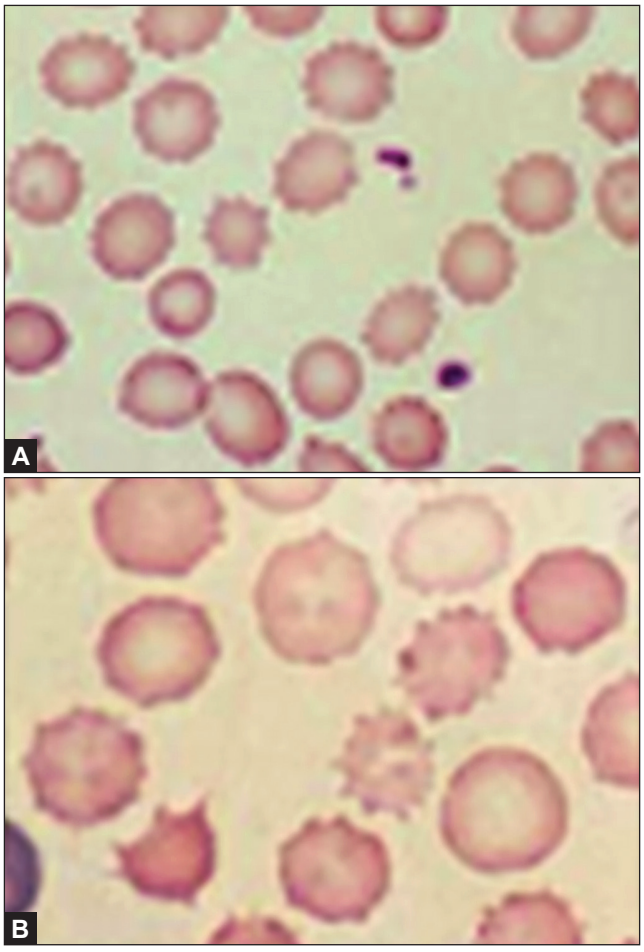
Reticulocyte count: The reticulocyte count often is within normal limits,[35] but it may be moderately increased.[36] The highest values were observed with extreme azotemia (BUN, 300 to 350 mg/dL).

Leukocyte count: The leukocyte count typically is normal, but slight neutrophilic leukocytosis may be observed.[35]

Platelet count and function: The platelet count is either normal or slightly increased,[35] but platelet function may be severely impaired resulting in defective hemostasis disease (×1500).

Bone marrow examination: The bone marrow tends to be hypercellular and slight erythroid hyperplasia may be observed. The myeloid-to-erythroid ratio averaged 2.5:1.0.[35] Erythroid maturation remains normal. In some instances, especially when renal failure is relatively acute, hypoplasia of erythroid elements is noted.

Liver function test: The serum bilirubin level is usually within normal limits but the hemolytic index (a measure



Figs 2A and B (A) Crenated cells in renal; (B) Burr cells in renal disease (X 3000)

of urobilinogen excretion in relation to total circulating hemoglobin) may be increased in ~40 percent of patients.[37]

Serum iron: It is normal in mild renal failure. Serum iron decreases in severe disease, or hyperferremia. The gastrointestinal absorption of iron is also reduced in patients with chronic renal failure.[38] FEP may be normal or moderately increased, but the increased values seem to occur only in patients with hypoferremia.[39] The erythrocyte lactate dehydrogenase level is within normal limits.

HbA_{1c}: The glycated fraction (A_1) of hemoglobin tends to increase in chronic renal failure. Hemoglobin A_1 value averaged 10.8 percent in patients with uremic patients compared with 7.1 percent in nonuremic individuals.[40] In uremic patients treated with dialysis, the value averaged 8.8 percent. The increase is thought to result from carbamylation of the hemoglobin molecule by urea-derived cyanate; it can be detected by using column chromatography. The increase in the A_1 fraction may continue after successful renal transplantation has brought the azotemia under control because of disturbed carbohydrate metabolism.[41]

Pathogenesis

The three factors involved are:
1. Erythropoietin deficiency
2. Suppression of marrow erythropoiesis
3. Shortened red cell survival.

- As renal function deteriorates, renal erythropoietin secretion decreases.[42] Measured erythropoietin values may be lower than normal, higher than normal, or normal.[42]
- Suppression of bone marrow
  - Retained uremic toxins depress erythropoiesis directly.[36,43]
  - Hyperparathyroidism may also contribute to marrow suppression. It is effects by causing marrow fibrosis.
  - Cytokine-mediated anemia mechanisms typically associated with ACD may be active in renal failure.
  - 20 to 70 percent of uremic patients show shortened red cell survival related to degree of azotemia.[36] In some patients, splenic sequestration of red cells may be a contributory factor.
  - In 20 percent patients, red cell pentose phosphate pathway is impaired.[44]
  - Oxidant drugs, such as primaquine or sulfonamides, produce a Heinz body hemolytic anemia in patients with the pentose phosphate pathway defect. Contamination of dialysate water by chloramines, which inhibit phosphoglyceromutase and thus cause accumulation of glycolytic intermediates, may worsen this defect.[44,45]
  - Impaired erythrocyte glycolysis has been found in uremic patients. Hemoglobin oxygen affinity is reduced,[46] because of increased erythrocyte adenosine triphosphate and 2, 3-DPG levels.
  - Neocytolysis, the selective hemolysis of newly formed red cells, has been reported after erythropoietic with-drawal in dialysis patients and may contribute to shortened red cell survival in dialysis patients.

Management and Course

- *Recombinant erythropoietin:* Recombinant human erythropoietin has been available for treatment of anemia of renal disease which has revolutionized the approach to this disorder. Erythropoietin can be administered intravenously or subcutaneously. Although erythropoietin was originally given three times weekly (to coincide with dialysis schedules), single weekly doses are similarly efficacious if the total weekly dose is increased appropriately.[48] A standard starting dose would be 100 to 150 U/kg/week, given as a single or in divided doses. Higher doses generally result in faster correction of anemia; target hemoglobin is typically attained within 6 to 8 weeks.[47] Iron supplementation is necessary, particularly in patients on hemodialysis. The target hemoglobin/hematocrit range is usually 12 g/dL/0.360.
- *Recombinant human erythropoietin (rHuEPO):* Determine the baseline serum erythropoietin and ferritin levels prior to starting rHuEPO therapy. If ferritin is less than 100 ng/mL, give ferrous sulphate 6 mg/kg/day aimed at maintaining a serum ferritin level above 100 ng/mL and a threshold transferrin saturation of 20 percent.
- Start with rHuEPO treatment in a dose of 150 units/kg/day subcutaneous three times a week.
- Monitor blood pressure closely (increased viscosity produces hypertension in 30 percent of cases) and perform complete blood count (CBC) weekly.

Titrate the dose:
If no response, increase rHuEPO to 300 units/kg/day SC three times a week.

↓

If hematocrit (Hct) reaches 40 percent, stop rHuEPO until Hct is 36 percent and then restart at 25 percent dose.

↓

If Hct increases very rapidly (>4% in 2 weeks), reduce dose by 25 percent.

Folic acid 1 mg/day is recommended because folate is dialyzable.

Side Effects/Adverse Reactions

- Erythropoietin is generally safe. When used for anemia in renal disease, hypertension is an important complication which is transient, confined to the first 3 to 6 months of treatment.[49] Rarely, the hypertension is abrupt and severe with encephalopathy and seizures. The pathogenesis is multifactorial. An increase in peripheral vascular resistance because of decrease in cardiac output, heart rate and stroke volume due to correction of anemia may be responsible.
- Anaphylaxis in response to erythropoietin has been described but is extremely rare.

Erythropoietin Resistance

The failure of patients to respond optimally to erythropoietin therapy or a requirement for unusually high doses is referred to as an erythropoietin resistance.

Iron deficiency is the most common cause.[50] Patients with serum ferritin levels <100 to 200 µg/L or with transferrin saturation values <20 to 25 percent[50,51] require iron supplementation. The best early predictors of erythropoietin response are serum TfR and serum fibrinogen. Response rates approaches 100 percent when both are low and 29 percent when both are high, reflecting both the patients iron status and the presence or absence of inflammation.

Inadequate hemodialysis is associated with erythropoietin resistance.[52] As discussed earlier, secondary hyperparathyroidism often accompanies renal failure and the associated marrow fibrosis may contribute to the anemia and to erythropoietin resistance.

Treatment with vitamin D_3 can decrease recombinant erythropoietin requirements and improve hemoglobin values.[53] If erythropoietin resistance is associated with an increased MCV, folate supplementation is warranted.

The use of angiotensin-converting enzyme inhibitors in renal failure patients may exacerbate erythropoietin resistance.[54] Splenectomy may be required in case of erythropoietin requirement increases in a patient with splenomegaly.

Anti-erythropoietin antibodies, including production of pure red cell aplasia, have rarely been reported.[55]

- *Darbepoetin (novel erythropoiesis-stimulating protein):* The long-acting erythropoietin analog Darbepoetin (novel erythropoiesis-stimulating protein) appears to be safe and effective in the anemia of renal failure.[56] The recommended starting dose is 0.45 µg/kg/week.
- *Iron:* Iron repletion and maintenance is the second pillar of anemia management in kidney disease. The current NKF-KDOQI recommendation for targets of iron therapy is to maintain serum ferritin at >100 ng/mL and TSAT at >20 percent in pediatric HD, PD and non-dialysis CKD patients.

Oral iron supplements, though cheap, are often insufficient to maintain iron stores, especially in HD patients, due to excessive blood loss, poor absorption, poor compliance with medications and gastrointestinal side effects.

The current NKF-KDOQI guidelines recommend oral iron therapy to be given in doses ranging from 2 to 3 mg/kg up to 6 mg/kg of elemental iron per day in two to three divided doses per day. Maintenance therapy aims to provide 1–2 mg/kg of elemental iron per week to achieve a TSAT between 20 and 50 percent and serum ferritin levels of 100 to 800 ng/mL.

RENAL REPLACEMENT THERAPY

Renal replacement approaches (transplantation and dialysis) aim to restore or substitute for lost renal function. As such, they may have some effects on anemia associated with renal failure.

RENAL TRANSPLANTATION

Renal transplantation is the complete and satisfactory treatment for renal insufficiency. Anemia is usually corrected over an 8- to 10-week period due to erythropoietin secretion by the grafted kidney.[57]

Two peaks of erythropoietin secretion have been documented. An early peak at 7 days and a second more sustained increase in erythropoietin levels, on approximately day 8, accompanied by reticulocytosis and a gradual increase in hemoglobin levels. Erythropoietin values return to normal when the hematocrit reaches 0.32.

Approximately, 80 percent of patients experience an increase in blood hemoglobin concentration after renal allograft.[57] Improvement in erythropoiesis occurs earlier when cyclosporin is used for immunosuppression rather than with antilymphocyte globulin (ALG).

DIALYSIS

Red cell production increases slightly in patients on hemodialysis, with small increases in hematocrit and decreases in transfusion requirement.[58]

As a general rule, anemia is less severe in patients receiving peritoneal dialysis, with consequently lower erythropoietin and transfusion requirements.[59,60]

Anemia in Cirrhosis and Other Liver Diseases

- Certain degree of anemia is commonly seen in patients with liver disease like alcohol-induced cirrhosis, biliary cirrhosis,[61] hemochromatosis, postnecrotic cirrhosis, and acute hepatitis.[62]
- The term *anemia of liver disease* refers to mild or moderate anemia associated with liver disease in the absence of any complicating factors such as blood

loss, marrow suppression by exogenous agents or nutritional deficiency.

- Some of the pathogenetic mechanisms of anemia include:
  - Shortened red cell survival and red cell fragmentation (spur cell anemia) in cirrhosis.
  - Hypersplenism with splenic sequestration in the presence of secondary portal hypertension
  - Iron deficiency anemia secondary to blood loss from esophageal varices in portal hypertension
  - Chronic hemolytic anemia in Wilson's disease secondary to copper accumulation in red cells
  - Aplastic anemia resulting from acute viral hepatitis (particularly hepatitis B) in certain immunologically predisposed hosts
  - Megaloblastic anemia secondary to folate deficiency in malnourished individuals.

Individuals with cirrhosis of any etiology are at increased risk for hemorrhage. Blood loss occurs in 24 to 70 percent of patients with alcoholic cirrhosis. The upper gastrointestinal tract is the major site of bleeding, but loss of blood from the nose, hemorrhoids, and uterus often occurs in association with coagulopathy of hepatic origins.

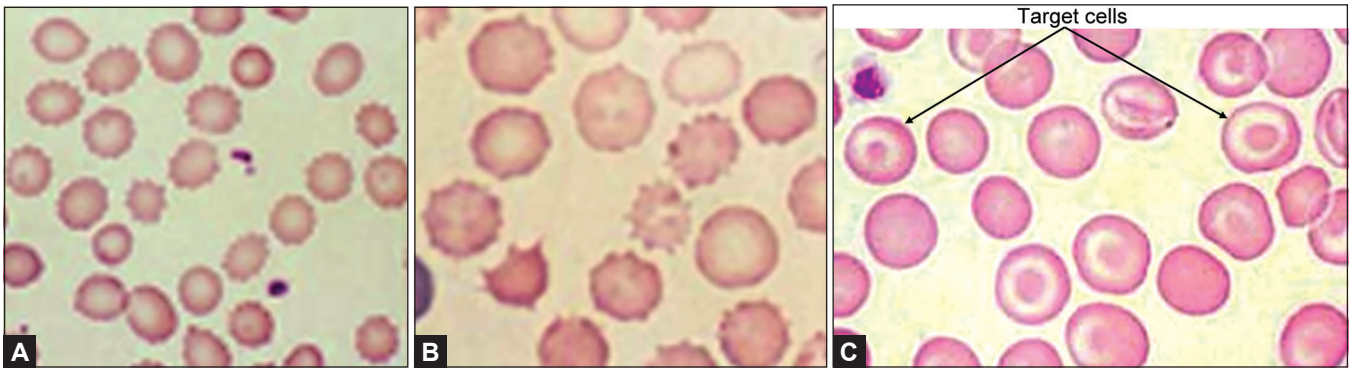
PREVALENCE AND CLINICAL MANIFESTATIONS

Approximately 75 percent of patients with chronic liver disease develop anemia as defined by a reduction in the hematocrit or hemoglobin level.[63] The whole blood volume in liver disease averages 10 to 15 percent greater than normal; thus, hemodilution tends to exaggerate the prevalence and degree of anemia.[64] Patients with more severe liver disease and bleeding tend to have reduced red cell mass.

The anemia is usually mild-to-moderate and hemoglobin level averages 12 g/dL or the hematocrit 0.36.[64] The hemoglobin level rarely falls below 10 g/dL in the absence of bleeding or severe hemolysis. Approximately, 5 percent of liver disease patients, with severe liver disease develop spur cell hemolytic anemia and hemoglobin concentrations <10 g/dL.[65] Episodic hemolysis when accompanied by jaundice and hyperlipidemia is known as Zieve's syndrome.

HEMATOLOGIC FINDINGS

- *PBF:* Anemia of liver disease is mildly macrocytic. Target cells and cells with increased diameters are evident on blood smear (Figs 3A to C). The cells appear hypochromic. These morphologic changes are accompanied by increased resistance to hemolysis in osmotic fragility tests.[66] When spur cell hemolytic anemia supervenes, characteristic acanthocytes—erythrocytes covered with five to ten spike like projections are evident.
- *Reticulocyte count:* The reticulocyte count often is increased, but sustained reticulocytosis of 15 percent or more is unusual in the absence of hemorrhage or spur cell anemia.
- *Platelet count:* Approximately 50 percent of patients with cirrhosis have mild thrombocytopenia, but values less than 50×10^9/L are uncommon.[63]
- *Total leukocyte count (TLC):* A variety of leukocyte abnormalities may be observed. Severe pancytopenia associated with splenomegaly in liver disease is known as Banti's syndrome.
- *Bone marrow aspiration:* Bone marrow cellularity is normal or increased.[63] Erythroid hyperplasia is observed. Red cell precursors have been described as macronormoblasts, a term that implies their size is increased, but their nuclear chromatin appears normal.[63,67] Frank megaloblastosis is seen in <20 percent of patients.



Figs 3A to C Anemia of liver disease. (A) Crenated cells in renal failure; (B) Burr cells; (C) Macrocytes and target cells in liver disease

PATHOGENESIS

- *Shortened erythrocyte survival:* Red cell survival is decreased in 33 percent of patients with alcoholic liver disease. Various causes are congestive splenomegaly, abnormal erythrocyte metabolism and hypophosphatemia with reduced erythrocyte adenosine triphosphate levels and consequent hemolysis.

  Characteristic alterations in red cell membrane lipids are found in patients with hepatitis, cirrhosis, and obstructive jaundice and may also be another contributor to shortened red cell survival.[68]
- In spur cell hemolytic anemia, the erythrocyte membrane accumulates excess cholesterol without a corresponding increase in lecithin, resulting in the characteristic morphologic abnormality. This change is accompanied by pronounced reduction in erythrocyte survival, probably because the distorted cells are less deformable than normal and thus become trapped by splenic macrophages.
- *Inadequate erythropoiesis:* The marrow response to the anemia in patients with liver disease may be inadequate. Serum from cirrhotic patients can suppress hematopoietic colony formation *in vitro*,[69] and cytokines implicated in inhibition of erythropoiesis have been found to be increased in patients with liver disease.[70] Dyserythropoiesis with morphologic abnormalities and intramedullary hemolysis has also been reported in severe liver disease.[71]

ANEMIA IN PATIENTS WITH CANCER

Much of the anemia commonly observed in patients with cancer can be attributed to the mechanisms involved in ACD. Erythroid precursors may be displaced from marrow by metastatic tumor, tumor-induced fibrosis, or tumor-associated marrow necrosis. The treatment of cancer can also produce or exacerbate anemia by a variety of mechanisms, including impaired erythropoietin production and cytotoxic effects of therapy on erythroid progenitors.

Typically, the serum transferrin is either low or low normal. The major differential diagnosis is iron deficiency anemia.

Treatment

Fewer than 30 percent of patients have anemia sufficiently severe to necessitate transfusion, and assessment of the symptomatic state should always be considered before administration of blood products.

Recombinant erythropoietin is effective and safe but expensive.[72]

Darbepoetin (also called novel erythropoiesis-stimulating protein) is an erythropoietin analog with modified glycosylation permitting a longer half-life. The results of studies comparing Darbepoetin and erythropoietin in anemic cancer patients vary depending on specific study endpoints, but both appear to be effective.[73]

It is debated whether or not to administer iron routinely to patients receiving therapy with erythropoietin products. Although there are reports of correction of ACD by intravenous iron without erythropoietin,[74] normalization of hemoglobin was only described in patients who were clearly iron-deficient. Anemic cancer patients treated with concurrent intravenous iron and recombinant erythropoietin appear to have a better response than those treated with no iron supplementation or with iron supplementation alone.[75]

ANEMIA ASSOCIATED WITH ENDOCRINE DISORDERS

A mild-to-moderate anemia commonly accompanies disorders affecting the thyroid, adrenals, parathyroids, gonads, or pituitary. It is usually not associated with symptomatology and in fact may reflect a physiologically appropriate hemoglobin concentration because the hormone deficiency often results in reduced oxygen requirements. Most individuals present as referrals for evaluation of moderate anemia with normal iron, B_{12}, and folate studies.

Hypothyroidism

A mild anemia with no other apparent etiology occurs in 10 to 25 percent of patients with hyperthyroidism.[82,83] Anemia is associated with severe or prolonged hyperthyroidism. Hemoglobin value falls but remains within normal limits.[82] MCV is either normal or modestly decreased. Hemoglobin A_2 levels are slightly increased but not as much as in thalassemia.[79] Both the anemia and the microcytosis are corrected when the hyperthyroidism is successfully treated. Erythropoiesis usually is accelerated but ineffective along with increased plasma erythropoietin levels.

Anemia is observed in 21 to 60 percent of hypothyroid patients. The anemia may be normocytic and normochromic, hypochromic and microcytic, or macrocytic. Hypochromic microcytic anemia found in association with hypothyroidism should be considered iron deficiency.[76,77] The microcytic anemia responds to iron therapy, even if thyroid hormone is not administered, but does not typically respond to thyroid hormone without iron.[76] Hypothyroid individuals are more likely to become iron-deficient because of predisposition to menorrhagia

and achlorhydria[77] and because thyroid hormone itself may be essential for normal iron absorption.[76] Severely, macrocytic anemia usually results from complicating deficiency of vitamin B_{12}[77] or folate.

Anemia usually affects children whose height is below the third percentile. The anemia usually is mild, with the hematocrit rarely falling below 0.35. The plasma volume often is decreased, which tends to make the reduction in hematocrit less than might be expected for a given decrease in red cell mass.[77] The degree of anemia is related to both the severity and the duration of the hypothyroidism.[78] The MCV may be increased in hypothyroid patients, even in the absence of anemia.[76] Acanthocytes are apparent in 20 percent of patients. Usually, the leukocyte and platelet counts are within the normal range, although both may be slightly reduced.[78] The bone marrow may be mildly hypoplastic, but the myeloid-to-erythroid ratio is not significantly altered. Hemoglobin A_2 levels are reduced slightly.

Pathogenesis

The anemia of hypothyroidism results from decreased red cell production. Plasma iron transport and erythrocyte iron turnover rates are reduced, indicating subnormal red cell production.[79] Erythropoietin secretion is reduced in hypothyroid patients,[80] and 2, 3-DPG levels are not increased[81] as occurs in most anemic and hypoxic states. The response of anemia of hypothyroidism to thyroid hormone is gradual. No striking reticulocytosis occurs, and the hematocrit returns to a normal value only gradually over approximately a 6-month period.[76,77]

Hyperthyroidism

A mild anemia with no other apparent etiology occurs in 10 to 25 percent of patients with hyperthyroidism.[82,83] Anemia is associated with severe or prolonged hyperthyroidism. Hemoglobin value falls but remains within normal limits.[82] MCV is either normal or modestly decreased. Hemoglobin A_2 levels are slightly increased but not as much as in thalassemia.[79] Both the anemia and the microcytosis are corrected when the hyperthyroidism is successfully treated. Erythropoiesis usually is accelerated but ineffective along with increased plasma erythropoietin levels.

Adrenal Insufficiency

Although anemia is common and nearly universal in adrenal insufficiency, it may be masked by the dehydration characteristic of this syndrome.[83] The red cells were normocytic and normochromic. Pernicious anemia is observed in 3 to 16 percent of cases of nontuberculous adrenal insufficiency and may complicate 13 percent of adrenal insufficiency cases associated with the polyglandular autoimmune syndrome type I.[84]

Androgen Deficiency

After puberty, values for the hematocrit, blood hemoglobin concentration, and red cell count average 10 to 13 percent higher in men than in women. In castrated men, these values fall to within the normal female range.[85] This is due to a difference in erythropoietin production. After the sixth decade, male hemoglobin values fall back toward those observed in women.[86] The anemia in these patients is corrected by androgen replacement.The differences in red cell parameters between the sexes are accounted for chiefly by the stimulating effect of androgens on erythropoiesis. The administration of androgens to castrated males restores male values for hemoglobin concentration. Androgens can also stimulate erythropoiesis in normal subjects. In normal men, testosterone enanthate induced an average red cell mass increase of 1.7 to 2.3. The increase in hematocrit was of smaller magnitude (from 0.456 to 0.494), probably because the plasma volume also increased. Androgens act by increasing renal synthesis of erythropoietin.[85] Estrogens produce anemia when given in large amounts which suggest that this effect results from suppression of hepatic synthesis of erythropoietin, but it may also simply represent opposition to androgen effects in general.

Hypopituitarism

Moderately severe anemia is seen as a feature of all types of pituitary insufficiency. Reduced hemoglobin levels are seen in Simmonds' disease, pituitary neoplasms and prepubertal pituitary dwarfs. The anemia usually is normocytic and normochromic and the red cells appear normal morphologically. Studies demonstrate reduced red cell production.[87]

The anemia of hypopituitarism results chiefly from deficiencies of the hormones of target glands controlled by the pituitary, especially the thyroid and adrenal hormones, but also from deficiency of androgens. In addition, lack of other pituitary factors, such as growth hormone,[87,88] prolactin, or factors characterized less clearly, may be of importance.

As suggested for the anemia of hypothyroidism, panhypopituitarism produces its effects on erythropoiesis chiefly by reducing tissue oxygen consumption.[88] The organism reacts to this decreased need for oxygen by secreting less erythropoietin and the red cell mass diminishes until a new equilibrium between oxygen supply and demand is established.

Treatment with a combination of thyroxine, cortisone, and growth hormone corrects both the anemia and the

marrow hypoplasia[88] and is more effective than any single hormone by itself. Administration of recombinant human erythropoietin (6,000 IU/day) was followed by correction of the anemia.

Hyperparathyroidism

Anemia is a rare complication of primary hyperparathyroidism.[89] The anemia is normocytic and normochromic and no evident reticulocytosis. Some authors conclude that parathyroid hormone decreases proliferation of erythroid precursors in culture. Marrow fibrosis may also be a result of excess hormone levels. Myelofibrosis is a common finding in bone marrow biopsy specimens, but the usual morphologic signs of myelophthisis are lacking.[89]

When hyperparathyroidism is secondary to renal disease, it is difficult to ascertain the relative importance of the hormone excess versus the erythropoietin deficit characteristic of renal failure as a contributor to the observed anemia. Medical treatment of hyperparathyroidism with vitamin D_3 can bring about improvement in anemia and decreased requirement for erythropoietin in some patients.[53]

Anorexia Nervosa

Anemia is observed in as many as 84 percent of patients with anorexia nervosa admitted to the hospital.[90] A moderate degree of leukopenia or thrombocytopenia may also be observed. The peripheral blood smear shows a striking composite process, and bone marrow examination shows gelatinous transformation with necrosis, as well as decreased cellularity in most cases.[90,91] These are essentially the findings observed in starvation, and they return to normal with improved nutrition.[90,91]

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CHAPTER 17

Thalassemia Syndromes

Mamta Vijay Manglani, Ambreen Pandrowala, Ratna Sharma, MR Lokeshwar

Thalassemia syndromes are a heterogeneous group of single gene disorders, inherited in an autosomal recessive manner, prevalent in certain parts of the World posing a major health problem. In populations from the Mediterranean basin, Indian subcontinent and South-East Asia, β-Thalassemia is the most common genetic variant associated with Thalassemia major. However population migration these days has, no longer, restricted the gene frequency to above tropical areas in which it was first observed, and hence it is seen all over the world.[1-4]

HISTORICAL REVIEW[1-6]

Thalassemia was first described by *Cooley and Lee in 1925*,[1] (Fig. 1) cases of severe anemia occurring in Italian children with hepatosplenomegaly, growth retardation, discoloration of skin and sclera, with peculiar bony changes, during the Transactions of "American Pediatric Society". It was then called as Cooley's anemia.[1]

The term *thalassemia* was first used in 1932, by Whipple and Bradford.[2] The word was taken from the Greek language which means great *sea* (anemia around the sea). As it was first described around Mediterranean countries. It was also called as *Mediterranean anemia*.[1,2] However, it was soon realized that it also occurs in South-East Asia, Indian subcontinent and Middle-East and not only around Mediterranean regions. The first case from India was reported by Dr M Mukherjee[5] from Campbell Medical School Calcutta India in 1938.[4] Dr PK Sukumaran[5] from Mumbai did pioneering work in the field of diagnosis of thalassemia syndromes in India.

Currently, as the life span of affected patients has been considerably prolonged by improvement in supportive care, the age distribution of patient population has dramatically altered. Furthermore, in many countries, the use of DNA based prenatal diagnosis has substantially reduced number of births of affected individuals, accentuating the trend of increasing age of thalassemia patients.

Fig. 1 Dr Thomas Cooley

EPIDEMIOLOGY[3,7-17]

Thalassemia Incidence: World Scenario

All over the world there are more than 250 million (1.5% of world population) carriers of β-thalassemia gene, and in South-East Asia, there are 40 million carriers of this gene (50% of these are in India alone, i.e. 20 million).[1-4]

Thalassemia syndrome: Indian scenario

The mean prevalence of the carrier status in India is 3.3% (ranging from 1% to 17% in various communities). If you draw a line between Mumbai (Bombay) and Kolkata (Calcutta) on the map of India, the region above the line has an incidence of 3–17 %, where as the region below the line has an incidence of less than 3%.

Thalassemia is more common in the following communities

North India	*Sindhis, Punjabis, Khatris, Kukrejas,* migrants from Pakistan
Gujarat Maharashtra	*Bhanushalis, Kutchis, Lohanas Mahars, Chamars, Buddhas, Navabuddhas, Kolis, Agris, Kunbis*
Andhra Pradesh and Karnataka	*Reddys, Gowdas, Lingayats, Kurgs*
Goa	Goud Saraswats
Other	Certain Muslim and Christian communities

It is estimated that every year approximately 100,000 children with thalassemia major are born world over.

With the birth rate of 22.8 per 1000 in India, it is estimated that there are about 65,000 to 67,000 β-thalassemia patients in our country and about 9,000 to 10,000 cases being added every year.

The prevalence of thalassemia varies in different communities, religions and ethnics groups.

The thalassemia belt stretches across the African continent, the Mediterranean regions, Middle-east, Indian subcontinent, South-east Asia, Thailand, Cambodia, Laos, Vietnam, Malaysia, Singapore, Southern China, and Melanesia[1] which is the same as malaria belt. The observation that the prevalence of thalassemia and falciparum malaria was similar, suggested the hypothesis that nature developed genetic mutation to overcome mortality and morbidity of malaria (Fig .2).

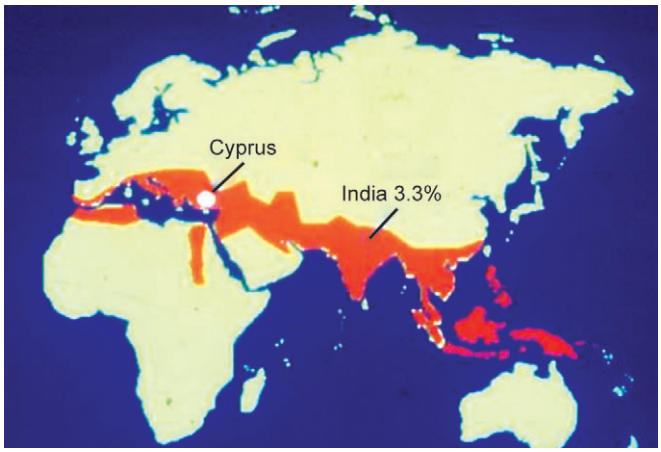
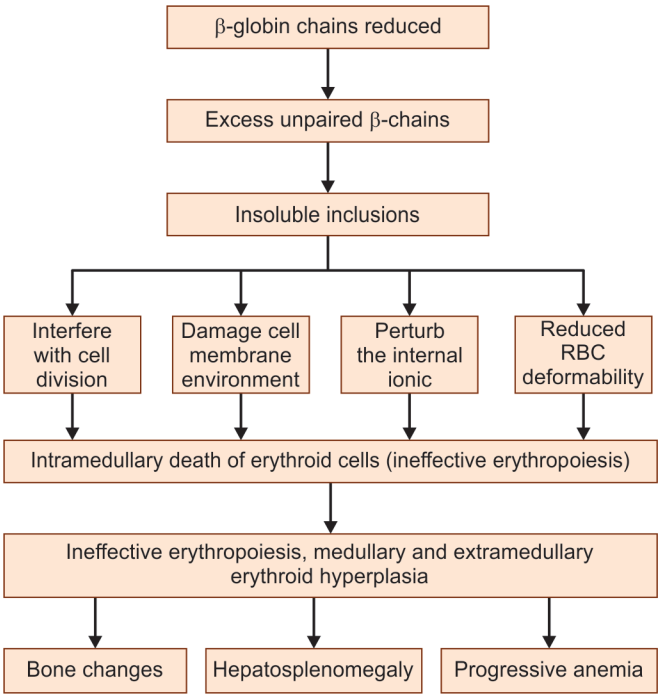


Fig. 2 Thalassemia Belt—World Scenario

Flow chart 1 Pathophysiology of β-thalassemia



PATHOPHYSIOLOGY (FLOW CHART 1)[18-21]

Normally hemoglobin consists of two pairs of amino acid chains:

- Adult hemoglobin HbA consists of two pairs of α-chains and β-chains each.
- **HbA_2** consists of two pairs of α-chains and δ-chains.
- **HbF** fetal hemoglobin is constituted by two pairs of α-chains and γ-chains.

Thalassemia is an inherited disorder of hemoglobin.

Thalassemia syndromes refer to a group of blood diseases characterized by defects in the synthesis of one or more of the globin chains that form the hemoglobin tetramer. This results in reduced or complete absence of production of one or more of the globin polypeptide chains of the hemoglobin molecule leading to imbalance in α and non-α chains of hemoglobin.

In β-thalassemia, decreased (β^+) or absent (β^0) chains lead to excess of unpaired α chains, that have no complementary non-α-chains with which to pair, form insoluble inclusions that precipitate on red cell membrane to form insoluble inclusion bodies, leading to damaged cell membranes, perturbed internal ionic environment of

the cells, decreased red cell deformability and interference with egress from the bone marrow spaces. This leads to premature destruction of RBCs in bone marrow (Ineffective erythropoiesis) and in peripheral circulation, particularly in reticuloendothelial system of spleen (Extravascular hemolysis) resulting in progressive anemia.

To compensate for the reduced hemoglobin, the synthesis of gamma chains persist after fetal life even beyond 6 months of age. The normal switch mechanism leading to reduction in β-chain synthesis does not occur. This leads to higher fetal hemoglobin $\alpha_2\gamma_2$(HbF) in postnatal life.

Increased fetal hemoglobin (HbF) with its high affinity for oxygen leads to tissue hypoxia, which in turn stimulates erythropoietin secretion leading to both medullary and extramedullary erythropoiesis (expansion of bone marrow space) causing characteristic hemolytic faces with fronto-parietal and occipital bossing, malar prominence and malocclusion of teeth and complications that include distortion of ribs and vertebrae and pathological fractures of the long bones, splenomegaly and its complication—hypersplenism, hepatomegaly, gallstones and chronic leg ulcers, etc.

The precise mechanism controlling the switch from fetal to adult hemoglobin (α_2b_2) is not fully understood.

CLASSIFICATION[3,22,23]

Thalassemias are classified depending on, which globin chain is defective—α(alpha), β(beta), γ(gamma), δ(delta), etc.

Classification of α-thalassemia (Tables 1 and 2)

The gene for α-globin chains is duplicated on chromosome 16 with each diploid human cell containing four copies of the α-globin gene. The four α-thalassemia syndromes with increasing clinical severity include silent carrier, α-thalassemia trait, HbH disease and hydrops fetalis due to 1,2,3 and 4 gene defects respectively.

The β-globin genes are represented one on each chromosome 11.

The β-thalassemias also include four clinical syndromes of increasing severity:

1. Silent carrier
2. Thalassemia trait
3. Thalassemia intermedia
4. Thalassemia major (Table 3)

The former two result from a single gene defect (heterozygous) whereas the latter two from both genes affected (homozygous).

Molecular Genetics (Figs 3 and 4 and Table 4) [5,24-43]

More than 200 mutations have been described that are responsible for thalassemia. The β-globin genes are clustered on chromosome 11 and are arranged over approximately 60,000 nucleotide bases (Fig. 3). These mutations occur in both introns and exons, and outside the coding regions of the genes. Most types of β-thalassemia are due to point mutation affecting the globin gene but some large deletions are also known. However, within each geographical region, few common mutations are responsible for over 90 percent of β-thalassemia.

Table 1 Classification based on the chain affected

• α-thalassemia	• α-chain is affected
• β-thalassemia	• β-chain is affected
• β^0-thalassemia	• β-chain is absent
• β + thalassemia	• If β-chain is partially present
• Double heterozygous states	• Sickle cell thalassemia, HbE thalassemia, HbD thalassemia, etc.

Table 2 Classification of α-thalassemia syndromes[18,22,23]

Syndrome	*Clinical features*	*Hemoglobin pattern*	*α-globin genes affected*
Silent carrier	No anemia, normal red cells	1–2% Hb Bart's (γ^4) at birth	1
α-thalassemia trait	Mild anemia, hypochromic microcytic red cells	5–10% Hb Bart's (γ^4) at birth	2
HbH disease	Moderate anemia, hypochromic, microcytic, red cells	5–30% HbH (β^4)	3
Hydrops fetalis	Death *in utero* caused by severe anemia	Mainly Hb Bart's, small amounts of HbH	4

Table 3 Classification of β-thalassemia[18,22,23]

Syndrome	*Clinical features*	*Hb pattern*	*β-globin genes*	*Inheritance*
Silent carrier	No anemia, normal, asymptomatic	Normal	1	Heterozygous state
Thalassemia trait	Mild anemia, hypochromic, microcytic red cells	Elevated HbA_2	1	Heterozygous state
Thalassemia intermedia	Moderate anemia, requires some transfusion occasionally and not dependent on blood transfusion for their survival	HbF elevated	2	Homozygous state/ Double heterozygous
Thalassemia major	Severe anemia, dependent on regular blood transfusion for survival	HbF elevated	2	Homozygous state

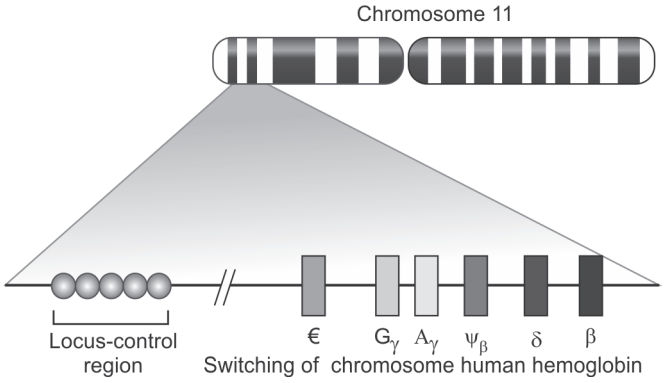


Fig. 3 The globin gene cluster on short arm

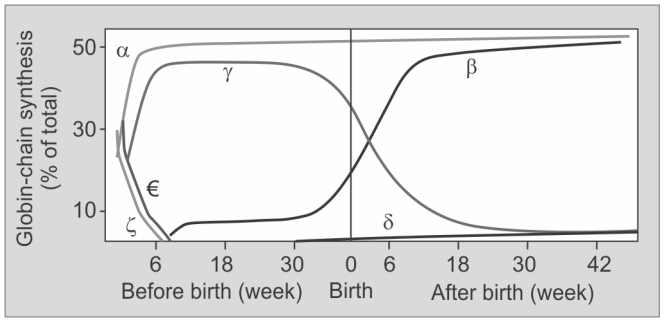


Fig. 4 Timing and normal development switching of chromosome human hemoglobin

Inheritance of Genes (Figs 5A and B)

- Inherited by autosomal recessive pattern
- If the child inherits one normal and one abnormal gene from each parent, child will have no disease (carrier/ minor)
- If both parents are carriers, i.e. thalassemia minor (single gene affected), there is a 1 in 4 (25%) chance of having a thalassemia major child in each pregnancy. This is depicted in Figures 5A and B.

Table 4 Various mutations have been found in the Indian population with β-thalassemia

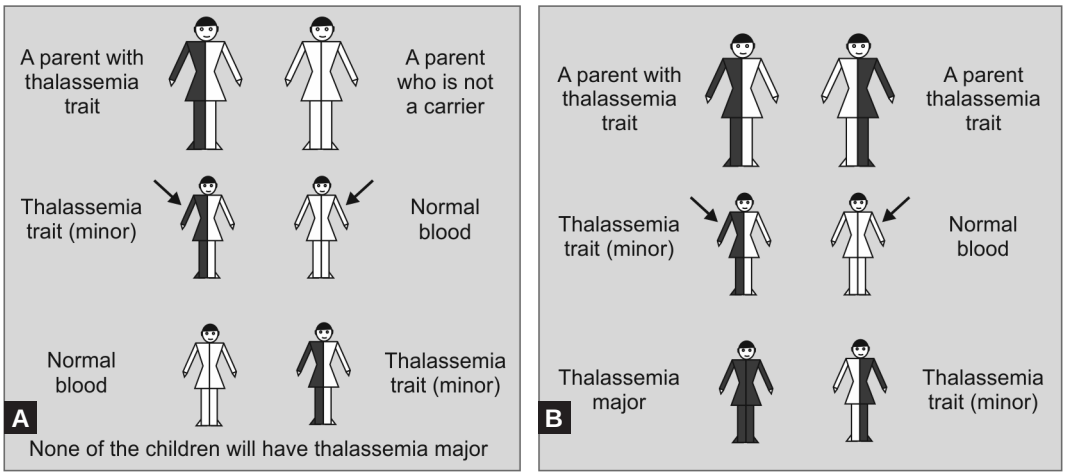
5 most common mutations in Indian population	*Newer mutations*
1. 619 bp deletion	• Codon 15 (TGG—TAG)
2. IVS 1-5(G-C)	• Codon 4/5 and 6 (ACT CCT GAG—ACA TCTTAG)
3. IVS 1-1(G-T)	• Codon 47/48 (+ATCT)
4. FS 8/9 (+G) and	• Codon 55 (+A)
5. FS 41/42 (-CTTT)	• IVS 2-837 (T to G)
	• Codon 88 (+T)
	• Codon 5 (-CT)
	• IVS 1-110 (G-A)

Clinical Heterogenecity of Thalassemia due to Diversity of Mutations

In populations in which thalassemia is prevalent, different types of mutations, affecting genes of either α- or β- or both globin clusters, may co-exist. Frequently, patients are compound heterozygotes for these mutations. The relative degree to which globin chain synthesis is impaired reflects this genetic heterogeneity and determines the clinical phenotype.

Mild β-thalassemia

- *Silent carrier:* Silent carriers of β-thalassemia have normal levels of HbA_2. However, they often reveal mild microcytosis, or a slight impairment in the β-globin synthesis in the peripheral blood reticulocytes. Several patients who are homozygous for the silent carrier β-thalassemia gene have been described. These children rarely require transfusions. They have significant hepatosplenomegaly and have HbF of 10 to 15 percent with an elevated HbA_2 (as seen in traits).



Figs 5A and B Inheritance of thalassemia

Two point mutations in the β-globin gene regions have been linked to the silent carrier phenotype.

- The -101 promoter mutations seen in Italians, Bulgarians and Turkish population.
- *+1 Cap site Inv mutation in an Indian family.*

β-*thalassemia trait:* Individuals with thalassemia trait are heterozygous for β-thalassemia, i.e. expression of one β gene is impaired by mutation, whereas that of the other gene is normal. Individuals with thalassemia trait exhibit mild microcytic, hypochromic anemia with basophilic stippling, target cells and elliptical cells on peripheral blood smear. Characteristically, HbA_2 and/or HbF are elevated in β-thalassemia trait, though various combinations may result depending upon the genetic mutation.

- *High* HbA_2 β-*thalassemia trait:* This is the most common form of β-thalassemia trait. HbA_2 levels vary from 3.5 to 8.0 percent whereas HbF from 1 to 5 percent.[27]
- *Thalassemia trait:* Individuals heterozygous for these mutations have increased HbF levels (5–15%) and low HbA_2 levels.
- *High* HbA_2 *high HbF* β-*thalassemia:* This form of thalassemia trait is associated with deletions of the globin gene that leave the δ- and β-goblin genes intact.
- *Normal* HbA_2 β-*thalassemia trait:* This should be distinguished from the silent carrier state. Both have normal HbA_2, however, individuals with normal A2 β-thalassemia trait have red cells which are hypochromic, microcytic in contrast to near normal red cells in a silent carrier.[25]

Severe β-thalassemia

Thalassemia major suggests severe homozygous β-thalassemia requiring regular transfusion to sustain life. Patients who are homozygous for β-thalassemia mutations, based on family studies, but maintain a hemoglobin concentration of 6 to 10 g% without transfusions are termed as thalassemia intermedia.

Various factors affect the severity of β-thalassemia. But the most important factor is the imbalance between α- and total non-α-globin synthesis.

Four factors determine this ratio:

1. The mutations (β^0 or β^+) in both globin genes.
2. Abnormalities in the α-globin gene cluster that increase or decrease α-globin gene expression.
3. The genetic capacity to produce HbF.
4. Co-inheritance of Xmn1 polymorphism.

- *Dominant* β-*thalassemia:* Rarely, heterozygote state of β-thalassemia may be associated with severe transfusion-dependent disease. Mutations involving the Exon 3 of the β-globin gene have been associated with such thalassemia.
- *Severe* β-*thalassemia trait:* Increased production of α-chains may lead to severe expression of β-thalassemia trait due to increase in unbalanced α-chains. Co-inheritance of triplicated α-globin gene chromosomes may result in severe β-thalassemia trait manifesting like thalassemia intermedia.[25]
- *Increased HbF synthesis:* β-globin gene deletions with mutations in γ-globin promoters are associated with increased HbF production and are shown to ameliorate the clinical course of thalassemia. This has

led to pharmacologic manipulation of HbF production to manage thalassemia patients.

Management of Thalassemia: Principles of Therapy

- Confirmation of diagnosis
- Transfusion therapy—packed red cell transfusions
- Management of complications
- Transfusion related complications
- *Iron overload:* Removal of iron with iron chelating agents
- *Hypersplenism:* Role of splenectomy
- *Transfusion transmitted infections:* Hepatitis B and C HIV, *Yersinia* spp, malaria, CMV
- Gallstones and leg ulcers
- *Curative treatment:* Bone marrow transplantation
- *Future treatment:* Pharmacologic manipulation of HbF/Gene therapy
- Prevention of the disease by antenatal diagnosis and genetic counseling

Confirmation of Diagnosis

Clinical manifestations of β-thalassemia:[1-4,6,7,9,11-17]

- Children with thalassemia major are generally diagnosed between 6 and 18 months of life. In India, many children born with thalassemia major die undiagnosed due to lack of facilities or ideal treatment. The spectrum of clinical manifestation of β-thalassemia varies widely. Severe β-thalassemia usually becomes manifest in the first year of life when the fetal hemoglobin (α_2,γ_2) starts declining and adult stable Hb cannot be formed.

The clinical syndromes associated with thalassemia, arise from the combined consequences of:

- Inadequate hemoglobin production
- Unbalanced accumulation of one type of globin chain.

The former causes anemia with hypochromia and microcytosis whereas the latter leads to ineffective erythropoiesis and hemolysis.

The high affinity of HbF for oxygen leads to tissue hypoxia, which in turn stimulates erythropoietin secretion leading to both characteristic hemolytic facies—with frontoparietal and occipital bossing, malar prominence and malocclusion of teeth.

At one end of the spectrum is the serious homozygous form (Thalassemia major) that presents in early infancy (6–18 months) with progressive pallor, failure to thrive, irritability, intercurrent infections and bony changes and hepatosplenomegaly. Ninety percent thalassemia children do not survive beyond 3 to 5 years of age if they remain undiagnosed and are not given red blood cell transfusions. Whereas at the other end of the spectrum is a heterozygous form (Thalassemia minor) in which the patient can lead a practically normal life except for a mild persistent anemia not responding to hematinics. They have a normal life span.

In between these two extremes are forms with varying degrees of clinical manifestations of anemia, splenohepatomegaly and bony changes who maintain their life fairly comfortably and are not dependent on blood transfusions for their survival and are called thalassemia-intermedia (they are also homozygous).

Untreated or irregularly treated children develop significant hemolytic faces including frontoparietal bossing with a hot-cross-bun appearance of the skull (caput quadratum with "hair-on-end" appearance on X-ray skull), depressed bridge of nose, malar prominences and malocclusion of teeth with protrusion and malocclusion of maxillary teeth.

LABORATORY DIAGNOSIS OF β-THALASSEMIA SYNDROMES (TABLE 5)

For a reliable diagnosis of thalassemia, it is advisable to correlate clinical profile and ethnicity of the individual with various laboratory investigations to confirm the diagnosis. Hemoglobin electrophoresis is the confirmatory test for diagnosis of most cases of thalassemia syndromes. However, a complete blood count and examination of the peripheral smear provide very vital information and are an important primary screen in thalassemia syndromes.

Thalassemia Major/Intermedia (Figs 7A and B): Complete Blood Count

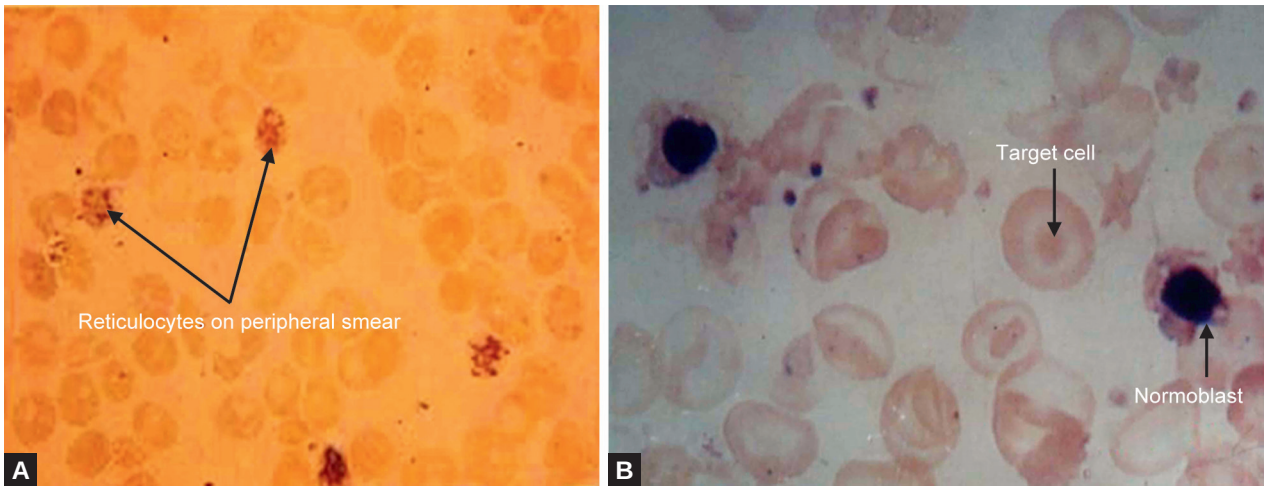
Complete blood count (CBC), examination of PBF and a reticulocyte count can help in suspecting and identification of hemoglobinopathies. CBC reveals generally severe anemia, a high leukocyte count (due to immature myeloid cells as well as nucleated red cells—also known as a "leukoerythroblastic reaction"). WBC and platelets may decrease if there is accompanying hypersplenism.

The red cell indices reveal a severe hypochromia with microcytosis. Patients in whom diagnosis is delayed there is significant macrocytosis due to relative folate depletion.

The red cell distribution width (RDW) in thalassemia major is significantly high (ranging from 30 to 40% for a normal value of 12–16%), suggesting a very high degree of anisocytosis. The peripheral smear shows a striking and characteristic bizarre picture with hypochromic, microcytic

Table 5 Clinical and hematological features of the β-thalassemia syndromes[1-4,6,7,9,11-17]

	Major	*Intermedia*	*Minor*
Severity of manifestations genetics	++++ Homozygotes, double heterozygotes	Homozygotes, double heterozygotes, rarely heterozygotes	++ Heterozygotes
Splenomegaly	++++	++, +++	+, 0
Jaundice	++	++, +	0
Skeletal changes	++++, ++	+,0	+,0
Anemia (Hb, g/dL)	<7	7–10	>10
Hypochromia	++++	+++	++
Microcytosis	+++	++	+
Target cells	10–35%	++	+
Basophilic stippling	++	+	+
Reticulocytes (%)	5–15	3–10	2–5
Nucleated red cells	+++	+, 0	+



Figs 6A and B Peripheral smear showing (A) Reticulocyte; (B) Target cell and normoblast

as well as macrocytic red cells, anisopoikilocytosis, target cells, polychromasia (more common in thalassemia intermedia), basophilic stippling, nucleated red cells and sometimes immature myeloid cells (Figs 6A and B) .

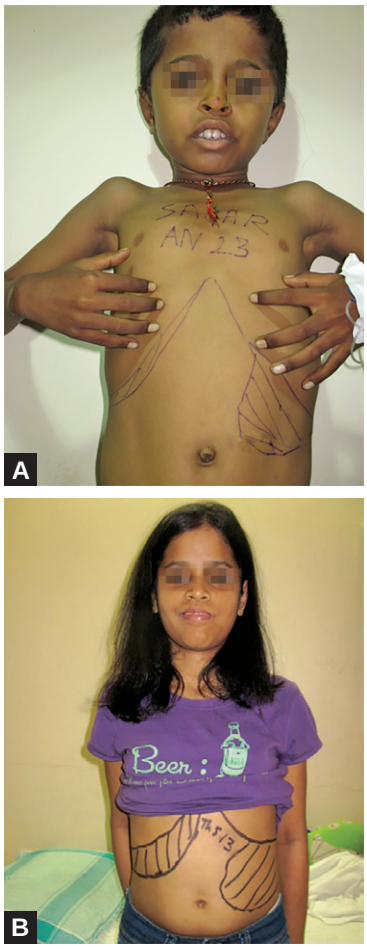
Thalassemia Minor

The CBC in thalassemia trait is associated with high red cell count relative to hemoglobin concentration and hematocrit, resulting in a marked fall in mean cell volume (MCV), mean cell hemoglobin (MCH) as well as mean cell hemoglobin concentration (MCHC). RDW is normal in thalassemia trait.

Reticulocyte count: Reticulocyte count is generally low to normal in thalassemia major, whereas in thalassemia intermedia, it is increased to 3 to 6 percent. The reason for a low reticulocyte count in thalassemia major is significant ineffective erythropoiesis preventing the precursor red cells from maturing to reticulocyte stage to be thrown into peripheral blood. In thalassemia intermedia, since the ineffective erythropoiesis is milder, the reticulocytes are increased in peripheral blood due to the anemia.

Naked-eye Single Tube Red Cell Osmotic Fragility Test (NESTROFT) for Thalassemia Minor[44-46]

Many investigators have studied naked-eye single tube red cell osmotic fragility test. The test has a high sensitivity of



Figs 7A and B (A) Thalassemia major on irregular treatment; (B) Thalassemia intermedia

95 percent, but its poor precision, interobserver variability and low specificity has precluded it from becoming a robust test.

Iron Studies[47]

Serum iron and transferrin saturation would be normal to increased (increased especially in multiply transfused children) in thalassemia major, whereas the total iron binding capacity (TIBC) would be decreased. It is generally normal to high even in thalassemia minor. Though iron deficiency is extremely uncommon in thalassemia minor, in our country, due to a high incidence of IDA, concomitant iron deficiency may be present in children with thalassemia minor. In such patients, serum iron level would be low with reduced transferring saturation and a high TIBC.

Serum ferritin is high in children with thalassemia major and normal to increased/decreased when concomitant iron deficiency associated in those with thalassemia minor.

In children with thalassemia major, the values of ferritin are proportionate to iron overload due to multiple transfusions. Serum ferritin estimation is used as the most common test for diagnosing iron overload. However, ferritin can be affected by various clinical situations—it may be elevated in acute infections as an acute phase reactant, in chronic diseases and chronic inflammatory disorders, etc.

Quantitation of Various Hemoglobins (Figs 8 and 9)

Separation of hemoglobins either by electrophoretic mobility or chromatographic separation is the confirmatory investigation for diagnosis of thalassemia syndromes.

Quantitation of various hemoglobins can be done by the following methods

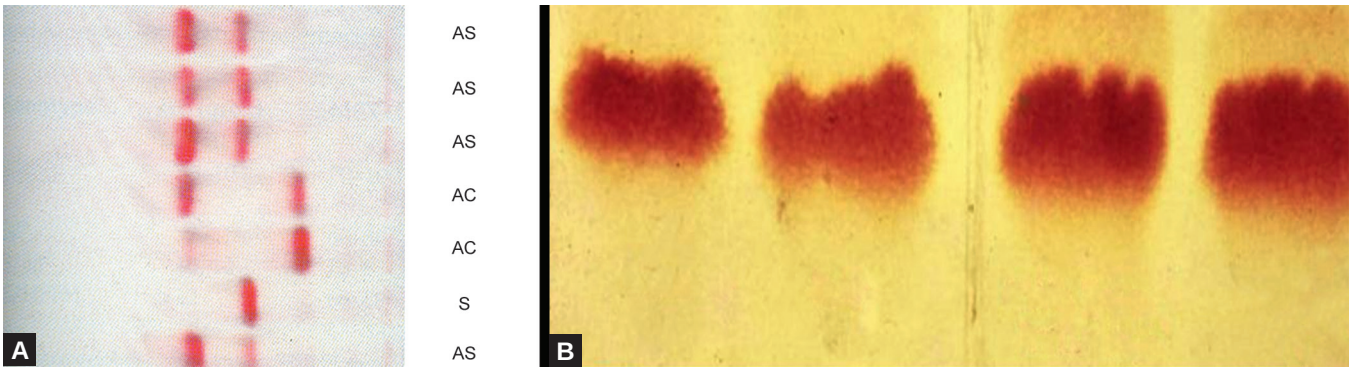
- Isoelectric focussing
- Microcolumn chromatography
- High performance liquid chromatography
- Both anion and cation—exchange HPLC
- Cellulous acetate electrophoresis (Fig. 8A)
- Paper electrophoresis (Fig. 8B)
- In untransfused patients with thalassemia major, hemoglobin pattern reveals 20–100% HbF, 2–7% HbA_2 and 0–80% HbA, the quantities varying depending upon the genotype. Thalassemia minor is characterized by elevated HbA_2 of more than 3.4% on paper electrophoresis, and more than 4% by certain other method.

- Microcolumn chromatography and HPLC,[48,49] by automated machines (Fig. 9A) are now becoming increasingly popular due to the ease of performing the test, less time consumption and greater reliability and reproducibility. It has thus become the gold standard for diagnosis of thalassemia syndromes and other hemoglobinopathies. It generates graphs depicting various abnormal and normal hemoglobins with quantification. HbA_2 value of > 9 percent indicates the presence of a co-eluting abnormal hemoglobin such as Hb E, Hb D Iran and Hb Lepore. Microcolumn chromatography and HPLC are currently used in most laboratories. Hemoglobins are separated graphically and quantified by photometer utilizing sophisticated computer (Fig. 9B).

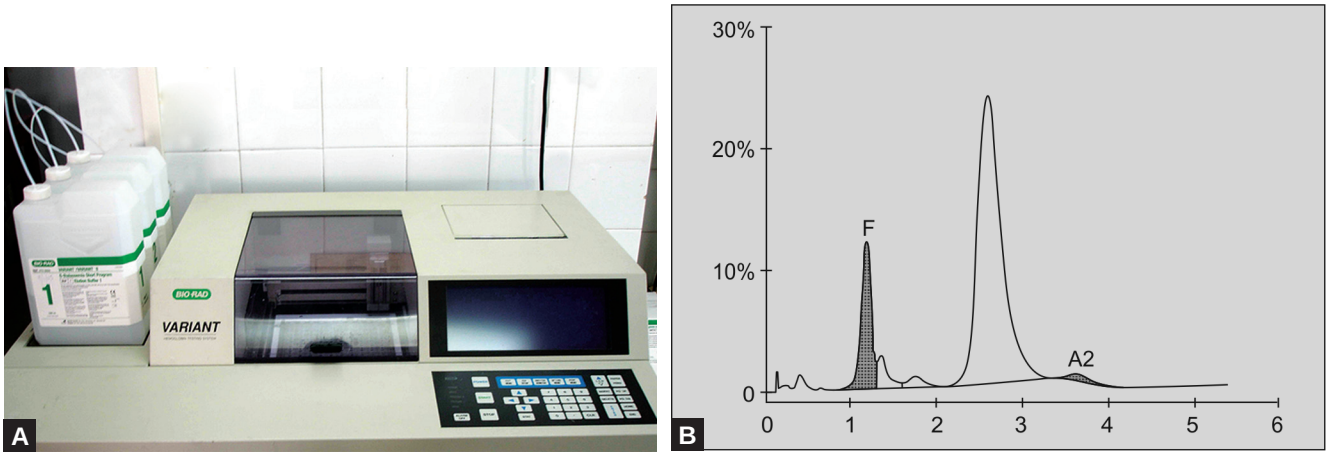
Radiological findings include widening of medulla due to bone marrow hyperplasia, thinning of the cortex and trabeculations and fracture are seen in long bones, metacarpals and metatarsals. X-ray—skull AP and lateral views show "hair on end"appearance (Figs 10A to C).

Tests Used for Estimating Iron Overload[50-53]

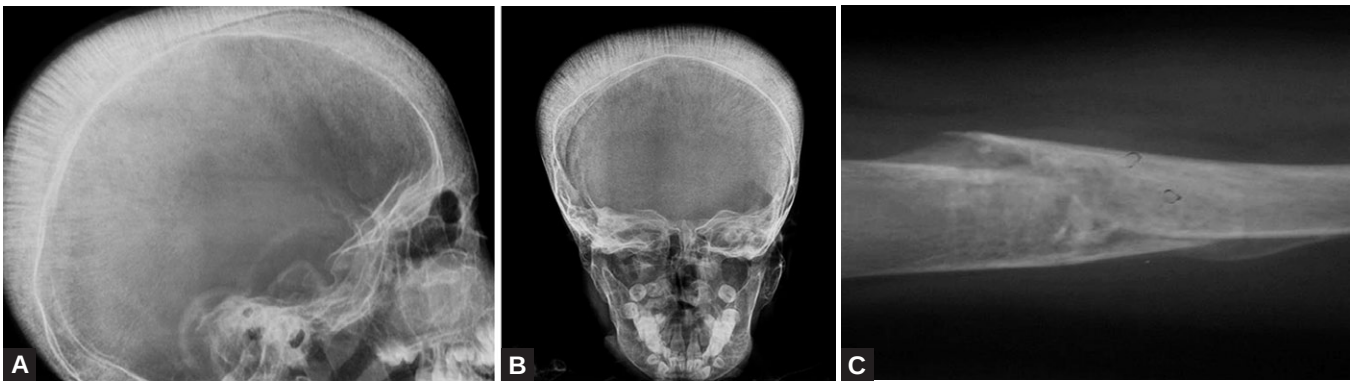
- *Liver iron concentration (LIC):* This is done on a liver biopsy tissue collected on a filter paper. Any level above



Figs 8A and B (A) Cellulose acetate electrophoresis; (B) Paper electrophoresis



Figs 9A and B (A) BIO RAD variant machine; (B) Pattern of hemoglobin in thalassemia minor/trait



Figs 10A to C (A and B) Thalassemia major X-ray skull; (C) Widening of medulla, thinning of the cortex and trabeculations, and fracture in the long bones

7 mg/g of dry liver tissue is indicative of significant iron overload. Levels above 15 mg/g is associated with cardiac iron overload.

- Superconducting quantum interference device (SQUID)[54,55] is a non-invasive method of estimating liver iron and has proven to be more accurate than serum ferritin in quantifying the total body iron overload.
- T2 weighted cardiac magnetic resonance imaging[56-60] helps in accurately determining the cardiac iron overload.

- Bone marrow examination not indicated for the diagnosis of thalassemia major but if done, it shows normoblastic erythroid hyperplasia with excessive iron on iron staining with Prussian blue.

Periodic tests for organ dysfunctions. While diagnosis of thalassemia major can be achieved by the tests, it is also mandatory to do baseline screening of various parameters:

Liver function tests (LFT) especially serum bilirubin, SGOT, SGPT, GGT, GTC (glucose tolerance curve).

- Baseline HBsAg, HIV and HCV antibody test
- Hormonal assays as and when required
- DEXA scan for density of the bones.

Clinical Consequences, Diagnosis and Management

Most of the complications of β-thalassemia are attributable to iron overload. Excess iron is toxic to the heart, liver, and various endocrine glands. In β-thalassemia, 70 percent of deaths are due to cardiac complications. Growth failure is seen in 30 percent of patients in the Western world and nearly all patients in our country, and is due to various factors including endocrine dysfunction. The other factors responsible for growth failure include: anemia, hypersplenism, desferrioxamine, and liver disease.

ENDOCRINE DYSFUNCTION[61-64]

The commonly affected endocrine glands include pituitary gland, thyroid gland, parathyroid gland, pancreas, gonads. Clinically, they may remain latent. Investigations should be done in all patients at regular intervals to detect these disorders and treat them appropriately.

- Diabetes may be seen as early as 5 years of age
- Short stature is commonly noted at 10 to11 years of age.

A glucose tolerance test, thyroid functions, serum calcium and phosphorus should be monitored every year, beginning at the age of 5 years and evaluation of height velocity, 10 years age onwards, goes a long way in early detection and treatment of these complications. Management would depend upon the specific abnormality found.

BONE DISEASE: OSTEOPENIA AND OSTEOPOROSIS (FIG. 11)[65-68]

Osteopenia and osteoporosis are major causes of morbidity in the aging thalassemic population, and it is suggested that prevalence is higher in men than in women. It is more severe in the spine than in the femoral neck.

Osteoporosis as defined by WHO as a "progressive systemic skeletal disease characterized by low bone mass and microarchitectural deterioration of bone tissue, with consequent increase in bone fragility and susceptibility to fracture".

- DEXA SCAN (Fig. 11) for assessing bone mineral density (BMD)—helps in identifying various bones with osteopenia and osteoporosis.
- Biochemical evaluation like serum calcium, serum phosphorus, serum alkaline phosphatase—help in identifying vitamin D deficiency as well as hypocalcemia, which may be due to hypoparathyroidism

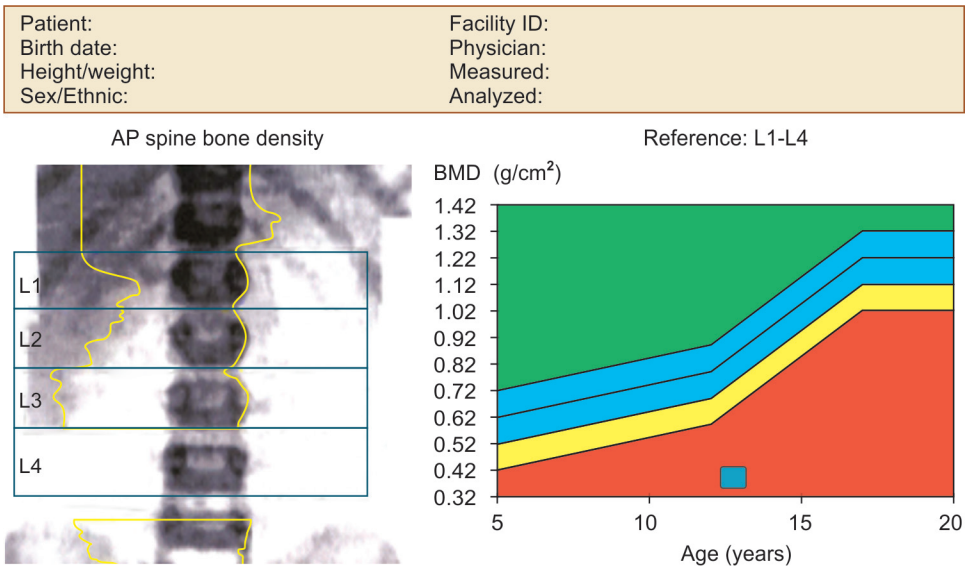


Fig. 11 DEXA scan in a child with thalassemia

in addition to calcium deficiency related to vitamin D deficiency.

Urinary calcium/Creatinine ratio >0.2 as well as urinary Phosphorus/Creatinine ratio >0.6 are useful screening parameters for suspecting osteopenia and osteoporosis. Genetic factors also play a role in bone mineralization, regulated by estrogen receptor gene, vitamin D receptor (VDR) gene, *COLIA1* and *COLIA2* genes. Furthermore, TGF-β1 has also been implicated as a possible mediator of coupling between bone resorption and formation. Testosterone, estrogen and progesterone are involved in the regulation of bone mineralization and contribute to the osteopenia and osteoporosis in thalassemic children.

Though studies have demonstrated iron deposition along the mineralizing perimeter of the bone, there are other factors influencing, such as anemia, which affects erythroid activity.

Cardiac Complications[69-71]

Most of the complications of β-thalassemia are attributable to iron overload. Excess iron is toxic to the heart, liver, and various endocrine glands. Iron overload causes deposition of iron in the ventricular walls, mainly in the left, relatively sparing the atria and the conduction system. In the ventricular wall, the epicardium contains most of the iron, the endocardium moderate with least iron in the intermediate layer. When iron accumulation increases, free iron damages the cells of the layers of the heart, inducing lipid peroxidation and lysosomal rupture.When iron accumulates in the cardiac tissue, free iron damages the cells of the layers of the heart, due to lipid peroxidation and lysosomal rupture. Cardiac failure and ventricular arrhythmias are the main cause of death in patients with β-thalassemia major and account for 70 percent of the deaths.

Cardiac iron overload related heart disease may be divided into three stages:

1. Preclinical
2. Early clinical
3. Advanced disease.

Early detection of cardiac involvement can be done by evaluation of serum ferritin level regularly and then doing the various tests to evaluate the cardiac functions like ECG, 2D echocardiogram, stress test, etc. However, these tests do not quantitate the cardiac function. T2 weighted cardiac MRI weighted cardiac MRI is the only method of assessing accurately the severity of cardiac iron overloading. In acute cardiac failure and arrhythmias, continuous subcutaneous desferrioxamine can reverse the ventricular dysfunction, whereas intravenous desferrioxamine can improve complicated arrhythmias and progressive heart failure.

In a study done at LTMG Hospital in 28 children with thalassemia major above the age of 10 years, 3 children had evidence of overt cardiomyopathy. Abnormalities such as left atrial dilatation, aortic root dilatation, reduced left ventricular internal dimension in systole and diastole were seen in 20 (71.42%), 11 (39.28%), 6 (21.42%) and 10 (35.7%) children respectively.

Ejection fraction was decreased in 16 out of the 28 (57.14%) patients. Of these, 33.3 percent were chelated adequately using deferiprone and 84.6 percent were unchelated. Four (14.28%) of the 28 children had decrease in fractional shortening.

Kaya SB et al.[80] also reported 3 out of 28 patients between the age group of 7 to 23 years with abnormalities on ECG.[51-53]

Since cardiac function has strong correlation with chelation early institution of appropriate and regular chelation and regular transfusion therapy to maintain pre-transfusion Hb above 10 gm% will lead to better cardiac function in these children. Early detection of cardiac involvement can be done by evaluation of ferritine level regularly and then doing the various tests to evaluate the cardiac functions like ECG, 2D echocardiogram, stress test, etc. However, these tests do not quantitate the cardiac function. T2 cardiac MRI is the only method of accessing accurately the severity of cardiac iron overloading.

Management of Thalassemia Major[72-76]

The management of thalassemia has undergone tremendous changes over the last 3 decades.

- If untreated, patients of thalassemia major die by the age of 3 to 4 years due to severe anemia.
- With transfusion therapy alone, patients with thalassemia major children died due to cardiac complications (related to iron overload) as early as 10 to 12 years of age. However, with the advent of chelation therapy as well as better screening procedures for transfusion related infections as well as leukodepletion through prestorage/bedside filtration, the lifespan of these children have improved considerably. If no complications occur, they live for an almost normal span with an improved quality of life.

Management of thalassemia involves a multidisciplinary therapeutic team approach and should be preferably done at a comprehensive thalassemia children care center with outdoor transfusion facilities (Table 6).

Transfusion Therapy in Thalassemia[77-79]

Packed red cell transfusions remain the cornerstone of therapy in thalassemia major. The decision to initiate lifelong regular transfusions in patients with β-thalassemia should be based on the molecular defect, severity of symptoms, and clinical criteria such as failure of growth, development and bone changes.

Table 6 Thalassemia outdoor center

Team Members	*Goals of Transfusion*
• Pediatric hematologist • Pediatrician • Dedicated nurses • Transfusion medicine specialist • Physiotherapist • Endocrinologist • Psychologist and social worker	• To obviate anemia • To reduce hepatosplenomegaly by reducing ineffective erythropoiesis • To reduce hemolytic faces • To improve tissue oxygenation • To improve growth

Progress in the Concept of Transfusion Therapy for Thalassemia

- In the 1960s, Wolman et al. proposed a palliative transfusion therapy[77] for children with thalassemia major. It was aimed at maintaining the hemoglobin at the level of 8.5 gm%. This led to improved survival, but the chronic illness, bone disease and cardiomyopathy persisted.
- To overcome these problems, Piomelli and workers suggested maintaining the hemoglobin.[78] A above minimum of 10 gm%. These vigorous regimens were termed as hypertransfusion, although Normo-transfusion may be a more descriptive term. Hypertransfusion promotes normal growth and development, prevents the onset of severe hepatosplenomegaly and hemolytic facies, lowers the absorption of gastrointestinal iron and reduces the anemic cardiomyopathy changes.
- In 1980, Propper and colleagues introduced a further improvised regimen[79] called supertransfusion, and maintained a pretransfusion hemoglobin of above 12 gm%. However this did not prove significantly superior to hypertransfusions and was given up. Hypertransfusion remains the most accepted regimen in most parts of the world. However, in Europe, a yet newer regimen termed the "moderate transfusion regimen" has been adopted and recommended by the Thalassemia International Federation. In this regimen, pretransfusion hemoglobin is maintained between 9 and 10.5 gm%.

INITIATION OF TRANSFUSION THERAPY

Before embarking on a lifelong transfusion therapy, it is essential to establish the diagnosis firmly with DNA analysis. This would help to know the severity of thalassemia as well as would help in prenatal diagnosis for future pregnancies.

One can ascertain the diagnosis of thalassemia Intermedia by observing the rate of fall of hemoglobin without transfusions. If the hemoglobin drops to below 7 gm% without transfusion, in absence of any concurrent illness, it is imperative to put the child on a regular transfusion program. If the child maintains hemoglobin above 7 gm%, the diagnosis of Thalassemia Intermedia has to be considered.

Whenever possible, it is equally important to know the compete genotype of the red cells to prevent red cell alloimmunization following repeated transfusions. However, this is not feasible in India and the alternative to this is Coomb's cross-match for each transfusion to prevent alloimmunization.

TYPE OF TRANSFUSIONS (FIGS 12 AND 13 AND TABLE 7)

The most ideal way to transfuse thalassemics is using group and type specific packed red cells that are compatible

Fig. 12 Cold centrifuge

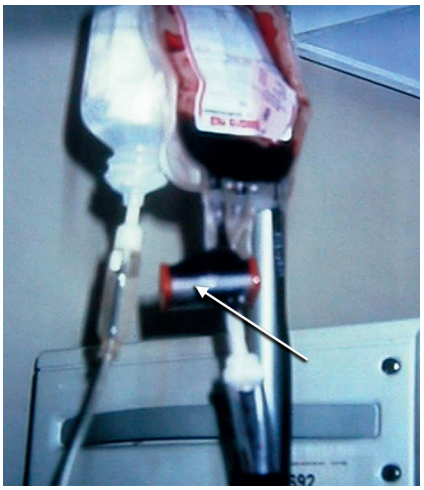


Fig. 13 Leukodepleting filters at bedside

Table 7 Various transfusion regimens (Progress in transfusion therapy)

Year of regimen	*Transfusion regimen*	*Pretransfusion Hb*
1960s	Palliative	Hb to 8.5 gm% (Wolman et al.)
1970s	Hypertransfusion	Hb 10–12 gm% (Piomelli and workers)
1980	Supertransfusion	Hb > 14 gm% (Propper and colleagues)
2005	Moderate transfusion	Hb 9–10.5 gm% (European regimen)

by direct antiglobulin test. The hematocrit should be standardized to 65 to 75 percent. This maintains the desired viscosity as well as aids in calculating the yearly requirement in a given patient. It is ideal to use prestorage leukodepleted blood, however, the next best is to use leukodepleting filters at bedside but they are also costly. An alternative to this is use of triple saline washed red cells. The red cells should be fresh, not more than 4 to 5 days old to maintain adequate levels of 2,3-DPG. Various other methods of leukodepletion are available, including use of frozen red cells (highly expensive and impractical in developing countries), filtration in the blood bank, use of apheresis, etc.

Neocyte transfusions to improve the survival of red cells after transfusion, have been tried but with limited success.

Amount and Rate of Transfusions

Approximately 180 mL/kg of red cells are required to be transfused per year in nonsplenectomized, nonsensitized patients to maintain the hemoglobin above 10 gm%, whereas splenectomized patients require 133 mL/kg per year. Even without hypersplenism, the requirement is 30 percent higher in nonsplenectomized patients (Table 7).

Rate of Transfusion

These red cells should be transfused 10 to15 mL/kg at the rate of 3 to 4 mL/kg/hour every 2 to 4 weeks to maintain the hemoglobin. Patients with cardiac decompensation should be given red cells at the rate of not more than 1 to 2 mL/kg/hour.

Adequacy of Transfusions

It is important to check the adequacy of transfusions to achieve best results and manage thalassemics ideally.

- In the first decade of life, normal growth confirms adequate transfusions.
- Also the number of normoblasts should be <5/100 WbCS in well-transfused children. This may be an indicator in older children. However, this is not applicable to those children who have not been initiated on therapy early and adequately in early life.

Advances in Transfusion Therapy

Daycare Transfusion Center

- Transfusions are given on an outdoor basis and hence no hospitalization is required.
- Children are more comfortable with the familiar staff members.
- There is less school absenteeism and parents lose fewer work days.
- The cost of the hospital stay is almost 5 times less.
- There is no threat of contacting infections from other patients in the wards.
- Parents and children are happy to be with other children and parents and discuss common issues of thalassemia management.

Management of Complications of Transfusion Therapy

A major problem encountered in the management of thalassemia is iron overload. Regular red cell transfusions to maintain hemoglobin and increased iron absorption from GI tract due to ineffective erythropoiesis and consequent low hemoglobin in irregularly transfused children is responsible for iron overload.

Transfusion Related Complications

- Iron overload
- Transfusion transmitted infections.

Iron Overload and Chelation Therapy

Clinical Consequences, Diagnosis and Management

Most of the complications of β-thalassemia are attributable to iron overload. Excess iron is toxic to the heart, liver, and various endocrine glands. In β-thalassemia, 70 percent of deaths are due to cardiac complications. Growth failure is seen in 30 percent of patients in the western world and nearly all patients in our country, and is due to various factors including endocrine dysfunction.

Iron Chelation Therapy[80-112]

The aims of chelation therapy are to reduce the serum ferritin to below 1000 ng/mL and hepatic iron concentration below 268 mmol/g of dry weight of liver. In addition, iron chelation also promotes growth and gonadal function. Established iron induced dysfunction of heart and liver may improve with effective chelation therapy, however, pituitary failure does not reverse. Improvement in thyroid and glucose tolerance is seen.

Chelation therapy should be started when:

- Serum ferritin level > 1000 ng/mL or above.
- More than 10 transfusions
- Hepatic iron concentration > 3.2 mg/g dry weight.

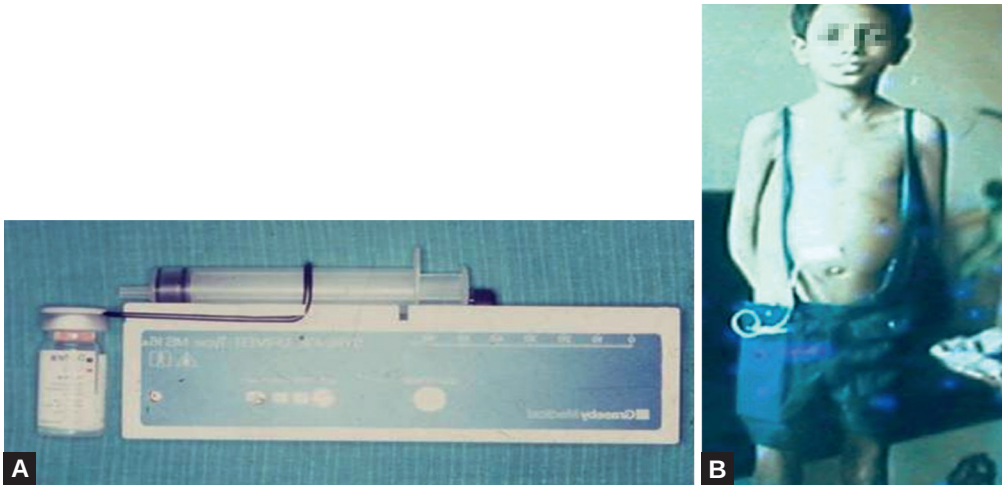
The standard available chelators used are:

- *Desferrioxamine (Desferal, DFO) (Figs 14A and B):* This was introduced in the early 1960s,[82-90] but its impact on the survival curves in thalassemic patients was recognized 15 to 20 years later. This is a hexa-dentate chelator and cannot easily mobilize iron from intracellular compartment due to its high molecular weight. It slowly binds iron to form Ferrioxamine and does not bind with iron from transferrin. 1 g of desferrioxamine binds 93 mg of iron. Desferrioxamine (DFO) is the gold standard therapy and is the most effective and safe iron chelator. Though, desferrioxamine is the gold standard it has not become popular particularly in the developing countries and is preferred by only 10 to 15 percent of thalassemia patients in our country. This is mainly due to its high cost and the need for continuous subcutaneous injection over 6 to 8 hours with the desferal pump. Hence, many of the thalassemic children develop complications related to iron overload and may require to be given high dose intravenous desferal, through the port or central line. This again is beyond the reach of many children in developing countries. The dose is 20 to 40 mg/kg/day given subcutaneously over 8 to 10 hours for 6 nights a week with the help of subcutaneous desferal infusion pump. In recent times, methods of administration have improved with better, more convenient smaller, lighter infusion pumps with LCD display. Balloon pumps, pre-filled syringes of desferal are available though they are prohibitively costly.

  Sixty percent of DFO chelated iron is excreted in urine and 40 percent in stool.

  Adverse effects include local reactions, auditory and visual toxicity, growth retardation, severe *Yersinia spp.* infections, etc. Rarely, pulmonary infiltrates and renal toxicity has been encountered.
- *Intravenous desferal (Table 8):* Intravenous desferal can be given particularly in those with very high iron overload through port-a-caths (central line). However, it is not easy to maintain the central catheter and infections are extremely common. However, high dose desferal (100 mg/kg, 3–9 g/day) can be given in severe hemosiderosis to prevent/reverse cardiac toxicity of iron overload.

  The dose for intravenous desferrioxamine is 50 to 100 mg/kg body weight. With depot preparation of desferal there is slow release of desferrioxamine leading to substantial plasma level of desferrioxamine in the blood for longer period and hence urinary iron excretion is sustained for longer period. This is more effective than the conventional subcutaneous infusion.



Figs 14A and B Desferal infusion pumps

Table 8 Indication for intravenous desferrioxamine is indicated in the following conditions

• Cardiac complications
• Very high ferritin level
• During pregnancy
• Prior to stem cell transplantation
• Rarely in patients with persistent local reaction at injection site

Toxicity of Desferrioxamine

- Minimal or no tachyphylaxis has been observed
- When given parenterally there may be liberation of histamine leading to bradycardia, hypo/hypertension, rigors, headache, photophobia, feeling cold and hot, etc.
- When given subcutaneously local pain, indurations, irritability and redness may occur
- Visual abnormality may occur in 4 to 10 percent of patients and includes decreased acuity of vision, peripheral field vision defects
- Defective dark adaptation, thinning of retinal vessels, retinal stippling, abnormal visual evoked responses and cataract
- High frequency sensory-neural hearing loss has been reported in 4 to 38 percent of patients
- Delayed linear growth accompanied by mild skeletal abnormalities such as short trunk, sternal protrusion and genu valgum.

As the auditory and visual toxicities are reversible, yearly slit-lamp examination and audiometry are mandatory to detect them early (Fig. 15).

Role of vitamin C: Ascorbic acid deficiency increases insoluble iron[90] (hemosiderin). Vitamin C helps in conversion of hemosiderin into ferritin, from which iron can be chelated. High doses of vitamin C can lead to increased free radical liberation and lipid peroxidation, resulting in tissue damage and rapid cardiac decompensation and even death. Addition of 100 mg of vitamin C daily, prior to DFO therapy increases iron excretion.

Oral Chelator

Deferiprone (L1) (Fig. 16) or 1,2-dimethyl-3-hydroxypyridin-4-one (Kelfer)[91-98] is a water soluble, bi-dentate molecule. It mobilizes iron from transferrin, ferritin and hemosiderin. It has been licensed for use in India since 1995. It is used orally and is less expensive. The recommended dose for deferiprone for effective iron chelation is 75 to 100 mg/kg body weight/day. Deferiprone/L1 has a better protective effect on myocardial tissue as shown in various studies. It also chelates zinc in addition to iron and therefore, zinc supplementation is mandatory in those receiving deferiprone.

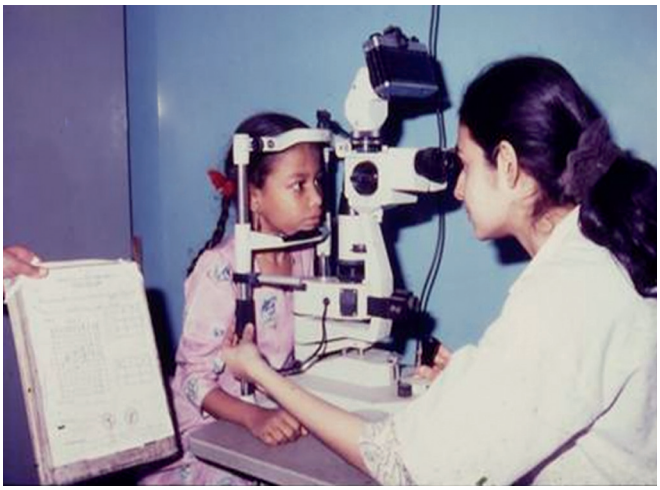


Fig. 15 Slit-lamp examination of eye

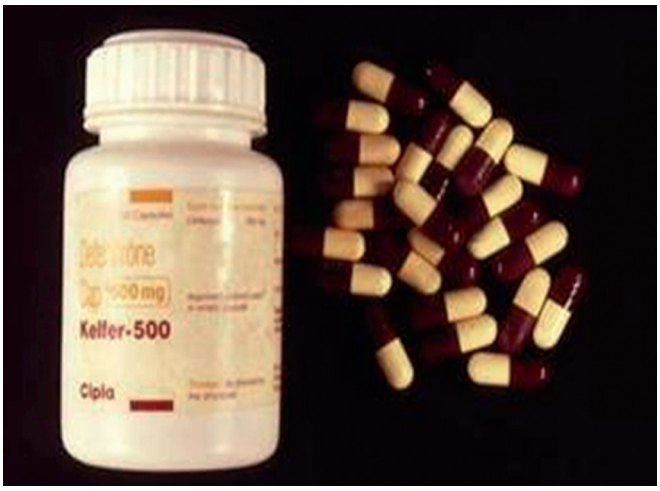


Fig. 16 Kelfer capsules

Side-effects of Deferiprone

- GI symptoms like nausea and vomiting
- Pain in abdomen and diarrhea
- Arthropathy
- Neutropenia/Agranulocytosis.

Patients on deferiprone should be monitored with monthly CBC, LFT, RFT besides clinical monitoring for arthralgia/arthropathy. It should be stopped in children who develop arthropathy and cytopenias. It may be restarted once the counts recover. In children with arthropathy, it should be cautiously be restarted in a lower dose of 50 mg/kg/day. If symptoms and signs recur, it should be discon-

tinued permanently. There are some controversial reports of increased hepatic fibrosis due to L1.

Deferasirox[99-101] or ICL670 (4- [3,5-bis (2-hydroxyphenyl) -1,2,4-triazol-1-y1] benzoic acid), (Asunra, Desirox, Deferijet, Desifer) is a new class of tri-dentate chelators[65-72] with a high specificity for iron. It is an orally active chelating agent developed for the treatment of chronic iron overload. The compound is an N-substituted bis-hydroxyphenyl-triazole that was selected from more than 700 compounds that were screened as part of a drug development program. Deferasirox is able to mobilize iron from both the hepatocellular and reticuloendothelial source. It has ability to prevent myocardial cell iron uptake and removes iron directly from myocardial cells, the liver cells, the intracellular labile iron pool and the surface of reticuloendothelial cells where iron is handed over to transferrin. Iron is excreted predominantly in the bile and hence in the feces.

This novel agent is well tolerated, with no major safety concerns at doses up to 80 mg/kg/day. Iron excretion is dose-dependent. The plasma half-life (11–19 hours) supports the once daily oral dosing regimen used in subsequent clinical studies.

It is also approved by the US FDA for use in non-thalassemia related transfusional iron overload. The drug is given orally as a single dose preferably in the morning on empty stomach. The recommended dose varies based on the serum ferritin levels and ranges from 20 to 40 mg/kg once daily and requires monthly monitoring of CBC, liver functions, renal functions, urine protein estimation and annual audiometry and ophthalmic examination.

Adverse Events

- Include gastrointestinal disturbances causing abdominal pain, nausea, vomiting, diarrhea and occasionally constipation.
- Headache, fever, anxiety and sleep disorders.
- Hearing loss has been reported.
- Nephropathy in patients with compromised renal function can be life-threatening.

Newer Iron Chelators

Desferrithiocin[102]

Desferrithiocin (DFT) caused kidney damage in rats on prolonged oral DFT. The damage was believed to be due to toxic effects of the Fe^{3+} complex, ferrithiocin. One of its analogs, 4-OH-desaza-desmethyl-desferrithiocin, appears to be less toxic while remaining biologically active as an orally administered iron chelator in animal studies. This analog may enter Phase I human trials shortly.

Hydroxybenzyl-ethylenediamine-diacetic Acid[103,104]

Hydroxybenzyl-ethylenediamine-diacetic acid (HBED) was able to clear radiolabeled iron when administered parenterally and that it remained active after oral administration. However, further evaluation in both iron-loaded primates and humans revealed that the oral activity was too small to be of value in the treatment of iron overload. Recently Bergeron et al. continued the preclinical evaluation of the efficacy and safety of HBED monosodium salt for the treatment of both transfusional iron overload and of acute iron poisoning in animals. Na-HBED was as efficacious as DFO in iron-loaded monkeys, either as a subcutaneous (SC) bolus or a 20-minute intravenous infusion (IV). Na-HBED was about twice as efficient as DFO in excreting iron. Safety evaluation showed no systemic toxicity.

Pyridoxal Isonicotinoyl Hydrazone[105]

Pyridoxal isonicotinoyl hydrazone (PIH) was identified as an effective iron chelator in 1979. PIH is a tridentate chelator with a selectivity for iron comparable to that of DFO. Over the years, various PIH analogs have been synthesized and some of them, such as pyridoxal-benzoyl hydrazones, were as much as 280 percent more effective than PIH. Studies in iron-overloaded patients treated with 30 mg/kg/day of PIH have much less than required iron excretion. However, recently investigators have examined the chelating potential of two additional PIH analogs, alone or in combination with DFO in hypertransfused rats. The latter studies have shown that the orally administered analogs are about 2 to 6 times more effective than intraperitoneally administered DFO in mobilizing liver iron in rats.

GT56-252[106,107]

GT56-252 is a novel orally available iron chelator derived from DFT that forms a 2:1 complex with Fe^{3+}. Eighteen adult patients with β-thalassemia received 3 to 8 mg/kg in 2 doses with food or fasting. The compound was well tolerated, with no related serious adverse clinical events, laboratory abnormalities or changes in the electrocardiogram (ECG). Further studies are in progress to define the effect of GT56-252 on iron balance.

40SD02 (CHF1540)[108,109]

40SD02 is a new entity synthesized by chemically attaching DFO to a modified starch polymer. The resulting high molecular weight chelator has a prolonged half-life. A Phase I study in 10 patients with thalassemia and chronic iron overload showed that single doses of up to 600 mg/kg of the compound were safe and well tolerated, and stimulated a clinically significant amount of iron excretion.

Combination Therapy: The Shuttle Hypothesis[110,111]

Additive and synergistic effects of combination of iron chelators have been explained by the shuttle hypothesis. The theory is that a bidentate (L1) or tridentate ligand with access to a variety of tissues acts as a "shuttle" to mobilize the iron from tissue compartments to the bloodstream, where most exchanges with a larger hexadentate (DFO) "sink". The sink binds this iron irreversibly, promoting its excretion. Experiments using a DCI assay showed that simultaneous administration of L1 and DFO produced shuttling of iron from L1 (shuttle) to DFO (sink).[110,111] Clinical studies using DFO and L1 in combination have confirmed this hypothesis. Several other combinations exhibiting shuttle mechanism have been tried with success—HBED and L1 as well as ICL670A and DFO (in experimental cells).

Management of Bone Disease in Thalassemia Major: Osteopenia and Osteoporosis (Intervention Recommended Based on BMD Result) (Table 9)[65-68]

Hormone replacement therapy with estrogen in females and hCG for males improves bone density parameters. Calcitonin, an inhibitor of osteoclasts, can reduce osteoporosis and increase cortical thickness in thalassemic children. Hydroxyurea, oral biphosphonates or injectable pamidronate are other useful modalities.

Prevention of Osteopenia/Osteoporosis

Children with β-thalassemia should be encouraged to indulge in moderate- and high-impact activities such as walking, ballet dancing, aerobics, climbing, mild sports, jogging, running, etc. to prevent bone changes. The diet should be rich in calcium and vitamin D may be supplemented. Hormone replacement therapy for endocrine abnormalities needs to be administered.

Table 9 Management of osteopenia/osteoporosis

Category	*Treatment*
Normal	Diet + exercise
Osteopenia	Diet + exercise + calcium
Osteoporosis	Diet + exercise + calcium + bisphosphonates
Established osteoporosis	Diet + exercise + calcium + bisphosphonates + treatment of fractures

Management of Cardiac Complications[69-71]

Investigations such as Holter monitoring, 2D-echo-cardiography and stress test assist in evaluating, but do not quantitate, the cardiac function. T2 weighted cardiac MRI[58-62] is the only method of assessing accurately the severity of cardiac iron overloading.

In acute cardiac failure and arrhythmias, continuous subcutaneous desferrioxamine can reverse the ventricular dysfunction, whereas intravenous desferrioxamine can improve complicated arrhythmias and progressive heart failure.

Transfusion Transmitted Infections[113-126]

The common transfusion transmitted infections seen in these multiply transfused thalassemic children include the following:

- Hepatitis B and C
- HIV
- *Yersinia spp.*
- Malaria
- CMV.

Hepatitis B has been reported in 5 to 30.6 percent amongst multiply transfused individuals in various studies.

A study at LTMG hospital have reported hepatitis B infection—recent or past in 85 percent of cases at LTMG Hospital, Mumbai, Maharashtra, India.

- Stringent screening of donors and testing of all blood bags for HIV, HBsAg and Anti-HCV has reduced the rate of these transfusion-transmitted infections.
- Additionally, it has been possible to effectively control hepatitis B through transfusions by vaccinating with hepatitis B vaccine, all children at diagnosis, with double dose, intense regimen (0, 1, 2 and 12 months).
- Boosters may be given at 5-year intervals.

Hepatitis C

The prevalence of hepatitis C is quite high in thalassemics the world over, and more so in our country. Hepatitis C has been reported in 39 percent of transfused thalassemics (before it was mandatory to screen all blood bags for HCV antibodies), whereas Narang et al.[115] from Delhi have reported it in 69 percent, on the other hand, Wonke et al.[116] reported HCV positivity in 11.1 percent and Sarin et al.[117] in 53.3 percent of cases. Agarwal et al.[118] from Mumbai, have reported hepatitis C in 16.7 percent of cases.

Treatment of these patients is difficult, as it is highly expensive. Interferons and Ribavirin are the commonly

used therapies. Pegylated interferons are obtained by conjugating Intron A with polyethylene glycol. This prolongs the activity of interferon allowing weekly dosing instead of the conventional thrice weekly dosing.

Human Immunodeficiency Virus (HIV)

The occurrence of transfusion transmitted HIV has ranged from 0 percent by Chaudhary et al.[113,114] to 70 percent by Currimbhoy et al. It has been reported in 10 percent of children in one study by Manglani and Lokeshwar[24] from LTMG Hospital, Mumbai. Children with transfusion transmitted HIV progress to AIDS over a period of 5 to 10 years unlike perinatally infected children, who progress quite rapidly. They can be managed with antiretroviral therapy, as per the national guidelines for children and adults.

Other infections commonly seen in these children include malaria and CMV infections.[123,124] CMV infection can be prevented by reducing the leukocyte content of packed red cells, either by pre storage filtration or using bedside leukocyte filters. Also, use of red cells from CMV-negative donors and frozen red cells are other alternatives in the developed world. Malaria has to be treated as and when it occurs, as despite screening blood bags for malaria, it would not be possible to identify all donors harboring the parasite due to a low sensitivity and inter-observer variability of peripheral blood smear evaluation.

Yersinia spp. infection is known to occur in iron overloaded[125,126] and desferrioxamine-treated patients. If a patient on desferrioxamine comes with symptoms of abdominal pain, diarrhea and vomiting, desferrioxamine should be stopped immediately, appropriate stool culture or blood serology undertaken and treatment with co-trimoxazole or aminoglycoside given.

Gall stones[127,128]: With better management of thalassemia with regular and adequate transfusions, the incidence of gallstones has considerably decreased. However, those who do not receive optimal treatment, may develop gallstones. Prophylactic cholecystectomy during splenectomy has been recommended as a safe, standard procedure for these patients.

Leg ulcers (Fig. 17)[129]: Ulceration around the ankles is commonly seen in thalassemia intermedia and inadequately transfused thalassemia major. This occurs due to venous stasis, vaso-occlusion, anemia, and local trauma. Treatment includes bed rest, increased transfusions, wound care, and the use of local tissue factors. Local irrigation of the wound with granulocyte-colony stimulating factor (G-CSF)—1 vial diluted in normal saline has been found beneficial.

Hypersplenism (Fig. 18)[130-133]

- Hypersplenism may occur due to inadequate transfusions, alloimmunization, rarely autoimmune hemolysis complicating thalassemia major and chronic liver disease. Splenectomy is recommended when the transfusion requirement exceeds 200 to 250 mL/kg/year of packed red cells.
- Splenectomy should be deferred till 5 years of age.
- Following vaccines should be given 3 to 6 weeks prior to procedure
  - Meningococcal
  - Pneumococcal
  - *H. influenzae type b*
- Lifelong penicillin prophylaxis should be advised.

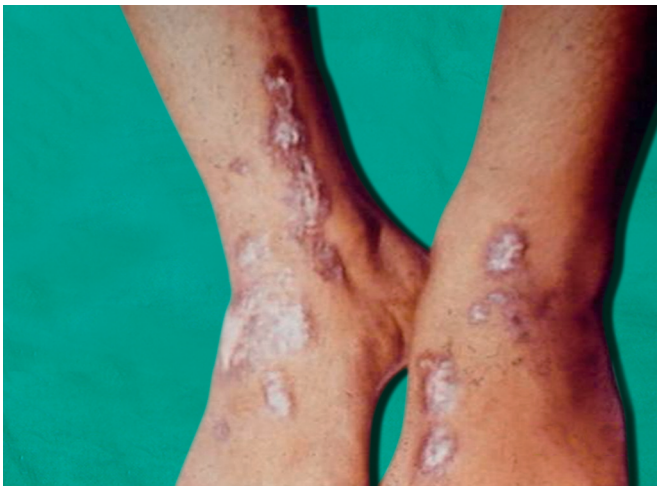


Fig. 17 Leg ulcer in thalassemia

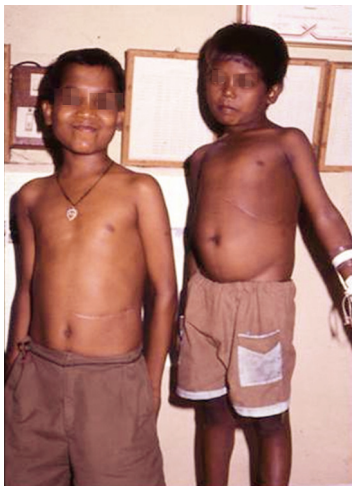


Fig. 18 Splenectomy

- Hospitalize promptly whenever required for cultures and antibiotic sensitivity and for IV antibiotics.
- Treat the infection promptly with appropriate antibiotics.

Curative Treatment

Stem Cell Transplantation (Fig. 19)[134-139]

Bone marrow transplantation offers the potential permanent cure, if an HLA-matched sibling donor is available. Though expensive, it is cost-effective when compared with the long-term ideal treatment.

Multiple pricks, blood transfusion hospitalization, can be averted. The outcome of this procedure depends upon the three factors:

1. Hepatomegaly
2. Hepatic fibrosis
3. Irregular chelation.

According to these factors, patients are classified into three classes as follows:

1. *Class I:* None of the above factors.
2. *Class II:* One or two factors.
3. *Class III:* All of the above.

For those with none of these factors (Class I), the success rate is about 93 percent, with one or two factors (Class II), it is 85 percent and with all three factors (Class III), it drops to a very low figure of 60 percent.

Umbilical Cord Stem Transplantation[140-142]

Umbilical cord stem cells collected from the cord of unaffected fetus at delivery, can be used for transplantation, even if partially matched. This has yielded better results in some centers.

- *BMT in utero (Fig. 20)*[143,144]*:* Research is underway on BMT *in utero*, at 14 weeks of gestation. Since the immune system is not developed at that time, there would be no rejection and mother's purified stem cells could be used, although the risk of GVHD would have to be looked into.
- *Pharmacologic manipulation of HbF*[145,146]*:* Various drugs, which induce production of HbF, have been tried in hemoglobinopathies with variable success. These include butyrates, hydroxyurea, 5-azacytidine, erythropoietin, etc.
- *Hydroxyurea*[147-150,154,155]*:* Few studies on effect of hydroxyurea in β-thalassemia major have been published and have shown variable responses. However, its efficacy in thalassemia intermedia has been established. Similarly, in double heterozygotes, the usefulness has been established.
- *Butyrates*[151-155]*:* Similar results, as seen with hydroxyurea, were seen with butyrates in β-thalassemia. Though an increase in HbF has been documented, no substantial increase in total hemoglobin or decrease in the transfusion requirement has been reported.

Fig. 19 Stem cell transplantation (*Courtesy:* MR Lokeshwar)

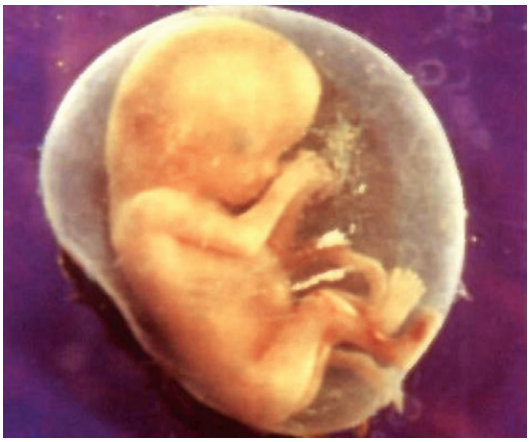


Fig. 20 BMT *in utero*

- *Erythropoietin*[156-158]*:* It has shown no benefit when used alone, but in combination with hydroxyurea, an additive effect has been found, resulting in increase in HbF as well as total hemoglobin.[40]

Gene Therapy[159-161]

Inherited disorders of hemoglobin remain desirable targets for genetically based therapies. These genetically based strategies aim at addition of a normal copy of the human globin gene along with key regulatory sequences to autologous hematopoietic stem cells. But this approach has been impeded by a difficulty of attaining high-titer vectors. Recent advances in vector construction have circumvented some of the problems limiting gene transfer efficiency and regulation of transgene expression. However, it will be some time before clinical application of this therapy becomes a reality.

Prevention[162-166]

- The cost of treatment of an average weight 4-year-old thalassemic child is around ₹ 90,000 to 100,000 annually in a private set-up. Therefore, not more than 5 to 10 percent of thalassemic children born in India receive optimal treatment. Stem cell transplantation as a curative treatment, which costs between 6 and 16 lac rupees is out of reach for majority of children.
- Besides bearing the cost of treatment, the psychological stress to both the patient and the parents/family is phenomenal.

It may be starling to know from a 15-year-old thalassemic child the account of what he has undergone so far. He has received around 250 units of packed red cells and 4000 injections of desferrioxamine. He has had a needle in his body for over 40,000 hours of his life. His family has already spent ₹ 16,20,000 for chelation alone. If this child lives for 50 years, then he would require 2000 units of packed red cells, 15,000 desferrioxamine injections, which translates into 1.5 lac hours of a needle in his body and ₹ 90 lacs for chelation alone (personal communication). This is besides the cost incurred by the hospital where he receives his regular treatment including packed red cell transfusions and other medical care.

The birth of a thalassemic child thus places considerable strain not only on affected child and family but on society at large. Therefore there is an emphasis for shift from treatment to prevention of birth of such children in future. This can be achieved by:

Population Education[167]

- Mass screening of high-risk communities for thalassemia minor
- Genetic counseling of those who test positive for thalassemia minor.

Prenatal Diagnosis[168,169]

The question that arises in the mind is whether prevention at a national level is cost-effective.

The answer to this is Yes, it is cost-effective and we should strive to prevent the birth of a thalassemic child.

Prevention of thalassemia, is practical, feasible and the answer to the agony of so many children, families and nations.

The methods would include creating awareness amongst high-risk communities about the prevalence and the difficulties in management of this condition. Screening young people amongst all high-risk communities before marriage is the right way to go. If screening is performed in childhood, it is often forgotten around the time, they get married. Hemoglobin electrophoresis is the confirmatory test to diagnose thalassemia minor or carrier status. All at-risk couples need to be counseled about the prenatal diagnosis to confirm the thalassemic status of the fetus. Thus, every baby born to two carriers of thalassemia trait should be screened *in utero* and termination should be advised for those fetuses who are found affected, so that no child or parent has to suffer the agony of management of thalassemia.

Future research is directed at improving the prevention strategies by diagnosis before the embryo is formed, to reduce the psychological trauma of termination of pregnancy. These newer methods include:

Preimplantation Diagnosis[170-174]

Biopsy of blastula: By washing uterine cavity after *in vivo* fertilization. Analysis of a single blastomere from an eight cell embryo after *in vitro* fertilization.

Preconception Diagnosis[175]

Analysis of the first polar body of an unfertilized egg and then. Distinguish between eggs which carry the defective/normal gene *in vitro* fertilization of normal egg.

SUMMARY

During the approach of a case with thalassemia, it is necessary to suspect thalassemia by clinical evaluation and doing simpler hematological parameter like CBC, Nestrof test and confirm the diagnosis by estimating HbF and HbA_2 and other abnormal hemoglobins by doing various test like hemoglobin electrophoresis, column chromatography, isoelectric focusing or microcolumn chromatography and high performance liquid chromatography using various instruments like Bio-Rad Variant. It is important to anticipate complication due to iron overload involving various organs and due to transfusion complications. It is therefore necessary to evaluate organ functions at regular intervals for early detection of complications.

Management of thalassemia involves a multi-disciplinary therapeutic team approach and should be preferably done at a comprehensive thalassemia children care center with outdoor transfusion facilities..

Packed red cell transfusions remain the cornerstone of therapy in thalassemia major. The decision to initiate lifelong regular transfusions in patients with β-thalassemia should be based on the molecular defect, severity of symptoms and clinical criteria such as failure of growth, development and bone changes.

Hypertransfusion remains the most accepted regimen in most parts of the world. *Moderate transfusion regimen* has been adopted and recommended by the Thalassemia International Federation. In this regimen, pretransfusion hemoglobin is maintained between 9 and 10.5 gm% (Pretransfusion Hb). The most ideal way to transfuse Thalassemics is using group and type specific packed red cells that are compatible by direct antiglobulin test. The hematocrit should be standardized to 65 to 75 percent. Best is to use leukodepleting filters at bedside.

Most of the complications of β-thalassemia are attributable to iron overload. Excess iron is toxic to the heart, liver, and various endocrine glands. The goal of iron chelation is to reduce the iron overload and subsequently maintain ferritin levels below 1000 ng/mL. The standard available chelators used are: Desferrioxamine (Desferal, DFO), given by subcutaneous route, with the help of Desferal pump over 6 to 8 hours 6 days in a week: Though ideal, its cost and mode of administration lead to non-compliance, especially in the developing world and more than 90 percent do not comply.

Oral iron chelators are available now deferiprone (L1) or 1, 2-dimethyl-3-hydroxypyridin-4-one,or kelfer and deferasiroxor, icl670(4-[3,5-bis (2-hydroxyphenyl) -1,2,4-triazol-1-y1] benzoic acid), (asunra, desirox), with minimal toxicity, can be given orally in multiple dose or single dose, thus improving compliance.

Stem cell transplantation is curative but out of reach of many because of cost and nonavailability of matching donor. Gene therapy for thalassemia is still under research.

Until last few decades, thalassemia was regarded as a uniformly fatal disease and death was expected during the second decade of life before adulthood. However, progress in the understanding and management of the disease in last 3 decades has improved prospects of survival such that they survive now into 3rd and 4th decades of life. This is possible provided, they receive the ideal treatment with good compliance. Thalassemia should no longer be, therefore, seen as a disease of childhood. Better management and improved survival has opened a new chapter in the management of thalassemia beyond transfusions and chelation therapy. Problems of adolescence, growth and development, attainment of puberty and full sexual potential, bone mineralization, proper education, suitable employment, marriage and parenthood are some of the concerns that require attention.

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CHAPTER 18

Sickle Cell Anemia in Children

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Sickle cell disease is a group of commonly inherited hematologic disorders. It is a single gene disorder, caused by a single point mutation in the β globin chain of hemoglobin leading to defective hemoglobin synthesis. Sickle cell disease involves the red blood cells or hemoglobin and their ability to carry oxygen, hemolytic anemia, painful crisis, end-organ failure and various complications. Sickle cell anemia is the first disease, described to be due to a molecular mutation. It is characterized by relentless pain and so the African tribes chose different names for sickle cell disease; chwechweechwe, hemkom, nuidudim all describing the relentless pain.

HISTORICAL ASPECT

Sickle cell anemia has been prevalent in Africa for the 5,000 years or more. It was first described in 1910, by Dr James Herrick in a dental student from Grenada who noticed abnormally shaped RBCs under the microscope. These cells looked like a sickle and so the name.[1] He also coined the term crisis to describe the acute episodes of pain.

Sydenstricker described the first pediatric case and recognized that this disorder was a hemolytic anemia.[2] It was only in 1952 that sickle cell anemia was reported in the tea garden laborers of Assam in India.

Types of Sickle Cell Gene Mutations

Sickle-cell gene mutation probably arose spontaneously in different geographic areas, as suggested by restriction endonuclease analysis.

There are five major mutations of the sickle cell gene. In Africa, four of the major sickle haplotypes are associated with different geographical areas:[3,4]

1. "Senegal" is located in Atlantic West Africa (5–15%)
2. "Benin" in central West Africa (50–70%)
3. "Bantu" (also known as CAR) is in central Africa (15–30%)
4. "Cameroon"
5. In India and parts of Saudi Arabia, the African haplotypes are not seen, but a unique "Arabian-Indian" haplotype is found.[5]

Their clinical importance, arises from the fact that some of them are associated with higher HbF levels, e.g. Senegal and Saudi-Asian variants, and tend to have milder disease.[6]

In Caribbean and in North American sickle cell patients of African origin, 50 to 70 percent of chromosomes are Benin, 15 to 30 percent of chromosomes are Bantu-CAR and 5 to 15 percent are Senegal.[7,8] In Los Angeles, 38 percent of patients are Benin homozygotes (Benin/Benin), 25 percent are Benin/Bantu CAR, 13 percent are Benin/Senegal, 5 percent are Bantu CAR homozygotes and 3 percent are Bantu CAR/Senegal.

Sickle cell disease is more commonly found in people from tropical regions particularly sub-Saharan Africa, India and Middle East which are endemic for malaria.[9] Disease is found with equal frequency in males and females.

Incidence and Prevalence of Sickle Cell Disease

The prevalence of the disease in the United States is approximately 1 in 5000, mostly affecting Americans of Sub-Saharan African descent, according to the National Institutes of Health.[10] One in 600 African-Americans will have homozygous sickle cell disease, one in 800 will have hemoglobin SC disease and one in 1700 will have Sickle-β Thalassemia Syndrome. The sickle gene has an

incidence of 8 percent in African-American individuals. The frequency of the gene can be higher in certain areas of Africa. In the US, it has been estimated that 1,000 children are born each year with sickle cell disease. One in twelve African-Americans has sickle cell trait.[10,11]

Sickle cell disease primarily affects those of African descent and Hispanics of Caribbean ancestry, but the trait has also been found in those with Middle Eastern, Indian, Latin American, Native American and Mediterranean heritage.

Sickle cell disease is prevalent in many parts of India, where the prevalence has ranged from 9.4 to 22.2 percent in endemic areas.[12]

Sickle Cell Anemia and Malaria

Sickle cell anemia is closely related to malaria. It is well known that sickle cell disease (SCD) is seen in those regions of the world where malaria is endemic. The presence of a sickle trait in an individual provides natural protection against malaria.[13] In persons with AS (heterozygotes), when the parasite infects the RBC, it causes the abnormal hemoglobin to sickle. This leads to preferential phagocytosis of the parasitized RBCs by the spleen[14,15] leading to a decrease in the parasitemia and therefore less severe disease, with a relative protection from dying of malaria. It is believed that this mutation arose by the process of natural selection.

Pathophysiology

The sickle mutation is a GAG-GTC conversion. The resultant sickle hemoglobin differs from normal adult hemoglobin by alteration of one amino acid in the β-globin subunit at the sixth position of the β-globin chain.[16] Sickle β chain has hydrophobic valine instead of hydrophilic glutamic acid in the sixth position of the β-globin chain. The properties of hemoglobin S result in the clinical manifestations of sickle cell disease.

Normal red blood cells, owing to their elasticity, can deform while passing through the capillaries. In sickle cell disease, presence of hypoxia, acidosis, deoxygenation, high percent of Hb S, high MCHC, dehydration, fever, acidosis promote red cell sickling due to formation of gel-like substance containing Hb polymers called tactoids. Repeated episodes of sickling, damage the cell membrane and decrease the elasticity of the red blood cell.

Thus, the pathophysiology of sickle cell disease can be based on:

1. Hb S polymerization
2. Increased adhesion of sickle RBC to endothelium
3. Hemolysis

Hb S Polymerization

As the RBC passes through the microcirculation there is deoxygenation which causes conformational changes in the Hb molecule. Due to the hydrophobic valine, and interactions between two valine molecules, a polymer is formed which later progress into helical fibers which group together, stiffen and give rise to the characteristic sickle shape. So, the polymerization of hemoglobin S is the primary event in the molecular pathophysiology of sickle cell disease and results in distortion of the shape of the red blood cell and a marked decrease in the deformability of the red blood cell. The resulting loss of red blood cell elasticity plays a key role in the clinico-pathologic manifestations of sickle cell disease. These cells thus assume a rigid sickle shape and are unable to regain the normal biconcave shape even after reoxygenation. Consequently, these rigid blood cells are unable to deform while passing through narrow capillaries resulting in vascular occlusion and ischemia and are also hemolysed. These rigid sickle cells are responsible for the vaso-occlusive phenomenon and hemolysis which are characteristic of this disorder.

Data regarding the initial trigger for vaso-occlusive crisis shows that there is contribution of the vascular endothelium, complex cellular interactions and a global inflammation mediated cell activation. The presence of anemia is due to hemolysis of abnormally shaped red blood cells. The presence of other hemoglobins in the red blood cell, such as hemoglobin F, hemoglobin A, hemoglobin C, and hemoglobin O, has an effect on the sickling phenomenon and on the polymerization of hemoglobin S. Hemoglobin F has the most profound anti-sickling effect, followed in order by hemoglobin A, C, and O. Elevated levels of hemoglobin F can modify the clinical and hematological effects of sickle cell disease and prevent polymerization of hemoglobin and thereby sickling of red cells.

Increased Adhesion of RBCs to Endothelium

Abnormal adhesion of sickle RBCs is initiated by young RBCs called stress reticulocytes. These cells are thrown out prematurely into the circulation from the bone marrow. By virtue of their surface receptors, alpha 4 beta1 integrin or VLA-4 (very late antigen 4), these cells bind directly to the VCAM1 on the endothelial surface. CD 36 on the RBC as well as on the endothelium are bound via a molecular bridge the plasmatic thrombospondin.

It is not only the RBCs but the polymorphs and platelets also that participate in the slowing of blood in the microcirculation.

Hemolysis

In addition to causing anemia, it also has other deleterious effects. Heme is the most powerful scavenger of NO or nitric oxide which is a vasodilator. Hemolysis also releases erythroid arginase in plasma, which degrades L arginine, the substrate of NO producing enzyme endothelial NO synthetase. Thus, NO levels are reduced by increase in scavenging as well as a decrease in production all leading to vasoconstriction and slowing of blood. Hemolysis of abnormally shaped red blood cells during their passage in the microcirculation results in anemia. The bone marrow production of red blood cells does not match the rate of destruction. The sickle cells have a shortened life span of 10 to 20 days as compared to the normal RBC lifespan of 90 to 120 days.

Genetics of Sickle Cell Anemia

Sickle cell disease is an autosomal recessive disorder caused by a point mutation in the sixth codon of the β-globin gene found on chromosome 11 coding for valine instead of glutamine. The disease includes various genotypes containing at least one sickle gene where Hb S constitutes > 50 percent of all hemoglobin.

Sickle Cell Syndromes

The common sickle cell syndromes are:

- Heterozygous sickle cell anemia (AS)
- Homozygous sickle cell disease (SS)
- Sickle-beta thalassemia (β^0 or β^+)
- Compound heterozygotes sickle cell disease
  - SD Punjab disease
  - SO Arab disease
  - Sickle E syndromes
  - Sickle α syndrome
  - Sickle C syndrome.

Sickle Cell Trait or Carrier State

Heterozygous form (HbAS), where only one β-globin gene is affected, i.e. one parent is a carrier of Hb S mutation and the other is normal, all the children will be phenotypically normal, however 50 percent will have sickle trait or will be carriers. Sickle cell trait is usually asymptomatic with Hb S constituting 40 percent of the total hemoglobin.

Homozygous Sickle Cell Disease

Sickle cell anemia is the homozygous form (Hb SS) where both β-globin genes carry the mutation. It is the most severe type and Hb S is as high as 90 percent of the total hemoglobin. Thus, if both parents are carriers, they are at risk for passing the gene on to a child—there is a 25 percent chance of having sickle cell anemia, 50 percent chance of being a carrier like the parents and a 25 percent chance of being unaffected. This means that there is a three in four chance or 75 percent chance for the child to not have sickle cell disease.

Compound Heterozygotes

Where one β-globin gene is affected with sickle cell mutation and the other gene includes mutations associated with Hb C (HbSC), Hb β-thalassemia (Hb S-β°thal/Hb S-β^+ thal, Hb D, Hb O Arab, hereditary persistence of fetal Hb with variable clinical manifestations.

Sickle-β Thalassemia Syndrome

Has two forms, Sickle-β° thalassemia and Sickle-β^+ thalassemia. The sickle-β° thalassemia individual is not able to produce any hemoglobin A due to a complete deletion of the beta-globin gene. In sickle-β^+ thalassemia, there is a partial deletion of the hemoglobin gene and the patient is able to make a variable amount of hemoglobin A. The amount of hemoglobin A will be less than the amount of hemoglobin S.

In general, hemoglobin SS disease is the most severe of the sickle syndromes followed by hemoglobin S-β^0 thalassemia, hemoglobin SC and finally hemoglobin S-β^+ thalassemia. The typical hematological values of these sickling syndromes are shown in Table 1.

Diagnosis of Sickle Cell Anemia

CBC and Routine Laboratory Testing

- **Mild to moderate anemia (5–9 g/dL) with decreased hematocrit (20–30%).** The anemia is usually normocytic normochromic unless associated with alpha or beta thalassemia or iron deficiency in which case microcytosis and hypochromia may be present. **MCV may be on the higher side** due to reticulocytosis.
- **Peripheral smear:** The peripheral blood smear may show sickled red cells, typically irreversibly shaped sickle cells, polychromasia indicative of reticulocytosis, target cells and Howell-Jolly bodies (RBCs with nuclear remnants) indicative of asplenia.
- **Reticulocyte count is elevated (Fig. 1.1) (3–15%).**

Table 1 Sickle cell syndromes hematological values

Type	*Hb (g/dL)*	*MCV*	*Severity*
SS	7–8	nL	++++
Sβ°-thal	8–10	<70	+++
SC	10–11	nL	++
Sβ± thal	11–12	<70	+

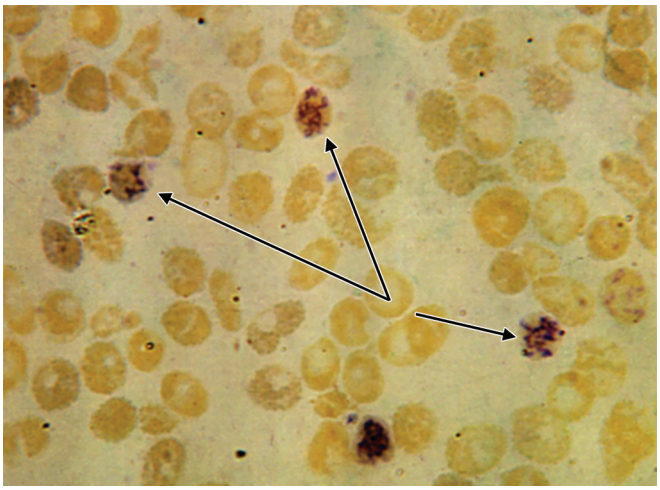

Fig. 1 Peripheral smear showing reticulocytes (*Courtesy:* MR Lokeshwar, India)

- There is unconjugated **hyperbilirubinemia.**
- Decreased **serum haptoglobin.**
- Increased **plasma hemoglobin levels.**
- Increased **serum lactate dehydrogenase levels.**

Hemoglobin Solubility Test

Hb S is an insoluble hemoglobin molecule. It forms tactoids or crystals when in the reduced state in high phosphate buffer solution. These crystals refract and deflect light rays and produce turbidity.

Sickling Test

Addition of sodium metabisulfite induces sickling of red cells, on the blood film (positive sickling test). It is done to diagnose sickle cell anemia or when there is an abnormal electrophoretic or chromatographic hemoglobin fraction in the position of Hb S, e.g. Hb D or G.

Method of sickling test: Sodium metabisulfite reduces the oxygen tension inducing the typical sickle shape of red blood cells.

The method is as follows: A drop of fresh anticoagulated blood is mixed with 1 drop of 2 percent sodium metabisulfite solution on a microscope slide. The solution is freshly prepared each time. A cover slip is placed and the edge is sealed with wax/vaseline mixture or with nail varnish. It is allowed to stand at room temperature for 1 to 4 hours. Under the microscope, in positive samples the typical sickle-shaped red blood cells are seen. False negative results may be obtained if the metabisulfite has deteriorated or if the cover slip is not sealed properly. It is important to examine the preparation carefully and in particular near the edge of cover slip.

However both these tests cannot distinguish between sickle cell disease and trait.

Hemoglobin Electrophoresis

It helps to differentiate individuals who are homozygous for Hb S (Hb SS) from those who are heterozygous by demonstrating either a single band of Hb S (in Hb SS) or Hb S with another mutant hemoglobin (in compound heterozygotes). This is the definitive test for sickle cell anemia. HbePP on cellulose acetate electrophoresis is the usual method for Hb electrophoresis. However Hb S, G and D have the same electrophoretic mobility. On citrate agar electrophoresis, at ph 6.2, Hb S is separated. The most commonly used method is by the variant machine.

- Only Hb S with an HbF concentration < 30 percent—Sickle cell anemia. A homozygous patient will have hemoglobin SS (80–90%), hemoglobin F (2–20%) and hemoglobin A2 (2–4%).
- A carrier patient will have Hb S (35–40%) and hemoglobin A (60–65%).
- Hb S is predominant, Hb F < 30 percent, Hb A2 is elevated - Hb S–beta-0 thalassemia.
- Hb S > A and Hb A2 is elevated—HbS–beta+thalassemia.
- Hb A2 level is normal—to consider the possibility of concomitant Hb SS and iron deficiency.
- Hb S and Hb C concentration in roughly equal amounts Hb SC disease.

The test may be inaccurate in a patient who has recently received blood transfusions.

Newborn Sickle Cell Disease Screening

The introduction of newborn screening has been a great advancement in the management of sickle cell disease as infants with sickle cell disease are healthy at birth and develop symptoms only after decline of fetal hemoglobin.

Such programs help in early recognition of affected infants, early intervention to reduce morbidity and mortality and to provide anticipatory guidance for parents. The most commonly used techniques for newborn diagnosis are thin layer/isoelectric focusing and high-performance liquid chromatography.

Repeat hemoglobin electrophoresis, if found abnormal and again at six months, to confirm the hemoglobinopathy. It is recommended to conduct a complete blood count and hemoglobin analysis on the parents to confirm diagnosis and offer genetic counseling. In newborn screening, the patterns of hemoglobin are reported in decreasing order according to the quantities detected.

- *FS pattern:* Newborns with sickle cell anemia (Hb SS) have this pattern with predominant Hb F and small amount of Hb S and no Hb A. It may also be found in

newborns with sickle cell-beta-0 thalassemia, sickle cell-hereditary persistence of fetal hemoglobin. Family studies help confirm diagnosis, in case of sickle cell-beta-0 thalassemia, one parent has sickle cell trait and the other beta thalassemia minor.

- *FSA pattern:* This pattern is supportive of sickle cell-beta+ thalassemia.
- *FAS pattern:* Newborns with sickle cell trait have this pattern.
- *FSC pattern:* This is supportive of a diagnosis of Hb SC.
- *AFS pattern:* This is suggestive of transfusion prior to the test or an error.

All patients screened to have either sickle cell disease or trait must be started on penicillin prophylaxis until the final diagnosis is determined. Due to clinical implications of a diagnosis of either sickle cell disease or trait, the need for repeat hemoglobin analysis at a later age must be emphasized.

Imaging Studies

- Radiological abnormalities and stroke evaluation are carried out as and when required to evaluate extent of the lesion. If a CT scan is ordered, it is preferable not use contrast until the hemoglobin S concentration can be reduced below 30 percent. If available, MRI is preferable.
- *Ultrasonography:* This can be used to visualize stones and detect signs of thickening gallbladder walls or ductal inflammation, indicating possible cholecystitis.

Prenatal Diagnosis

Parents with a child suffering from sickle cell disease or those at risk of having a child with sickle cell disease, often seek prenatal diagnosis and termination of pregnancy in case of an affected fetus. However, patients with sickle cell disease have a great variation in the symptoms despite having the same mutation and therefore, it is as yet not a very useful test.

Clinical Features of Sickle Cell Disease

There are great variations in the manifestations of sickle cell disease (Fig. 2). Clinical features of sickle cell disease are due to the intermittent episodes of vascular occlusion, tissue ischemia/reperfusion injury and hemolysis, all of which lead to multiorgan dysfunction as well as pain. Hypercoagulability, hyposplenia and infections also contribute to the clinical spectrum of sickle cell disease.

Symptoms do not develop in the first 6 to 12 months of age due to elevated levels of hemoglobin F, in circulation. After infancy, the red cells of a patient with sickle cell anemia will have 90 percent hemoglobin S (Hb S), 2 to 10 percent of hemoglobin F (Hb F) and normal amounts of minor fraction of adult hemoglobin A2 (Hb A2). Adult hemoglobin A (HbA), which increases at 3 months of age, is absent.

The most common clinical manifestation of sickle cell anemia is vaso-occlusive crisis. It occurs due to the sickled red blood cells obstructing the capillaries causing ischemic injury to the organ. Factors precipitating vaso-occlusive crisis include hypoxia, acidosis, dehydration, infection, changes in body temperature.

The clinical features of sickle cell anemia occur secondary to the physiological changes in the RBCs leading to various acute and chronic complications. When Hb S is deoxygenated the Sol (soluble) form of Hb changes to Gel form of Hb to form rigid, sickle shaped tactoids, and they polymerise forming insoluble structure. RBC membrane becomes more fragile. Upon reoxygenation the sickle cell initially resumes normal configuration, but with repeated cycles of sickling and unsickling, fixation of membrane occurs leading to irreversible sickle cell formation and hemolysis.

Manifestation of Sickle Cell Anemia

Anemia

Patients are well compensated due to chronic nature of anemia. Anemia may be complicated by secondary folate deficiency. This is due to increased RBC turnover and folate utilization. Periodic episodes of hyperhemolysis may occur. All patients are universally anemic and are hemolytic in nature. Repeated cycles of deoxygenation and morphologic sickling irreversibly damage the red cell membrane and result in hemolysis. Bone marrow increases red cell production but is unable to compensate for the rate of hemolysis. This results in moderate-to-severe anemia. Thus patients may simply be found to be jaundiced or pale. Patients may show few manifestations of anemia as they readily adjust by increasing their heart rate and stroke volume (compensated). Though they may be able to carry out daily activities in a normal fashion, their tolerance for exertion is limited.

- Splenic sequestration is an anemic crisis. It is a life threatening medical emergency in children with sickle cell anemia. It occurs due to pooling of blood and trapping of deformed RBCs in the narrow splenic vessels. This causes rapid, painful enlargement of spleen, precipitous fall in hemoglobin, hypovolemic shock and persistent reticulocytosis. It tends to occur with higher frequency in infants and has been reported as early as few weeks of age. Spleen suddenly becomes enlarged and traps blood cells. The platelet

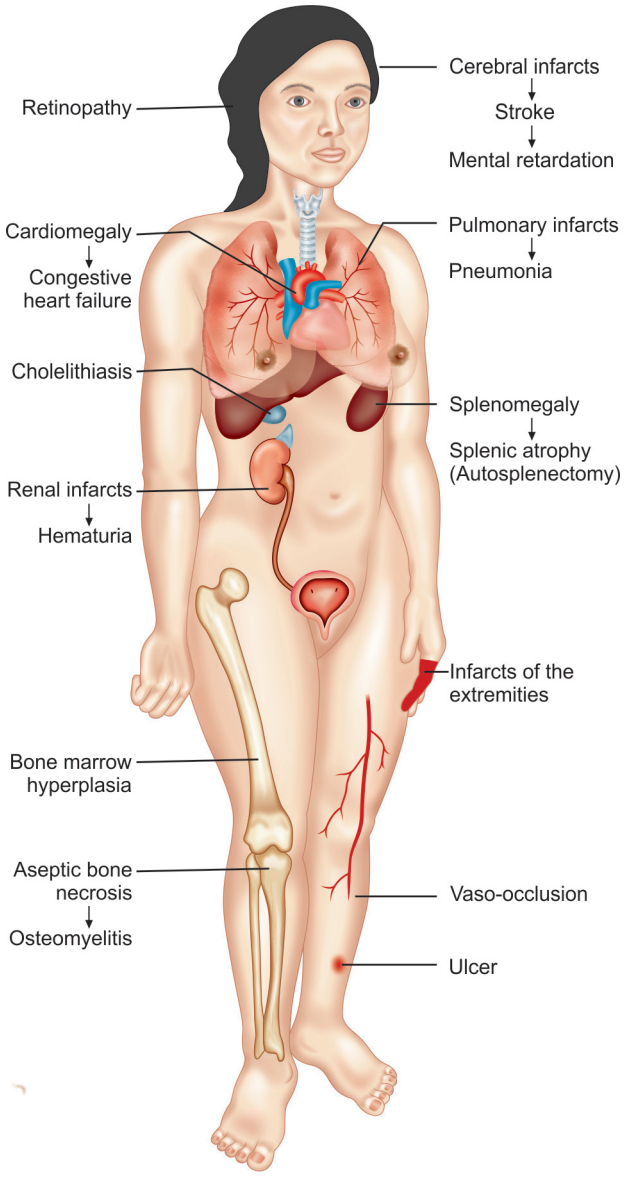

Fig. 2 Site of manifestations of sickle cell disease (*Courtesy:* Swati Kanakia, India)

count often is slightly decreased. It occurs due to pooling of blood and trapping of deformed RBCs in the narrow splenic vessels. Hypovolemic shock occurs if a large volume of blood is trapped or sequestered. Hemoglobin levels dramatically drop from baseline values and the reticulocyte count is elevated. In Figure 3, a child showing the sickle cell anemia with hepatosplenomegaly.

Treatment includes early diagnosis and aggressive management in the form of intravenous fluids and blood transfusion. The mortality rate may be up to 10 to 15 percent before transfusion therapy. Sequestration crisis may be recurrent in 50 percent of the survivors and hence splenectomy is recommended after the first

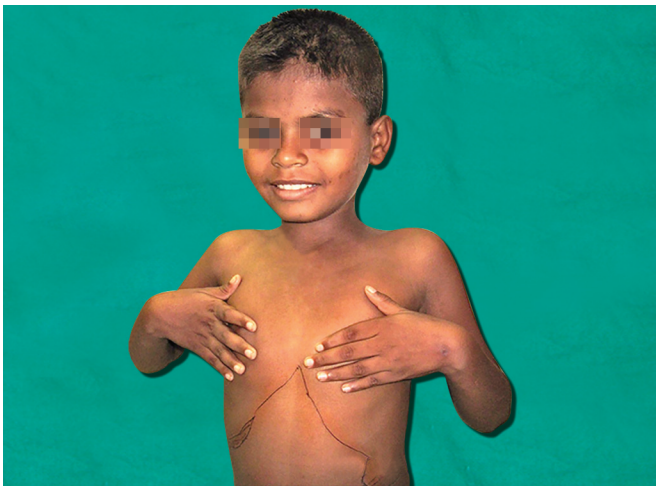

Fig. 3 Sickle cell anemia with hepatosplenomegaly (*Courtesy:* MR Lokeshwar, India)

acute event.[17] Parents should be educated about the signs and symptoms of sequestration and to palpate the spleen to recognize sudden increase in the size of the spleen so that early treatment may be sought.

- Aplastic crisis is also an anemic crisis. This is a serious complication leading to worsening of anemia resulting in pallor, tachycardia and fatigue. Aplastic crisis is precipitated by parvovirus B-19 infection which infects the red blood cell precursors in the bone marrow thereby affecting red blood cell production. Impaired red blood cell production that lasts for a few days can cause an abrupt life threatening crisis in patients with sickle cell anemia due to decreased lifespan of RBCs (10–20 days). Initial reticulocytopenia is followed by brisk reticulocytosis as bone marrow spontaneously recovers in a few days to a week. The spleen usually is not enlarged over baseline. Management includes packed red cell transfusion.

Vaso-occlusive Crisis

Pain resulting from vascular occlusion and ischemia and can occur abruptly. Pain may be accompanied by malaise, fever, and leukocytosis. It is the leading cause of emergency department visits and hospitalizations, causing disruption of daily life with pain lasting for several hours to days and occurs in any part of the body particularly extremities, bones, chest, abdomen. Bone pain occurs due to bone marrow infarction particularly due to obliteration of nutrient arteries of bone. Hence the site of involvement changes with the site of maximum bone marrow activity. Factors precipitating vaso-occlusive crisis include hypoxia, acidosis, dehydration, infection, changes in body, bone marrow infarction, etc. and since marrow activity changes with age, site of infarction also changes.

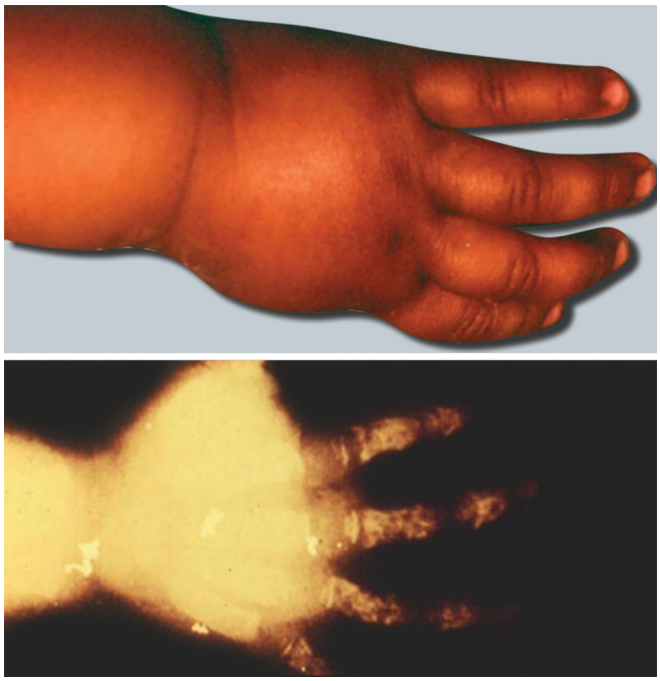

Fig. 4 Dactylitis (Hand-foot syndrome)
(*Courtesy:* MR Lokeshwar, India)

- *Dactylitis (Hand-foot syndrome) (Fig. 4):* The usual presentation is symmetric or unilateral swelling of the hands and/or feet which may be relieved with pain medication such as acetaminophen and codeine and supportive measures. Osteomyelitis must be ruled out in all cases as they may have similar presentation but require different line of management. It is one of the earliest clinical manifestations of pain in children with sickle cell anemia. Up to 18 months of age, the metacarpals and metatarsals are active part of erythropoiesis and are involved presenting as dactylitis or hand-foot syndrome. Occurs in 50 percent of the children by 2 years of age. Episodes are frequently recurrent. Clinical involvement may be limited to a single phalanx or metacarpal or may involve all small bones of all small extremities.

  Radiologic changes become evident after a few days and include patchy areas of osteoporosis and sclerosis, periosteal new bone formation, and occasionally apparent disappearance of a bone. Resolution is usually complete both radiologically and clinically although occasionally premature fusion occurs causing permanently shortened, deformed small bones. The long bones of the extremities which retain marrow activity as the child grows older are affected during childhood. During adolescence, as the marrow activity recedes further, pain involves the vertebral bodies of the lumbar region.

- *Abdominal pain:* Patients may present with acute abdomen due to severe pain which may be due to mesenteric vein thrombosis, referred pain or secondary to underlying organ or soft tissue infarction.

Majority of the pain episodes are short lived and can be managed at home with pain medications and other comfort measures (heating blanket, relaxation technique and massage). Specific therapies for pain include acetaminophen and NSAIDs in conjunction with oral/IV opioids and their derivatives. Optimal maintenance oral/intravenous fluids to maintain hydration must be initiated in hospitalized children. The 2003 BCSH guidelines recommend use of oral analgesia for treatment of pain although severe pain is best managed with parenteral analgesics.[18] Morphine is the drug of choice and dosing must be individualized for each patient. It should be given hourly followed by three hourly dosing once effective analgesia is achieved. Patient-controlled analgesia may also be helpful. Sleeping through the night may be an indication to decrease dosing by 20 percent the next morning. Decreasing analgesia dose at night is not advisable as pain is often worse at night. After 24 to 48 hours, when pain is controlled, patient may be shifted to sustained-release oral morphine and discharged from the hospital with gradual tapering of dose over several days. Pain must not be undertreated due to fear of opioid addiction or dependence as they seldom occur due to brief duration of painful episodes (5–7 days). Blood transfusion does not help to decrease intensity or duration of a painful episode and is indicated in patients with hemodynamic instability due to decreased hemoglobin. Hydroxyurea may decrease frequency and severity of pain episodes.

Neurological Complications

It is most prevalent in childhood and adolescence though it may be found as early as 1 year of age. Involvement of the nervous system due to sickle cell anemia may have a varied presentation such as headaches, seizures, cerebral venous thrombosis and reversible posterior leukoencephalopathy syndrome of which stroke is the most serious manifestation in 10 to 15 percent patients of SCD. Infarcts are usually ischemic in children and hemorrhagic in adults.[19] While it is unusual for children to have strokes, approximately 11 percent of patients with sickle cell anemia have strokes before they reach the age of 20 years. Hemiparesis is the usual presentation.

Overt stroke, occurring in 11 percent of the children, is defined as the presence of focal neurological deficit lasting for > 24 hours and/or evidence of a cerebral infarct on T2 weighted MRI of the brain corresponding to the deficit.

The presence of a cerebral infarct on T2 weighted MRI of the brain in the absence of a focal neurological deficit

lasting for > 24 hours is defined as a silent stroke. These silent infarcts occur in 20 percent of the children and tend to cause progressive decline in cognitive function, affect learning and behavior and increased risk of epilepsy as compared to general population.[20]

Management of a patient presenting with focal neurological deficit includes:

- Oxygen administration to maintain saturation > 96 percent.
- Blood transfusion initiated within 1 hour of presentation to increase hemoglobin up to 10g/dL.
- Transfusion therapy to maintain Hb S level < 30 percent is the mainstay of therapy for primary and secondary prevention of strokes.[21] This strategy results in 90 percent reduction in the rates of overt strokes. Transfusion therapy must be continued indefinitely for there is increased risk of stroke on stopping.[22]
- Convulsions frequently are associated with stroke.
- Primary prevention of strokes can be achieved by transcranial Doppler (TCD) measurement of the blood velocity in the circle of Willis and values of > 200 cm/sec suggest a high risk of stroke even before lesions become evident on MRI.
- Chelation therapy must be given after 2 to 3 years for iron overload from repeated transfusions.

Acute Chest Syndrome

Acute chest syndrome (ACS) is defined as combination of respiratory symptoms, along with fever, cough, respiratory distress, chest and/or back pain, and new lung infiltrates.[23,24] It is the second most common cause for hospital admission in children with sickle cell anemia and a common cause of death.[23,25] In young children, it is usually due to infection. Older children and adults have infarction more often. ACS is most frequently preceded by a painful episode requiring opioids.

Emergency management of ACS includes supplemental oxygen and simple or exchange transfusion. Continuous pulse oximetry monitoring is required and oxygen therapy is initiated when room air saturation is < 90 percent. The aim of blood transfusion is to reduce Hb S level < 30 percent and when administered early may help prevent further respiratory complications and need for supplemental oxygen.

The etiology of ACS is multifactorial including pulmonary infarction, fat embolism due to bone marrow infarction and more commonly infection. Due to similar clinical presentation of pneumonia and ACS and infection being a common causative factor, it is essential to start empiric antibiotic therapy with a third generation cephalosporin and a macrolide. *S.pneumoniae*, *Mycoplasma* and *Chlamydia* species are common organisms.[26] Other treatment measures include adequate analgesia and fluid management. Patients with coexistent asthma should be treated promptly with bronchodilators and steroids. Incentive spirometry and chest physiotherapy can help to reduce the frequency of episodes of acute chest pain. Prevention of recurrent episodes of ACS can be achieved with chronic transfusions and hydroxyurea which reduces the rate of episodes by 50 percent.

Avascular Necrosis of the Femoral or Humeral Head

Avascular necrosis (AVN) of the femoral head presents a greater problem due to weight bearing (Fig. 5). Vascular occlusion can result in avascular necrosis and subsequent infarction and collapse at either site. Subjects with high-baseline hemoglobin are at increased risk. Approximately 30 percent of all patients have hip pathology by age 30 years.

Infection

Infection is a major cause of morbidity and mortality in patients with sickle cell anemia. These children are prone to life threatening infections as early as 4 months of age due to hyposplenia or functional asplenia. These patients are more prone to infections by encapsulated organisms such as *Streptococcus pneumoniae* and *Haemophilus influenzae* type b and *Salmonella* responsible for osteomyelitis. Sickling of red cells within the spleen results in multiple episodes of splenic infarction, leading to functional asplenia (autosplenectomy) which occurs in most children by 5 years of age. In addition, they may also have abnormal IgM and IgG responses, defects in the alternate pathway complement fixation and opsonophagocytic dysfunction.[27,28]

The two major measures in preventing infections in these children are penicillin prophylaxis and immunization for all patients.

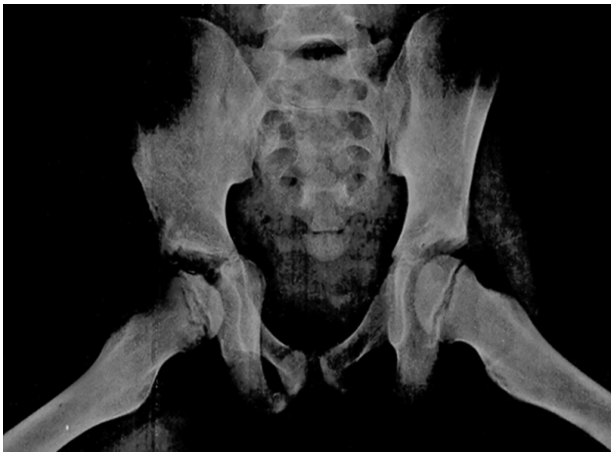

Fig. 5 Avascular necrosis (AVN) of the femoral head (*Courtesy:* MR Lokeshwar, India)

- Oral penicillin V in a dose of 125 mg twice daily should be started at 2 months and given till 3 years of age after which it is increased to 250 mg twice daily till 5 years of age.
- Most clinicians stop prophylaxis at 5 years of age provided the child has not had prior pneumococcal infection or splenectomy and has received pneumococcal vaccine.[29]
- Patients who are allergic to penicillin should be given erythromycin 10 mg/kg twice a day.
- Children with sickle cell anemia should receive all the routine childhood immunizations including those against *Streptococcus pneumonia*, *Haemophilus influenzae* type b, *Neisseria meningitidis*, seasonal influenza, hepatitis B. Fever may be the first sign of bacterial infection in these children prone to fulminant and life threatening infections.
- Thus, fever must be considered a medical emergency requiring prompt medical attention and antibiotic therapy. The factors with high risk for invasive infection requiring inpatient management:[30]
- Children younger than 2 years with hemoglobin SS (Hb SS) or hemoglobin S-β° thalassemia
- Temperature >40°C
- WBC > 30,000 mm^3 or < 5000 mm^3
- Hemoglobin < 5g/dL
- History of previous bacteremia (due to increased risk of recurrence)
- Presence of indwelling catheter.
- Signs of systemic toxicity, hemodynamic instability and/or meningitis
- Prior treatment with vancomycin or clindamycin instead of ceftriaxone due to short half life of these medications.

All patients must be immediately started on empiric antibiotic therapy with a 3rd generation cephalosporin and may be discharged after a 24 to 48 hours afebrile period with duration of antibiotic therapy titrated as per culture reports. Outpatient management may be considered in those without risk factors. Another distinct infection in these patients is osteomyelitis, most commonly caused by *Salmonella spp.* Hence all patients with persistent pain and fever or bacteremia due to *Salmonella spp.* must be evaluated for osteomyelitis with a definitive bone scan.

Unhealed Ulcers (Fig. 6)

Skin Ulcers (Unhealed Ulcers)

Skin ulcers are relatively infrequent. The most common site of skin ulcers is over the lateral malleoli. The ulcerations often have no clear-cut antecedent trauma and progress over a period of weeks. Lesions in children occur most commonly around malleoli where poor circulation along with sickling and microinfarcts leads to poor healing and infection.

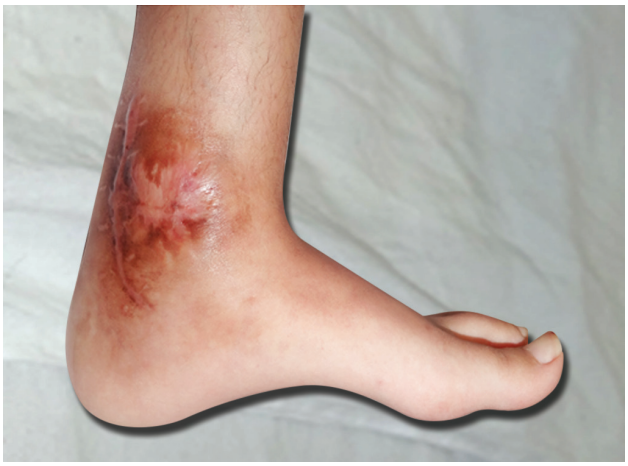

Fig. 6 Unhealed ulcers
(*Courtesy:* MR Lokeshwar, India)

Rest, elevation and dry dressings with antimicrobial ointments are the best approach to this problem. Attempts at skin grafting are frequently not successful due to poor blood flow to the affected region. Healing usually takes weeks to months.

The area should be protected against trauma. Socks or other clothing that cover the area should be avoided, to reduce friction injury. A simple dry dressing provides additional protection. Zinc supplementation has been traditionally thought to aid wound healing.

Other Complications

Priapism (Fig. 7)

It is defined as sustained, painful and involuntary erection of the penis lasting longer than 30 minutes. Priapism is not uncommon problem in sickle cell anemia and most patients experience their first episode by 12 years of age and by 20 years of age as many as 90 percent of the patients have experienced one or more episodes of priapism. Minor recurrent episodes are common during adolescence.

Occasionally, the problem is seen in pre-pubertal boys. Pain becomes severe if erection persists longer than 3 hours. Episodes may be described as stuttering or refractory. **Stuttering episodes** last for a few minutes but less than 3 hours and resolve spontaneously. **Refractory episodes** are prolonged, lasting longer than 3 hours. Acute therapy of prolonged episodes includes aspiration of blood from corpus cavernosa followed by irrigation with dilute epinephrine to sustain detumescence to be done in consultation with pediatric urologist. Supportive measures like sitz bath and pain medications may be tried.

Hydroxyurea may help to prevent recurrent episodes. A sympathomimetic drug with mixed alpha and beta actions, etilefrine seems promising for secondary prevention of episodes.[31] Evidence for the role of transfusion therapy for

Fig. 7 Priapism
(*Courtesy:* MR Lokeshwar, India)

acute or preventive therapy is lacking.[32,33] Prolonged and/or recurrent episodes of priapism may lead to fibrosis and impotence.

Renal Disease

Renal involvement is common in sickle cell anemia and contributes to the morbidity of the disease with renal failure in up to 18 percent of the patients. The primary event is occlusion of vasa recta capillaries in renal medulla where there is low oxygen concentration and high osmolarity thereby increasing the concentration of Hb S.

The renal manifestations of sickle cell anemia:

- Enuresis secondary to hyposthenuria.
- Painless hematuria due to papillary infarcts.
- Proteinuria and hypertension. Asymptomatic albuminuria is a precursor of progressive renal disease.[34,35]
- Renal infarction, papillary necrosis, and renal colic.
- Nephrogenic diabetes insipidus that can lead to polyuria.
- Focal segmental glomerulosclerosis that can lead to end-stage renal disease; dialysis is well tolerated and increasing numbers of patients are being treated with renal transplantation.
- Renal medullary carcinoma is a malignancy found almost exclusively in black patients with Hb SC disease or sickle cell trait.

Pulmonary Hypertension

Pulmonary hypertension occurs due to formation of microinfarcts and microthrombi in the pulmonary vasculature due to circulation of deoxygenated blood which promotes sickling. Depletion of nitric oxide may also contribute to the pathogenesis independent of vaso-occlusive episodes. Various studies have found that more than 40 percent of adults with SCD have pulmonary hypertension that worsens with age and is a major risk factor for death.

Cholecystitis

Due to chronic hemolysis, cholelithiasis is common in children and 40 percent of adolescents with sickle cell anemia are affected with cholecystitis or common duct obstruction can occur. Consider cholecystitis in a child who presents with right upper quadrant pain, especially if associated with fatty food.

Consider common bile duct blockage when a child presents with right upper quadrant pain and dramatically elevated conjugated hyperbilirubinemia.

Retinopathy

The retina is primarily affected with manifestations such as proliferative retinopathy, retinal artery occlusion, retinal detachment and hemorrhage.[36,37] The vaso-occlusions may begin in childhood and progressively lead to loss of visual acuity. Prophylactic photocoagulation may have a role in the treatment of proliferative sickle retinopathy. Regular eye checkups are recommended.

Growth and Development

Growth failure and sexual maturation are delayed in patients with sickle cell anemia. Normal height may be achieved by adulthood but weight remains lower for age as compared to controls. Neurodevelopment and skeletal maturation are also delayed.[38,39]

Cardiac Involvement

The heart is involved due to chronic anemia and microinfarcts. There is chamber enlargement due to compensatory increase in cardiac output. Arrythmias may be an important cause of death in older patients. As per a study, coronary artery dilatation is common in children with sickle cell anemia.

Cardiac complications are usually related to high output stress due to anemia and manifest in the form of congestive cardiac failure and hemosiderosis due to iron overload because of chronic blood transfusion. Chelation therapy must be given after 2 to 3 years for iron overload from repeated transfusions. There is no specific cardiomyopathy in sickle cell anemia.

Pregnancy

Pregnancy in patients with sickle cell anemia is associated with fetal and maternal complications.

Fetal complications are related to compromised placental blood flow and include spontaneous abortion, intrauterine growth restriction, fetal death *in utero*, and low birth-weight. Maternal complications occur in as many as one-half of pregnancies, including acute chest syndrome, bacteriuria, urinary tract infection, pyelonephritis, endometritis, pre-eclampsia, thromboembolic events, and the use of cesarean section.

As a result of these complications, careful monitoring during the antenatal period to ensure maternal and fetal well-being is essential.

Treatment

Comprehensive medical care must be provided to these patients with the team effort of physician, disease specific specialist and pediatric hematologist to treat the clinical symptoms and complications.

Hydroxyurea Therapy

Sickle cell disease (SCD) is a potentially devastating condition, which results in the vaso-occlusive phenomena and hemolysis. The severity of the complications that occur with this disorder are widely variable, but overall mortality is increased and life expectancy decreased when compared to the general population.

Hydroxyurea is a chemotherapy agent (myelosuppressive agent) currently approved by US Food and Drug Administration (FDA) for the treatment of sickle cell disease. It acts by increasing the fetal hemoglobin as its metabolism leads to NO related increase in cGMP which increases gammaglobin synthesis.[40] This in turn decreases sickling of red blood cells. These effects have been found to reduce the incidence of pain episodes, acute chest syndrome episodes and blood transfusions by 50 percent.[41]

Starting dose: Hydroxyurea can be started at a dose of 10 mg/kg orally, on a daily basis. The patient's hematological status should be monitored to rule out fall in the neutrophil count to less than 2,500 per cubic millimeter or platelet count to less than 80,000 per cubic millimeter.

Dose escalation: The dose of hydroxyurea can be increased at a rate of 5 mg/kg/week as long as the hematological values remain in an acceptable range, and the patient shows no other evidence of side-effect from the HU. Maximum dose is 25 mg/kg/day. Higher doses have been given at some institutions 35 mg/kg/day.

Indications for Hydroxyurea Therapy

- Patients who are hospitalized more than 4 or 5 times per year with painful vaso-occlusive crises
- History of acute chest syndrome
- History of other severe vaso-occlusive events
- Severe symptomatic anemia
- Severe unremitting chronic pain that cannot be controlled with conservative measures
- History of stroke or a high risk for stroke.

Transfusion Therapy

Chronic transfusion with red blood cells decreases the concentration of HbS in patients with sickle cell disease by three mechanisms:[42,43]

- Addition of Hb A from the blood of normal donors dilutes the Hb S in the blood
- The rise in hematocrit following blood transfusion suppresses erythropoietin release thereby reducing the production of new RBCs containing Hb S
- Longer circulating lifespan of Hb A containing normal RBCs as compared to sickle RBCs decreases the levels of Hb S.

Transfusion therapy for individuals with sickle cell disease can be categorized as therapeutic or prophylactic. Accepted indications for transfusion therapy in individuals with SCD include:[44,45]

Therapeutic: Acute use of transfusions for acute stroke, acute chest syndrome, acute multi-organ failure, sudden severe drop in hemoglobin (splenic sequestration, aplastic crisis, hyperhemolytic crisis), acute symptomatic anemia (e.g. onset of heart failure, dyspnea, hypotension, marked fatigue).

Prophylactic: Use of periodic red cell transfusions for primary or secondary stroke prevention.

Transfusion: Related complications include alloimmunization, infection and iron overload. Matching for minor antigens such as C, E, Kell, JKB (Kidd) and Fya (Duffy) antigens can significantly reduce alloimmunization.

A decision of simple versus exchange transfusion must be made on case to case basis.

Simple blood transfusion is used when the aim is to restore blood volume or oxygen carrying capacity in an acutely ill child. Partial exchange transfusions are recommended when an acute or chronic reduction in the concentration of Hb S is required without an increase in viscosity and iron burden (e.g. for acute emergencies such as multi-organ failure, suspected stroke, acute chest syndrome, primary and secondary prevention of stroke and prevention of recurrent painful episodes). The upper limit of hemoglobin should be kept at 10g/dL to prevent hyperviscosity and decreased oxygen delivery.

Transfusion and surgery: In patients with sickle cell disease undergoing surgery, events like hypoxia, dehydration, hypothermia may result in intra or post

operative complications. Blood transfusion with the aim of maintaining hemoglobin concentration at 10g/dL and HbS concentration < 30 percent given preoperatively may help to reduce complications. An exchange transfusion may be required in cases with Hb > 10g/dL.

Chelation Therapy

Iron overload occurs as a result of repeated transfusions in patients with sickle cell disease resulting in heart and liver failure along with other complications. MRI is a more accurate and non-invasive method of estimating tissue iron load and response to chelation. The chelating agents commercially available and approved for use are: desferrioxamine, deferasirox.

Desferrioxamine needs to be given parenterally or subcutaneously by prolonged infusion and nearly every day (5 days a week), which has limited its effectiveness in many patients. Deferasirox is an effervescent tablet that is dissolved in liquid and taken orally daily.

Erythrocytapheresis

This technique involves automated red cell exchange that removes blood containing Hb S from the patient while simultaneously replacing that same volume with packed red cells free of Hb S. Transfusion usually consists of sickle-negative, leuco-reduced, and phenotypically matched blood for minor red cell antigens C, E, K, Fy and Jkb.

The procedure is performed on a blood cell processor (pheresis machine) and is a better technique than manual exchange transfusion. This technique has the advantage of minimum iron accumulation; however it is less commonly performed due to requirement of expertise and equipment.

Bone Marrow Transplantation

Allogenic bone marrow transplantation (BMT) is the only known cure for sickle cell disease. Many risks are associated with BMT, and the risk-to-benefit ratio must be assessed carefully. The lack of availability of a matched donor may limit the utility of BMT.

Novel Therapies

New agents have been developed which are now in clinical trials based on the pathophysiology of sickle cell anemia.

- Induction of hemoglobin F
  - *Decitabine:* It is a nucleoside analoge that hypomethylates cellular DNA without cytotoxicity. This results in re-expression of γ-globin genes and induces γ-globin synthesis.[46,47]
  - *Butyrate:* It is a short chain fatty acid that inhibits histone deacetylase (HDAC) thereby affecting chromatin structure and transcription rates of γ-globin gene.[48]
  - *Trichostatin A:* HDAC inhibitor which significantly inhibits pulmonary vein expression of vascular cell adhesion molecule (VCAM) and tissue factor (TF) in mouse models.[49]
  - *Pomalidomide:* Thalidomide derivative that stimulates erythropoiesis, F cell production, total hemoglobin and Hb F synthesis in human CD34+ cells.[50]
- *Prevention of red blood cell dehydration:* Senicapoc is a potent and selective blocker of the Gardos channel (potassium efflux pathway) which prevents the loss of solutes and water maintaining the hydration of sickle RBCs which is critical to the rate and degree of polymerization.[51]
- *Nitric oxide (NO):* Sickle cell anemia is characterized by a state of NO resistance, NO inactivation and reduced NO availability. Options under investigation to increase the supply of NO include direct administration of inhaled NO, increasing the substrate availability with arginine supplementation, treatment of phosphodiesterase-5 inhibitor sildenafil to prevent breakdown of endogenous NO.
- *Anti-inflammatory agents:* Statins have potent anti-proliferative and anti-inflammatory actions and stabilize endothelial barrier function. Steroids have been reported to reduce the duration of severe pain episodes and severity of acute chest syndrome in children and adolescents, however more studies are required to assess the benefits and risk of this therapy.
- Treatment with tinzaparin (LMWH) may help in painful episodes as it decreases coagulation activation and adhesion of cell to vessel wall. Use of eptifibatide (glycoprotein IIb/IIIa inhibitor) in acute pain episodes are currently being studied.
- *Other agents:* Nix-0699 (Niprisan), a phytomedicine has shown to inhibit RBC sickling and produce a left shift of the oxygen dissociation curve of HbS on *in vitro* studies.[52] Safety and efficacy of intravenous immunoglobulin (IVIg) in these patients with acute pain episodes is being studied. Studies to evaluate the use of Bosentan (endothelin receptor antagonist) which have shown results in mouse models must be considered.

Supportive Therapy

Supplementation with folate (1 mg/day), multivitamin without iron, oral calcium and vitamin D along with nutritional management are recommended.

Addressing psychological issues like depression, scholastic backwardness, neurocognitive dysfunction with appropriate therapy and rehabilitation.

Morbidity and Mortality

The following three prognostic factors have been identified as predictors of an adverse outcome:[53]

- Hand-foot syndrome (dactylitis) in infants younger than 1 year
- Hb level of less than 7 g/dL
- Leukocytosis in the absence of infection

The mortality rate in infants and young children who have access to comprehensive health care has decreased dramatically. Due to routine use of antibiotic prophylaxis and immunization, acute chest syndrome and multi-organ failure are replacing bacterial sepsis as the leading cause of death. In regions where comprehensive care is available, the disease has shifted from a fatal pediatric illness to a chronic disease often associated with progressive deterioration in quality of life and organ function.

Patient Education

Patient education regarding the nature of the disease is essential. They must be taught to recognize early signs of complications to obtain prompt treatment and identify environmental factors that may precipitate a crisis. The importance of hydration, prophylactic penicillin, immunization and drug therapy and regular follow-up must be emphasized. Patients (including asymptomatic heterozygous carriers) must be explained the genetic basis of the disease and made aware of genetic counseling and prenatal diagnosis. Genetic testing can identify parents at risk for having a child with sickle cell disease. Families must be encouraged to join support groups for information of newer drugs and therapy such as of gene therapy, bone marrow transplantation and the usage of cord blood stem cells and psychosocial support.

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CHAPTER 19

Antenatal Diagnosis of Hemoglobinopathies

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BACKGROUND

The hemoglobinopathies and thalassemia syndromes are a group of autosomal recessively inherited disorder resulting from either the qualitative (structurally abnormal globins-*hemoglobinopathies*) or quantitative defects (abnormal synthesis of one or more of the globin chain-*thalassemia syndromes*). It is one of the common group of single gene disorder with a carrier frequency of >5%. In India, the carrier frequency varies from 3% to 17%, highest being in Pakistani Sindhi and Punjabi populations.

Thalassemia is an example of a best studied disease of a known molecular mechanism, and one of the first human genes cloned and sequenced. It is classified according to the abnormal synthesis of the globin chain. It is known as α-thalassemia or α-thal if the α chain is affected or the β-thalassemia/β–thal if β chain is affected. β-thalassemia affects HbA whereas α-thalassemia affects both HbA and HbF. Hemoglobins with any of the four identical globin chains are completely unstable and incompatible with life for example HbH disease with four β chains (β 4) or Hb Bart's disease with four γ chains (γ4). Until 1970s, prenatal diagnosis for hemoglobinopathies was possible by analyzing globin chain levels on fetal cord blood, but with gradual advancements in DNA based technology the testing gradually shifted to the more reliable and efficient PCR based studies on chorionic villi DNA to provide earlier fetal diagnosis. Recent technological advancements have lead to the possibility of fetal diagnosis on maternal blood as well.

MUTATIONS IN GLOBIN GENES

Although globin gene is one of the small gene, till date more than 1000 hemoglobin variants with single amino acid substitutions in one of the globin chains are known. Many of them are associated with hemoglobin disorders of different types and severity. Interaction of various thalassemia mutations and abnormal hemoglobins produces complex genotypes and an extremely wide spectrum of clinical and hematological phenotypes. The three main categories of these interactions that are associated with severe disease states and require further genetic counseling and prenatal diagnosis are as follows:

- β-*thalassemia syndromes* (includes δβ thalassemia and E/β mutations)
- *Sickle cell disease* (HbSS and variant interactions e.g. Hb S/C, Hb S/β thalassemia, Hb S/D Punjab, Hb S/O Arab, HbS/Lepore, Sβδ)
- Hb Bart's and HbH hydrops fetalis syndrome.

Alpha Thalassemias

The α thalassemias are a group of disorders characterized by α reduction in a globin synthesis. They can be divided into the severe types (α^1 or α° thalassemias) with typical microcytic hypochromic blood picture in heterozygotes, and the milder form (α^2 or α^+ thalassemias) which is usually silent. Deletion mutations are the common in α thalassemia, although α^+ thalassemia can also be caused by point mutations (nondeletional α thalassemia or α^T).[1] Deletion of all 4 globin chains results in the most severe type of α^0 thalassemia which is known as Hb Bart's hydrops fetalis syndrome. In the absence of α globin, HbF and HbA are not synthesized and fetal blood contains abnormal hemoglobin Bart's (γ4) leading to severe anemia, hydrops fetalis and fetal death. Alpha^0 thalassemia is particularly common in South East Asia whereas α^+ thalassemia predominates in Africa and India. The compound

heterozygous condition of $\alpha^{\circ}/\alpha^{1}$ thalassemia (- - / - α), α^{0} thalassemia and nondeletion α^{+} thalassemia (- - / $\alpha^{T}\alpha$) or homozygous nondeletion α^{+} thalassemia ($\alpha^{T}\alpha/\alpha^{T}\alpha$) results in moderately severe to severe HbH (β4) disease. In India, HbH traits have been reported among Bengali's, Malayalis, and Tamils, Gujaratis and Sindhis in Singapore. Other common structural hemoglobin variants are hemoglobin Constant Spring and Koya Dora from the populations of Southeast Asia[2] and Andhra Pradesh[3] in India respectively. High prevalence of hemoglobin Constant Spring (both heterozygotes and homozygotes) have also been reported among the coastal people of Orissa.[4]

Box 1 Disorders of globin chain synthesis warranting prediction in a fetus and prospective parents

Disorders of globin chain synthesis that should be predicted in a fetus

- β-thalassemia major (including E/β^{0} mutations)
- β-thalassemia intermedia (including E/β^{0} mutations)
- Sickle cell disease
- Hb Bart's hydrops fetalis syndrome (Homozygous α^{0} thalassemia) and (rarely) HbH hydrops fetalis syndrome ($\alpha^{0}/\alpha^{T}\alpha$)
- Compound heterozygous sickle cell states (including Hb S/C, Hb S/β-thalassemia, Hb S/D Punjab, Hb S/O Arab, HbS/Lepore).

β-Thalassemia[5]

The β-thalassemia are a heterogeneous group of disorder. These can be classified either as β^{0} thalassemia with the absence of β globin chain synthesis or β^{+}-thalassemia with reduced rate of beta chain synthesis. More than 200 mutations have been identified majority of them being the point mutations. About 10% of mutations are deletion mutations of varying size. Homozygous or compound heterozygous state of most β^{+} or β^{0} type of severe mutations result in β-thalassemia major, a transfusion dependent anemia. That usually present in later part of infancy or second year of life. Thalassemia intermedia is the milder clinical condition that present usually after second year of life. The type of mutation in the β globin gene determines the phenotype. Thalassemia intermedia too can be of varying severity depending upon the type of mutations and its interaction with other variant forms. In authors experience, due to the complex gene interactions and unpredictability of the phenotype prenatal diagnosis is often requested by the parents.

Sickle Cell Disease[5]

The sickle cell disease (SCD) is widely prevalent in India and has a variable clinical course. It is characterized by hemolytic anemia with acute crises due to infection or vaso occlusive episodes. An A to T substitution in codon 6 of the beta globin gene is responsible for SCD. Variable severity in SCD occurs due to the interaction with beta thalassemia and other structural hemoglobins. Homozygosity for HbS, HbS/HbD–Punjab, HbS/HbO–Arab; and HbS/β-thalassemia (severe mutations) results in moderate to severe clinical disease.

Box 1 summarizes the various globin chain disorders associated with severe disease states that requires prenatal diagnosis and genetic counseling. Clinically asymptomatic states are β thal trait, α thal trait (α -/α – or α α/--) or silent carrier state (α α/α-), HbE trait, homozygous HbE, HbC, HbD, deletional or nondeletional hereditary persistence of hemoglobin and $\delta\beta$ trait or Hb lepore trait.

- *Inheritance of hemoglobinopathies:* In most of the common hemoglobinopathies like alpha, beta thalassemias and sickle cell anemia the mode of inheritance is autosomal recessive. Autosomal recessive disorders manifest if both the parents are carrier of the mutant gene. The disease usually occurs in one generation only. The risk of occurrence and recurrence is 25% in such cases (Fig. 1). Identification of mutation in such couples or proband is the prerequisite for prenatal diagnosis. There is no risk of having an affected child if only one partner is a carrier or is affected (Figs 2 and 3). If only one partner is a carrier there is a 50% chance of the baby being a carrier and all babies will be carriers if one partner is affected with an autosomal recessive hemoglobinopathy. Identification of carrier status is easy and is possible by measurement of HbA_2 which is almost always high in carriers except in a rare situation of silent carrier status when HbA_2 may be normal and carrier status can only be confirmed by molecular studies.
- *Diagnosis of hemoglobinopathies*[5-7]: Hematological and biochemical investigations are basic screening investigations that are widely available for diagnosis as well as carrier screening. DNA studies are important for fetal diagnosis and making genotype phenotype correlation.
  - *Basic hematological parameters*:
    i. Complete blood count including Hb, RBC, MCH, MCV, and RDW
    ii. Hemoglobin electrophoresis at pH 8.6 using cellulose acetate membrane for common hemoglobin variants i.e. HbS, C, DPunjab, E, OArab and Lepore Hb
    iii. Hemoglobin electrophoresis at pH 6 using acid agarose–Distinguishes between C, E, and

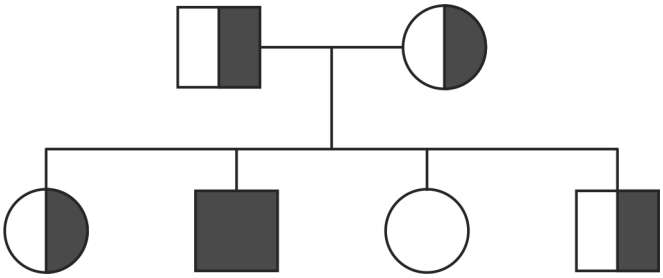


Fig. 1 Twenty-five percent risk of affected child if both parents are carrier

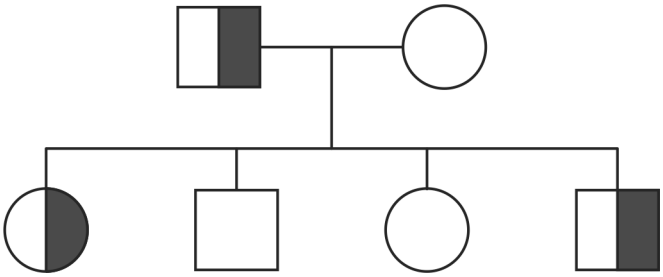


Fig. 2 No risk of affected child if only one parent is carrier

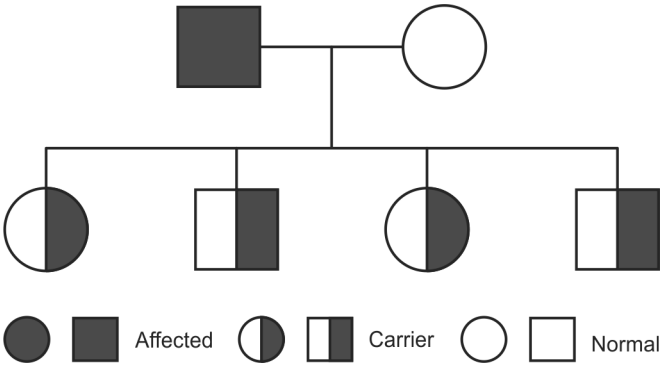


Fig. 3 No risk of affected child if one parent is affected

OArab from each other and also HbS and, DPunjab from each other.

iv. HPLC for simultaneous detection and quantitation of hemoglobin fractions.

– *Supplementary hematological methods*
  i. S, Fe and Ferritin, transferrin saturation
  ii. Globin chain synthesis
  iii. Immunological measurement of fetal cells for HPFH. Distinguishes between heterocellular and pancellular distribution
– *DNA analysis:* An analysis or the research conducted at various places across the globe reveals that hemoglobinopathies are caused due to the result of a broad spectrum of mutations (N>1000). These include point mutations, frame shift mutations, large deletions and rearrangements. Various techniques such as oligonucleotide specific hybridization, oligonucleotide specific amplification, oligonucleotide specific ligation, gap-PCR, restriction endonuclease analysis of amplified product, and real-time PCR are being used to identify these alterations. Each of these techniques has its inherent advantages and disadvantages. Every laboratory selects its approach of mutation detection on the basis of the mutation that is prevalent in that particular population, ethnicity of that particular family and running cost, suitability to routine diagnosis and reliability of the technique.

Some laboratories have started using direct sequencing as the first approach to identify a large number of different mutations in at-risk populations. Indirect mutation tracking using restriction enzymes having polymorphic sites linked to mutations becomes the second choice where mutations are not identified in previous affected child and family has requested for prenatal diagnosis in next pregnancy.

Source of DNA: For molecular diagnosis in parents and in affected child, preferable source of DNA is blood. The 5 mL blood in EDTA can be transported to the laboratory at room temperature within 24–48 hours for further testing. However blood spots and buccal swabs can also be used in case the child is very young or parents are apprehensive of venipuncture.

On the other hand, for prenatal diagnosis fetal DNA can be obtained from chorionic villi sample, amniotic fluid and cord blood. Chorionic villi sample is usually preferred over amniotic fluid and cord blood because it can be done at earlier gestation and gives better yield of DNA. The 20–25 mg of tissue in heparinized saline provided by laboratory/RPMI culture medium can be transported to the laboratory within 24–48 hours at room temperature, where fetal tissue is carefully separated from maternal tissue by looking under inverted microscope.

Antenatal screening followed by prenatal diagnosis in carrier couples and selective termination of affected pregnancies is the only modality available which could significantly reduce genetic load of hemoglobinopathies in various countries. Cyprus for instance, has reduced their incidence of thalassemia and thus stands as an ideal for other countries. It has progressed from reporting the highest occurrence of thalassemia in the world to nil within an impressive span of just 11 years (1991–2002).[8] In addition to the incidence, the carrier frequency has also been reduced by 1.89% in 24 years.[9] Today, in India also

many centers have opened up which are actively involved in providing prenatal diagnosis.

Carrier screening and prenatal diagnosis: The best way to decrease the burden of hemoglobinopathies is to perform carrier screening followed by prenatal diagnosis and selective termination of affected pregnancies. Before prenatal diagnosis was available, premarital screening and counseling did not affect the marriage and reproductive behavior in Greece and Cyprus, countries with very high carrier frequency. With the introduction of prenatal diagnosis along with mandatory premarital screening had a tremendous effect on reducing the birth of affected babies to almost zero. This was only possible by mass education program with involvement of religious leaders. The screening protocol which is usually followed is outlined in the Flow chart 1.

Prerequisites for prenatal diagnosis:

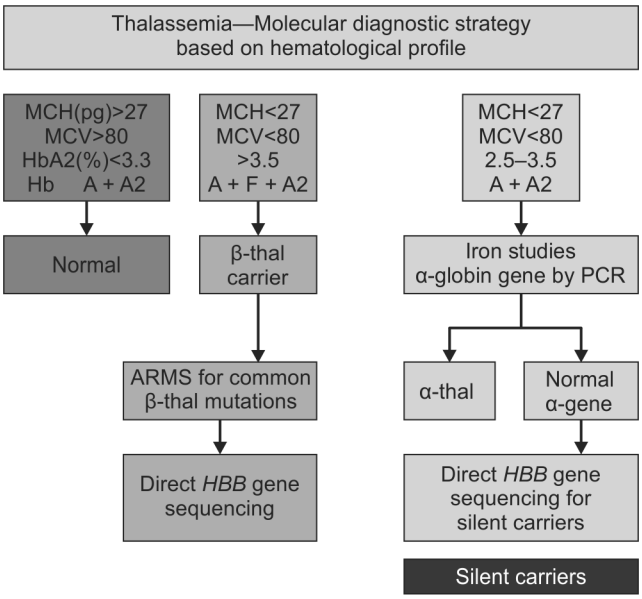
- Confirmation of carrier status in both partners and identification of disease causing mutations/informative linkage
- Counseling about recurrence risk of disease (25% in each pregnancy) and medical termination of affected pregnancy
- Explanation about procedural risk (i.e. chances of fetal loss).

Prenatal diagnosis is increasingly becoming available world over. Across India, many centers are now offering prenatal diagnosis using molecular techniques.[6,7] Once the carrier status of the partners is confirmed, it is followed by DNA studies to identify the mutant allele in both the partners. If the family has a previously affected child then the most important step in prenatal diagnosis is to first determine the mutation status of the affected child in the pretransfusion state along with the parents genotyping. If proband is not available then, parental DNA can be tested for thalassemia mutation followed by prenatal diagnosis. There are five common mutations reported in Indian population along with about 12 rare mutations. According to our experience mutations are identified in 95–97% of North Indian families.

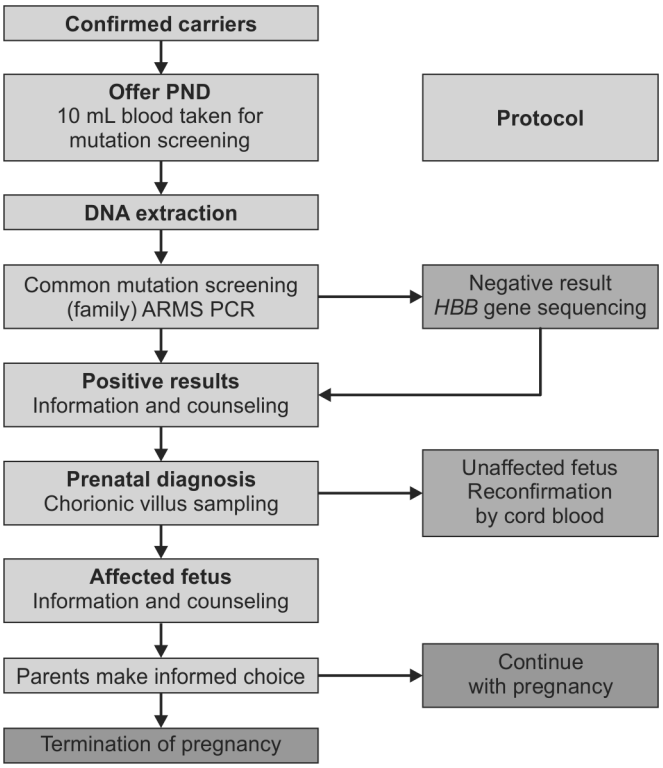
The technique used for identification of mutations is based on allele specific amplification and is called amplification refractory mutation system (ARMS) PCR in most centers in India and abroad and is very reliable.[10] If the mutations are identified chorionic villus sampling (CVS) is offered around 10-12 weeks of pregnancy after counseling. CVS is the most widely used sample as it gives good DNA yield and can be done early in pregnancy. The risk of fetal loss with CVS is around 2-3%. It usually takes about a week for initial mutation screening and the same time for CVS reporting. In situations when the mutations are not identified, use of linkage studies or cord blood analysis for globin chain synthesis or HPLC can be useful for prenatal testing.

Flow chart 2 shows the strategy being followed in our laboratory for prenatal diagnosis.

Flow chart 1 Thalassemia carrier screening protocol for thalassemia



Flow chart 2 Thalassemia prenatal diagnosis protocol



DNA Techniques Required for Hemoglobinopathies

Detection of Known Mutations

There are numerous PCR based techniques which are being used to detect known mutations, other than large deletions and rearrangements causing hemoglobinopathies. Commonly used techniques are amplification refractory mutation system, restriction enzyme PCR (RE-PCR) and reverse dot blot analysis with allele specific oligonucleotide probes.

Reverse Dot Blot Analysis

Reverse dot blot analysis is a nonradioactive technique in which allele-specific oligonucleotide (ASO) probes are immobilized on a nylon membrane. Separate probes complementary to each known mutant normal allele are spotted on a nylon membrane and subjected to hybridization with patient DNA. Signals are generated only for those particular mutations which are present in patient DNA. These strips are commercially available and can be customized according to mutation spectrum of any population. Prior knowledge of common and uncommon mutations in a population and simplicity of the technique make it affordable and suitable for routine diagnostic labs.

In India, this technique is commonly used to detect five common mutations and other variants like HbS, HbD and HbE in *HBB* gene. Whereas in other countries like Sicily it is also used to detect α-thalassemia and δ-thalassemia point mutations.

Amplification Refractory Mutation System

Amplification refractory mutation system (ARMS) is the most commonly used technique throughout the world to detect known common and few rare mutations causing beta-thalassemia. Its principle is based on the quality of perfectly matched primer over mismatched primer resulting more efficiency in annealing and primer extension. The 3' terminal nucleotide of these primers is specific to the desired allele. Hence annealing of this primer is possible only if the desired (Mutant/normal) allele is present in the tested DNA. So, for every patient two parallel reactions in two separate tubes containing normal and mutant primer each are set up. A homozygous mutant and wild sample shows amplification only in the tube containing mutant and normal primer respectively whereas a heterozygous sample shows amplification in both tubes.

Two modifications are generally practiced in most of ARMS PCRs. First modification is done at the time of primer designing. This includes the incorporation of a mismatched nucleotide at the -2/-3 position from 3' end to increase the specificity of these primers. Second modification is addition of another set of primers amplifying a different gene locus which acts as an internal control. This modification is introduced while setting up an ARMS PCR reaction. To detect five common Indian mutations the general practice is to set up four different reactions containing one mutant primer for each mutation naming IVS1-5(G>C), IVS1-1(G>T), Cdn8-9(+G) and Cdn41-42(-CTTT), a common reverse primer and also a set of primer specific to 5th common mutation (619 bp deletion). This primer set for 619 bp deletion serves dual purpose. First it serves as an internal control by amplifying a region of 861 bases and second it also detects patients having this deletion of 619 bases from that region by showing an additional band of 242 bp size. Figure 4A depicts the principle of ARMS PCR and Figure 4B shows an agarose gel photograph of a common beta-thalassemia mutation.

ARMS PCR is mostly preferred to detect known point mutations, small insertions and deletions over RFLP and direct gene sequencing due to its simplicity, cost effectiveness and less labor intensive nature.

Restriction Enzyme PCR

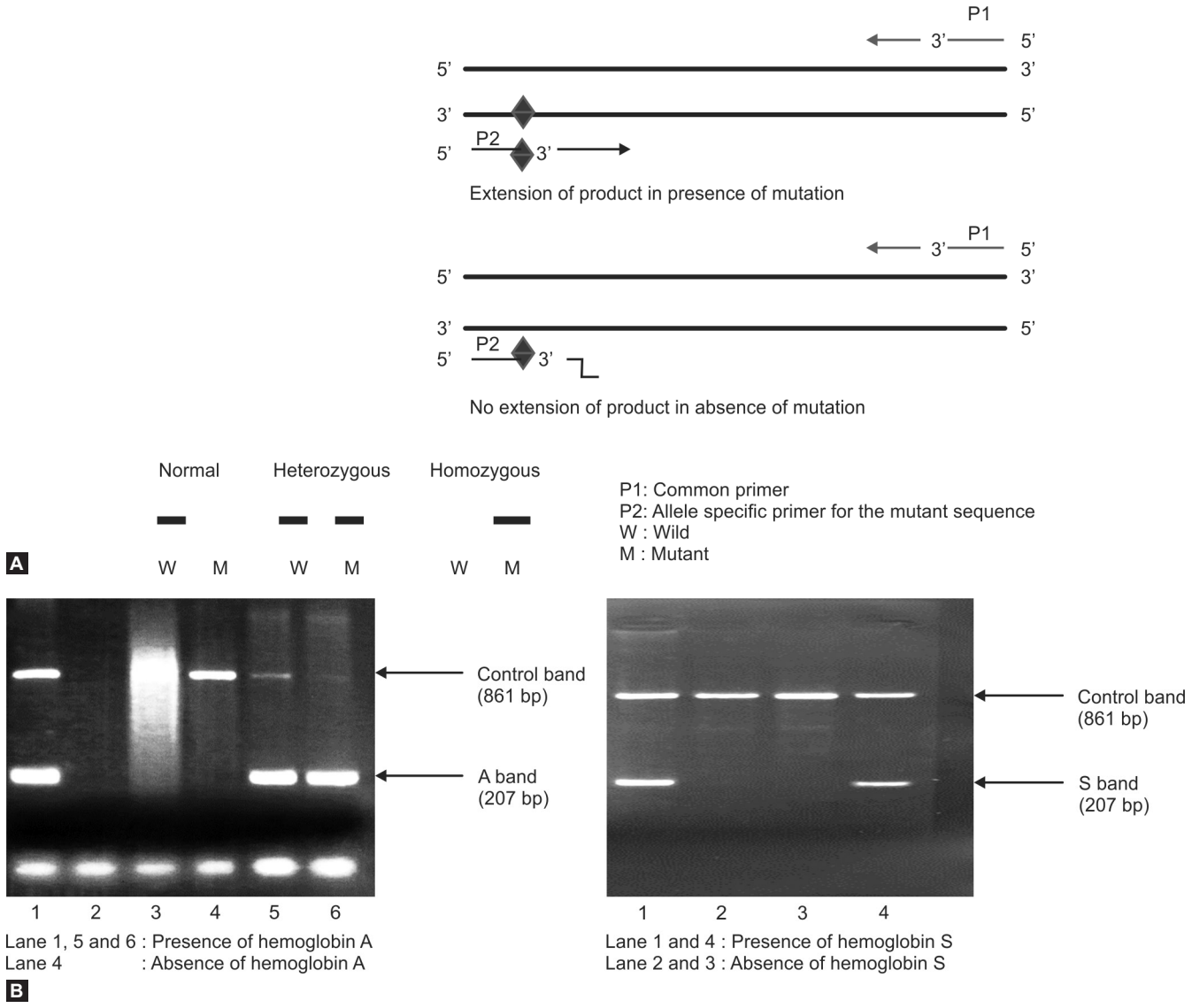
Restriction enzyme PCR (RE-PCR) is less preferred technique for molecular diagnosis of hemoglobinopathies because consumption of restriction enzyme for a large set of samples make it comparatively expensive than ARMS PCR and there are very few mutations in the list of hemoglobinopathies which creates or abolish a pre-existing restriction site. It was previously used very commonly for the detection of HbD, HbS and HbE mutations. Flanking sequence of a particular mutation is first amplified by PCR before cutting a mutant DNA with a restriction enzyme resulting in a different restriction map.

For example, a missense mutation (A > T) at codon 6 of *HBB* gene causing the substitution of glutamic acid by valine resulting in sickle cell anemia the most common hemoglobinopathy of tribal population abolishes a specific recognition site for the restriction endonucleases Dde I and Mst II.

Gap PCR

Detection of large genomic rearrangements, gross deletions causing deletional HPFH, δβ-, γ δβ-, εγ δβ – thalassemia and α thalassemia was previously based on a nonPCR technique called southern blot. But with time and technical advancements this technique has been totally replaced by Gap PCR.

If the deletion is less than 1 Kb then only two primers flanking deleted sequence are needed which generate



Figs 4A and B (A) ARMS PCR; (B) gel photograph of ARMS

two fragments of different sizes in heterozygous patients. The smaller one is due to deletion. But in case of larger deletions one more primer complementary to a part of deleted sequence is needed which gives an amplification in normal individuals, as distance between primers flanking deleted sequence is too large to amplify normal allele. On the other hand, in the presence of deletion these flanking primers produce an amplified product.

Gap PCR is much faster, simpler, cost effective and less labor intensive in comparison with southern blot hence most suitable for a routine diagnostic set up.

MLPA is another technique which is gaining in reputation day by day. The technique has an advantage over gap PCR because of its ability to detect unknown deletions and rearrangements in addition to the previously reported in literature. But its high cost limits its use in populations where frequency of large deletions and rearrangements is very low.

Detection of Unknown Mutations

Detection of unknown mutations was previously based on few screening techniques like denaturing gradient gel electrophoresis/single stranded conformation polymorphism/heteroduplex analysis followed by sequencing of that particular exon where a change was suspected by any of above mentioned screening technique.

However, these days, most laboratories resort to direct gene sequencing as a first hand test since the rapid advancement and competition within the biotechnology, sector has led to a significant fall in the cost of gene sequencing. This has made the process of reporting faster and high throughput. Direct sequencing analysis is particularly applicable to the globin genes which are compact and relatively small (1.2–1.6 kb) with the majority of the point mutations within the gene or its flanking sequences. Mutations in the *HBB* gene are not limited to the exons and their direct splice sites so the primers are designed in the manner that they cover deep intronic region and regulatory sequence.

Diagnosis Using Indirect Methods

Restriction Fragment Length Polymorphism (Fig. 5)

Linkage analysis can be an approach for prenatal diagnosis in families at risk where mutations could not be identified by using direct mutation detection methods. Before offering PND to these families it is mandatory to do heterozygosity screening of different intragenic markers like AVA II, BamH I, etc. in family members including parents, previous affected child/normal child. By establishing heterozygosity of these markers and following their patterns in parents and affected child mutated allele can be tracked indirectly in fetal DNA with no knowledge of causal mutations. The β-globin gene cluster is known to characterize at least 18 restriction fragment length polymorphism (RFLP). But nonrandom association of these sites result in only few haplotypes. Application of this technique is limited to only those families where at least two intragenic markers are informative and previous affected child/normal child's sample is available. The diagnostic error of this technique is a little higher (0.3%) than direct mutation detection methods due to chance of recombination between RFLP site and mutation locus.

Laboratories having no access to relatively advanced and expensive techniques of direct mutation analysis (e.g. gene sequencing and MLPA) can make use of this technique in families at risk of producing children with hemoglobinopathies.

Cord blood high performance liquid chromatography or globin chain synthesis in fetal blood may be useful in rare situations if the mutation is not identified.

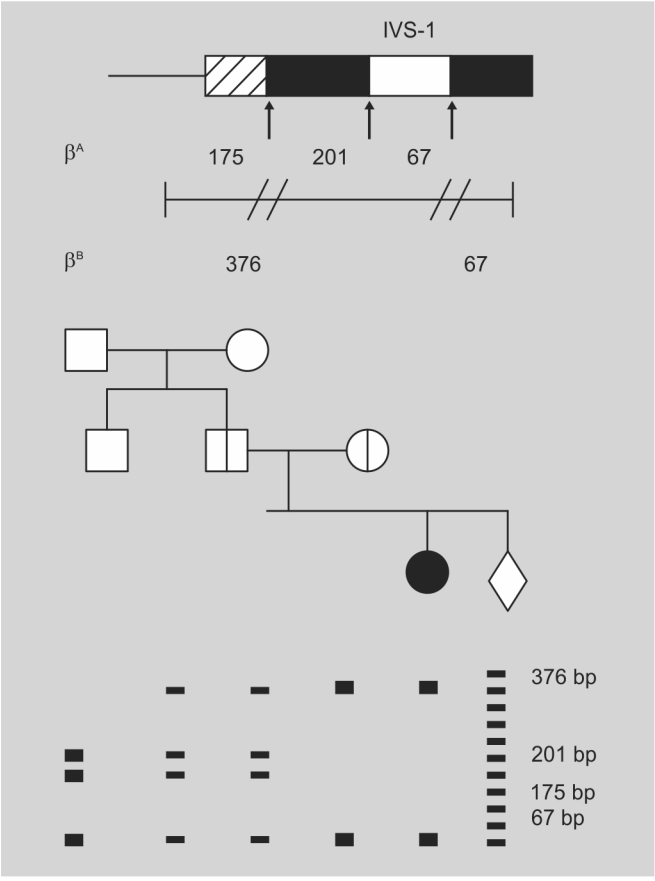


Fig. 5 Use of linkage studies for prenatal testing

PRECAUTIONS TAKEN WHILE DOING DNA ANALYSIS

A lab following reasonably good standards can also result in 1-2% diagnostic error. This can arise due to several technical reasons such as maternal DNA contamination, cross contamination causing false amplifications leading to false positive results, false paternity, genetic recombination, deficient endonuclease digestion, allele drop out, undiagnosed hemoglobinopathy in compound heterozygote state in parents leading to misdiagnosis of fetus and also due to human errors like sample exchange, mislabeling of samples, etc.

Following guidelines can help in minimizing these errors:

- Chorionic villus sample should be washed in saline to get rid of maternal blood and it should also be dissected under microscope to get rid of maternal decidua before sending to the fetal diagnostic laboratory
- Appropriate positive and negative controls should always be analyzed with fetal sample
- Fetal sample should always be run in duplicate
- PCR program should have a limited number of cycles to minimize amplification contaminating DNAs
- VNTR analysis using different polymorphic markers should always be run in parallel to rule out maternal contamination

- Every sample should have more than one identification code e.g. full name, lab number, date of birth, sample date, etc.
- The test that involves gene sequencing should always be done in both directions
- The type of DNA analysis method used and its inherent risk of misdiagnosis should be clearly mentioned in prenatal diagnosis report.

Noninvasive Methods

Owing to abortion risk to the fetus and the anxiety experienced by mothers undergoing any prenatal diagnostic procedure done to rule out hemoglobinopathies, focus has been shifted towards development of noninvasive tests which are not only accurate but rapid, and can be performed early in pregnancy for prenatal diagnosis.

There are two noninvasive approaches which have been followed till today. One involves analysis of cell free fetal DNA in maternal plasma and serum and the other approach utilizes fetal cells within maternal circulation as a source of fetal DNA. Determination of fetal gender and RhD status of the fetus with in RhD-negative pregnant women by using maternal plasma and serum has already been established in European countries.[11,12]

This approach, however is not suitable for the analysis of fetal loci that do not differ largely from the maternal allele (e.g. beta-thalassemia), due to the vast predominance of cell-free-maternal DNA in maternal samples.

To overcome this problem researchers have used size fractionation as a possible enrichment technique to enrich fetal DNA molecules. Use of peptide nucleic acids followed by allele specific real time PCR to suppress the amplification of wild type maternal allele is another approach which has been used extensively and has given promising results in detection of paternally inherited fetal point mutations of beta-thalassemia and sickle cell disorder.[13]

MS-SABER (mass spectrometry-based single-allele base extension reaction) and MS-ASBER (mass spectrometry-based allele-specific base extension reaction) have also been used in this field and have enabled sensitive differentiation of fetal specific alleles down to a single nucleotide level and has shown to be useful for the detection of certain beta-thalassemia mutations and HbE disease respectively.[14]

Moreover Gaibiati, et al. explored the applicability of COLD PCR (coamplification at a lower denaturation temperature polymerase chain reaction) to enrich paternally inherited mutated allele in maternal plasma which was then detected on a sequencer.[15]

Applicability of above mentioned techniques is limited to only those families where partners carry different mutations. To overcome this problem now focus is towards finding new SNPs which are linked to known beta-thalassemia mutations and can be used later as a marker for NIPD of beta-thalasssemia in couples carrying same mutations. Papasava, et al. used this approach in combination with arrayed primer extension (APEX) method in genetically homogenous population of Cyprus and screened 34 families to know the most informative paternally inherited single nucleotide polymorphisms (SNPs). Only 11 families were infomative for more than two SNPs.[16]

More recently a group in Netherland assessed the possibility of using pyrophosphorolysis-activated polymerization (PAP) technique for noninvasive prenatal diagnosis (NIPD) of β-thalassemia major and sickle-cell disease (SCD). Phylipsen, et al. developed this assay to detect SNPs specifically inherited from the father by linkage to the normal or mutant allele to determine the risk of having an affected fetus. This approach can provide NIPD to the families where both partners carry same mutation.[17]

Tracking associated SNP to the paternal allele is an indirect approach of knowing presence of father's mutation and has its own limitations. For instance, the low heterozygosity of these markers make the analysis more time consuming and allows only few families suitable for NIPD. Secondly, it does not give any direct information of the genotype of the fetus.

The introduction of massive parallel sequencing in the field of NIPD and recent demonstration of deep sequencing of maternal plasma genome augmented the applicability of CFF DNA to many more diseases unlike before. Applicability of this technique in screening aneuploidies has already been established but in the field of hemoglobinopathies research is limited to very few reports and world is still waiting for an accurate, safe, rapid, noninvasive tests for prenatal diagnosis of hemoglobinopathies. More recent technologies such as next generation sequencing (NGS) and whole genome sequencing (WGS) although have potential to replace currently practiced invasive procedures for both pre- and postnatal diagnosis in the near future. Although highly useful, these techniques will not be suitable to all laboratories performing prenatal diagnosis of hemoglobinopathies given their high cost and sophistication.

Full range of genetic disorders including hemoglobinopathies can be diagnosed noninvasively through examination of intact fetal cells circulating with in maternal blood. But due to scarcity of fetal cells within maternal blood, procedures to enrich the cells and enable single cell analysis with high sensitivity are the complexities associated with this technique. Recent advancements like lectin-based method to separate fetal cells from maternal

blood and autoimage analysis have reported better sensitivity. The noninvasive prenatal testing is already in clinical practice for aneuploides and the progress in the field of single gene disorders is promising.

COUNSELING

Counseling for hemoglobinopathies include exact determination of parental genotypes, proper understanding of their interaction and assessment of clinical severity of the disease in the fetus. It should also be accompanied with the available treatment and the overall prognosis. One should offer the prenatal diagnosis by available methods along with the adequate pretest and post-test counseling.

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CHAPTER 20

Red Cell Membrane Disorders (Spherocytosis, Elliptocytosis, Stomatocytosis)

Sunil Gomber, Pooja Dewan

Normal RBC survival span is 110 to 120 days (half life, 55–60 days). The plasma membrane of the red blood cell (RBC) consists of a complex ordered array of lipids and proteins stretched over the outer surface of the cell in the form of a lipid bilayer that is dotted by penetrating or surface proteins. Specialized interactions occur between specific membrane proteins or lipids, or both, to maintain the stability of the membrane. Various red cell membrane proteins are necessary to maintain the normal shape of an erythrocyte, which is a biconcave disc.[1,2] A deficiency of any of the components of this membrane can lead to distorted red cell morphology (Fig. 1), increased breakdown of red cells and anemia, i.e. intracorpuscular (intrinsic) hemolytic anemia (Table 1).

Common red cell membrane defects include:
- Hereditary spherocytosis
- Hereditary elliptocytosis
- Hereditary stomatocytosis

HEREDITARY SPHEROCYTOSIS

It is a genetically-transmitted form of spherocytosis, an autohemolytic anemia characterized by the production of red blood cells that are sphere-shaped rather than donut-shaped, and therefore more prone to hemolysis.

Prevalence

It is the most common red cell membrane defect and is especially common in people of North European or Japanese descent, where the prevalence may be as high as 1/5000.

Etiology

Hereditary spherocytosis is an autosomal dominant trait, although sometimes the mode of inheritance can be recessive, and an estimated 25 percent of cases are due to spontaneous mutations. A patient has a 50 percent chance of passing the disorder onto his/her offspring, presuming that his/her partner does not also carry the mutation.

Table 1 Major human erythrocyte membrane proteins

SDS gel band	*Protein*	*Location of protein in red cell membrane*
1	Alpha spectrin	Peripheral
2	Beta spectrin	Peripheral
2.1	Ankyrin	Peripheral
2.9	Alpha adducing	Peripheral
3	AE1	Integral
4.1	Protein 4.1	Peripheral
4.2		Peripheral
4.9	Demantin P55	Peripheral Peripheral
5	Beta actin Tropomodulin	Peripheral Peripheral
6	G3PD	Peripheral
7	Stomatin Tropomyosin	Integral Peripheral
8	Protein 8	Peripheral
PAS-1	Glycophorin A	Peripheral
PAS-2	Glycophorin B	Peripheral
PAS-3	Glycophorin C Glycophorin D	Peripheral Peripheral

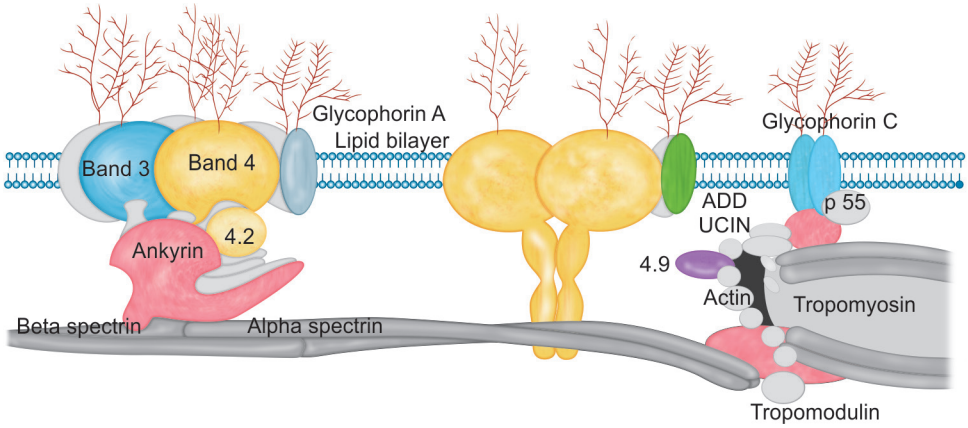

Fig. 1 The structural composition of the red cell membrane in vertical and horizontal interactions

Table 2 Common gene mutations in hereditary spherocytosis

Mutation	*Protein-coded*	*Inheritance*
ANK1	Ankyrin	Dominant/Recessive
AE1 (SL4A1)	Band 3	Mostly dominant
SPTB	β-Spectrin	Dominant
SPTA1	α-Spectrin	Recessive
EPB42	Protein 4.2	Recessive

- Hereditary spherocytosis is caused by a variety of molecular defects in the genes that code for red cell membrane. The protein that is most commonly defective is ankyrin (dominant and recessive defects). A recessive defect has also been reported in α-spectrin; dominant defects have been reported in β-spectrin and protein 3.

Table 2 depicts the common gene mutations seen in hereditary spherocytosis. These defects lead to loss of membrane surface area while the mean corpuscular volume is normal, and a consequent sphering of red blood cells is seen. An increased permeability of the red cell membrane to sodium occurs, which is compensated by an active transport of sodium out of the cell by a cation pump mechanism. There is an increased glycolysis to generate the adenosine triphosphate needed for the active transport of sodium out of the red cells. The spherocytes are also less deformable and hence are destroyed in the spleen during passage through the splenic cords to the splenic sinuses.[3,4]

Clinical Features

It has a varied clinical presentation, ranging from asymptomatic to severe hemolysis.[3,4] Anemia, jaundice and splenomegaly are the common clinical features of hereditary spherocytosis. The degree of anemia is extremely variable and may be absent, mild, moderate, or severe to the point of threatening life. The clinical severity of this condition is generally classified into three forms, as shown in Table 3.

Mild: It occurs in 20 to 30 percent of cases. These patients have no anemia, modest reticulocytosis, and little splenomegaly or jaundice, and may not be detected until adolescence or adult life. Increase in erythropoiesis is maintained via erythropoietin despite accelerated hemolysis. The stimulus for increased production of erythropoietin is not known but does not appear to be hypoxia.

When detected in the neonatal period, it is commonly accompanied by jaundice, requiring.

Treatment with phototherapy or exchange transfusion Hemolysis can be marked in the neonate due to an increased level of Hemoglobin F. Hemoglobin F binds 2,3-diphosphoglycerate poorly, consequently increased levels of 2,3-diphosphoglycerate destabilizes spectrin-actin-protein 4.1 interactions in the red cell membranes.

However, most neonates have little or no anemia, reticulocytosis, or spherocytosis on the peripheral blood smear; this is followed by a reduction in the hemoglobin concentration over the ensuing three weeks that is transient, but may be severe enough to require transfusions.[5]

In infancy and childhood the presentation is variable. Some children present with pallor, acholuric jaundice, icterus, exercise intolerance, and progressive splenomegaly (Minkowski-Chauffard syndrome). Pigmentary gallstones appear starting from 4 to 5 years of age. Hemolytic facies may be seen but are less marked than in thalassemia major. Radiography may show widened diploe of skull bones and oxycephaly. Chronic leg ulcers may be seen.

Table 3 Clinical severity of hereditary spherocytosis

	Trait	*Mild*	*Moderate*	*Severe*
Hemoglobin (g/dL)	Normal	11–15	8–12	6–8
Reticulocyte count (%)	≤3	3.1–6	≥6	≥10
Bilirubin (mg/dL)	≤1	1.0–2.0	≥2.0	≥3.0
Spectrin per erythrocyte	100	80-100	60–80	40–60
Osmotic fragility: • Fresh blood • Incubated blood	Normal Slightly increased	Normal/slightly increased Increased	Increased Increased	Increased Increased
Autohemolysis without glucose (%) Correctability (%)	<10 >60	≥10 >60	≥10 0–80	≥10 50
Splenectomy	Not needed	Usually not needed during childhood and adolescence	Necessary during school age before puberty	Necessary, delay till 6 years, if possible
Symptoms	None	None	Pallor, erythroblastopenic crisis, hyperbilirubinemia, gallstones	Pallor, erythroblastopenic crisis, hyperbilirubinemia, gallstones

These patients are particularly prone to aplastic crisis due to parvovirus infections.

Diagnosis

The diagnosis of hereditary spherocytosis is established by a combination of clinical examination, detailed history including family history, and laboratory tests.

- A positive family history is seen in up to 75 percent of patients.

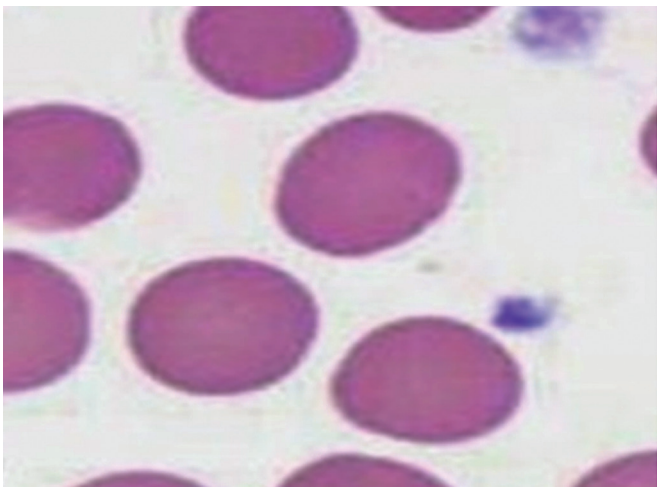

Fig. 2 Spherocytes, lacking, central pallor

- The red blood cells appear sphere shaped and lack the central pallor (Fig. 2).

Peripheral blood smear shows microspherocytes and polychromasia. The percentage of microspherocytes usually correlates with the severity of hereditary spherocytosis. The mean corpuscular volume is normal, and the mean corpuscular hemoglobin concentration is raised (36–38 g/dL). The red cell distribution width (RDW) is increased.

Evidence of hemolysis is in the form of reticulocytosis (3–15%), decreased haptoglobin, indirect hyperbilirubinemia and ultrasonic detection of gallstones may be seen. Coomb's test is negative. Increased red cell osmotic fragility is seen. Spherocytes lyse in higher concentrations of saline than normal red cells. This feature gets accentuated when RBCs are deprived of glucose for 24 hours at 37°C (Incubated osmotic fragility test). However, this test is not specific for hereditary spherocytosis, but may be positive in hereditary elliptocytosis. Also, the test may be negative in presence of iron deficiency, during recovery from aplastic crisis or in presence of obstructive jaundice. Also osmotic fragility cannot differentiate the immune and non immune causes of spherocytosis. The eosin-5-maleimide (EMA) binding dye test, which requires a flow cytometer is also positive, and may be used as a screening test in addition to cryohemolysis test. Gel electrophoresis analysis of erythrocyte membrane proteins may be used as confirmatory diagnostic test in selected cases.

Complications

- Hemolytic crisis
- Erythroblastopenic crisis
- Folate deficiency
- Gallstones
- Hemochromatosis
- Leg ulcers
- Growth retardation

Treatment

Transfusion: Continued transfusion dependence is unusual and it is important to avoid repeated transfusion whereas possible. Many older children with Hb levels of 5 to 6 g/dL do not require transfusion. Children who require one or two transfusions early in life frequently become transfusion independent.

- A regular follow-up is to be done once a child is diagnosed to have hereditary spherocytosis (HS). An annual visit to the physician is recommended even in the absence of symptoms. A hemogram is unnecessary in the absence of symptoms but a clinical examination including a general assessment, measurement of splenic size, growth, and exercise tolerance is needed.
- An ultrasonogram of abdomen, every 3 to 5 years is needed to look for gallstones, starting from the age of 5 years.
- In the presence of chronic anemia, there may be increased iron absorption in these patients, necessitating estimation of iron load during follow-up visits.
- Folic acid supplementation (2.5 mg/day up to 5 years age and 5 mg/day thereafter) is needed to meet the increased bone marrow requirements. In presence of erythroblastopenic crisis, leukocyte-depleted red blood transfusions are needed. Erythropoietin may be of benefit in reducing or avoiding transfusion, and can usually be stopped by the age of 9 months. Splenectomy is needed in moderate and severe cases.[4–6]

Indications for Splenectomy

- Severe anemia
- Reticulocytosis > 10 percent
- Repeated hypoplastic or aplastic crisis
- Faltering growth

Following splenectomy, there are chances of septicemia, and thrombosis. Therefore, where possible, splenectomy should be deferred till six years. Even in severe cases of HS who are transfusion dependent, it should not be done till 3 years of age to avoid risk of septicemia. Some people recommend partial splenectomy, where around 85 to 90 percent of spleen is removed, while 10 to 15 percent of spleen is left behind to preserve the immune functions of spleen. Usually the lower pole is removed while the upper pole is left *in situ*.[7] Sometimes, there is a failed splenectomy, when the child continues to be pale and transfusion dependent even after splenectomy. This is usually due to the presence of accessory spleens in the body.

Two to Three Weeks Prior to Splenectomy

- Children should be vaccinated against pneumococcal, meningococcal, and *Halmophilus influenza B* infections.
- Children should receive prophylaxis with penicillin (age < 5 year: 125 mg BD, age ≥ 5 year: 250 mg BD). Following splenectomy.
- There is disappearance of jaundice, anemia, and reticulocytosis.
- Peripheral smear shows the presence of Howell-Jolly bodies, target cells, acanthocytes and siderocytes
- Mean corpuscular volume of RBCs rises, while the mean corpuscular hem oglobin concentration falls.
- Pigment gallstones usually develop in patients of HS. In case of symptomatic gallstone disease, it is preferable to remove the gallbladder at the time of splenectomy.

HEREDITARY ELLIPTOCYTOSIS

Hereditary elliptocytosis are a heterogeneous group of inherited erythrocyte disorders, most of which are autosomal dominant that have in common the presence of elongated, oval, or elliptically shaped red blood cells (15–50% of RBCs) on the peripheral blood smear. Some conditions like thalassemias, and iron deficiency anemia, are also characterized by the presence of elliptocytes on peripheral smear but they constitute less than 10 percent of RBCs.

Prevalence

The prevalence of hereditary elliptocytosis in the United States is not greater than 2.5 to 5 per 10000. However, in West Africa and South-east Asia, where malaria is endemic, it may reach 1.6 and greater than 30 percent of the population, respectively. The hereditary pyropoikilocytosis variant of hereditary elliptocytosis is more frequent among black population, while the spherocytic elliptocytosis variant is reported only in Caucasians.

Etiology

It is usually inherited as, however, hereditary pyropoikilocytosis is a severe variant where there are two defective alleles. Usually, there is a defect in spectrin leading to defective spectrin heterodimer self-associations. Rarely, there may be abnormalities in protein 4.1 or glycophorin C.

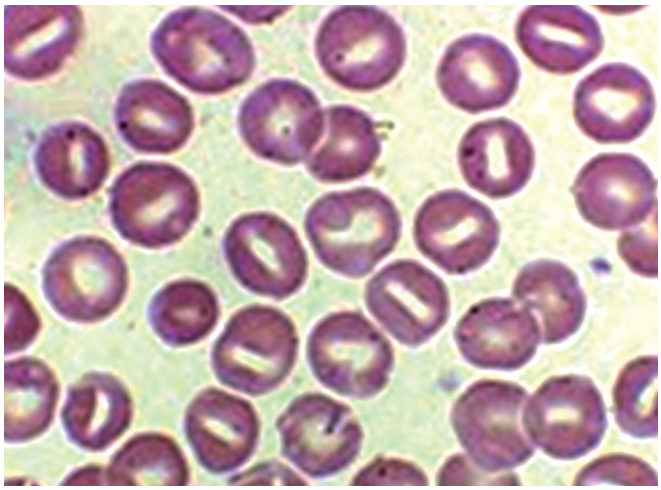

Fig. 3 Stomatocytes showing a slit-like gap in center of red cells

Elliptocytic RBCs are the characteristic finding on the peripheral smear (Fig. 3). The elliptocytic shape is conferred by the red cell membrane skeleton, as shown by persistence of the shape change in red cell ghosts and in skeletons prepared from ghosts by removal of the lipid bilayer. Elliptocytes form as the mature RBC ages *in vivo*, since RBC precursors in the HE syndromes are round and do not exhibit morphologic abnormalities. The elliptocytic shape change is thought to result from repeated episodes of elliptocytic deformation that all RBCs experience during each circulatory cycle, as they pass through capillary beds. Whereas normal RBCs regain a discocytic configuration by a process of elastic recoil, hereditary elliptocytosis RBCs appear to have disruption of the connections between the various cytoskeletal components, followed by the formation of new contacts that lock the cell into the elliptocytic configuration.

Types of Hereditary Elliptocytosis

The clinical features in generally fall into one of five categories:

1. Silent carriers
2. Common HE
3. Hereditary pyropoikilocytosis
4. Spherocytic elliptocytosis
5. South-east Asian ovalocytosis.[8,9]

Hemolytic anemia in these disorders ranges from absent to life-threatening. Severe hemolysis is usually a consequence of homozygosity or compound heterozygosity for one or more of the various membrane protein mutations associated with this disorder.

- Usual features of hereditary elliptocytosis like anemia, splenomegaly, cholelithiasis and In the newborn, hereditary elliptocytosis may manifest as hemolytic jaundice with presence of poikilocytes and pyknocytes on peripheral smear.
- The bone changes appear later.

South-east Asian Ovalocytosis is characterized by presence of ovalocytes in the peripheral smear, which are less elongated than elliptocytes. It is due defective protein 3. This condition offers protection from *Plasmodium falciparum malaria*. Hereditary pyropoikilocytosis is the most severe form characterized by presence of microspherocytes in blood smear whose mean corpuscular volume is decreased (MCV: 50–60 fL). This condition as the name suggests is characterized by increased thermal lability of RBCs and they lyse at 45 to 46°C, instead of 49 to 50°C.

Treatment

- Treatment is needed only if there is chronic hemolysis.[8,9]
- Folic acid supplementation (1 mg/day) is needed.
- Splenectomy may be indicated, if the hemoglobin is below 10 g/dL or there is reticulocytosis >10 percent.

HEREDITARY STOMATOCYTOSIS

Hereditary stomatocytosis is a group of autosomal dominant conditions which are characterized by the presence of cup-shaped red blood cells (Fig. 4). The red cell membrane leaks sodium and potassium ions. There is a defective protein 7.2 or stomatin on chromosome 9. A variety of variants of this condition have been described:

Overhydrated Hereditary Stomatocytosis

- Most severe variety, stomatin gene may be defective.
- *Dehydrated stomatocytosis (Hereditary Xerocytosis/ Hereditary hyperphosphatidylcholine hemolytic anemia):* It is the most common variety of stomatocytosis caused by defective protein 7.2 or stomatin gene.

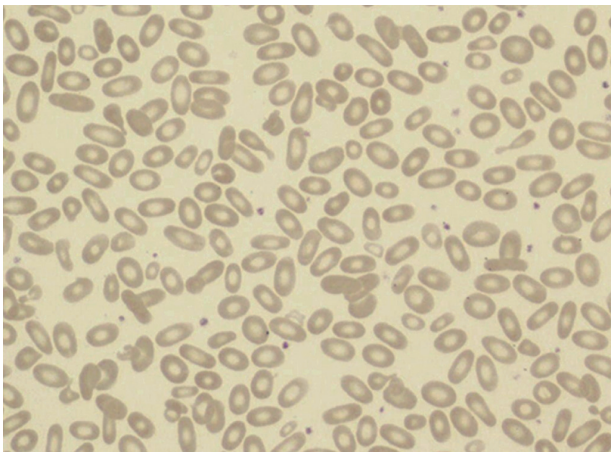

Fig. 4 Elliptocytes seen on peripheral smear

- Dehydrated stomatocytosis with perinatal ascites.
- *Cryohydrocytosis:* It is the mildest type where the RBCs lyse on cooling *in vitro* and it is associated with pseudohyperkalemia.
- Blackburn variant.

Treatment

Usually no treatment is needed. Splenectomy is not indicated as there is a greater tendency to life-threatening thrombosis following splenectomy due to thrombocytosis post-splenectomy coupled with abnormal adherence of stomatocytic RBCs to vascular endothelium.[8,9]

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CHAPTER 21

Red Cell Enzymopathy

Bhavna Dhingra, Dinesh Yadav, Jagdish Chandra

The hereditary hemolytic anemia resulting from altered red blood cell (RBC) metabolism due to defects in various enzymes associated with glycolytic pathway, hexose monophosphate shunt or pentose phosphate pathway are described as red cell enzymopathy or erythro-enzymopathy (EEP). These hereditary anemias are distinguished from hereditary spherocytosis by absence of spherocytosis in the peripheral blood. The most well known and widely distributed EEP is the deficiency of G6PD, which is involved in the initial reaction of pentose phosphate pathway.[1,2] Deficiency of pyruvate kinase and other enzymes of glycolytic pathway also result in hemolytic anemia but the magnitude of clinical problem resulting from deficiency of these enzymes is considerably less compared to G6PD deficiency.

G6PD DEFICIENCY

The main role of pentose phosphate pathway is related to metabolism of glutathione (GSH) through production of reduced form of nicotinamide adenine dinucleotide phosphate (NADPH). GSH is important for preservation of sulfhydryl group in many proteins including hemoglobin and to prevent the damage from oxidative radicals in general. Thus, GSH should be constantly available in the reduced form which is effected by, GSH reductase through NADPH, the later is provided by G6PD.[1] G6PD catalyses nicotinamide adenine dinucleotide phosphate (NADP) to its reduced form, NADPH (Fig. 1). NADPH protects cells from oxidative damage. As red blood cells (RBCs) do not generate NADPH in any other way, they are more susceptible than other cells to destruction from oxidative stress. Therefore deficiency of G6PD in red cells leads to various clinical manifestations in human beings. The level of G6PD activity in affected RBCs is lower than in other cells in the body.

Majority of mutations cause this enzyme deficiency in RBC by decreasing enzyme stability. The polymorphic mutations affect amino acid residues throughout the enzyme and decrease the stability of enzymes in the RBC, possibly by disturbing protein folding.[2]

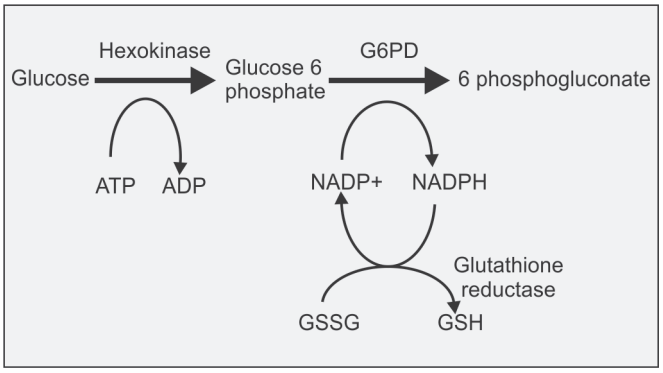

Fig. 1 Pentose phosphate pathway

Prevalence

G6PD deficiency is the most common EEP affecting approximately 400 million people worldwide.[3] The disorder is transmitted as a x-linked recessive trait. Though the distribution of G6PD deficiency is worldwide, highest prevalence is observed in Mediterranean countries, Africa and Asia. In south-east Asia, the prevalence varies widely in different ethnic groups—10 to 20 percent in certain Combodian groups to 1 to 3 percent in Vietnamese population groups.[4]

Table 1 Frequency distribution of G6PD deficiency in India

S. no.	Authors	Area/Caste/Tribes studied	Frequency
1.	Thakur and Verma,1992[8]	Bastar (Central India), Muria Gond tribes	M12.3% F–3.7%
2.	Handa et al, 1992[9]	Punjab, Bania	2.8 %
3.	Ramadevi et al, 1994[10]	Bengaluru (neonates)	7.8%
4.	Kaeda et al, 1995[11]	Odisha	3–15%
5.	Balgir et al, 1999[12]	Odisha (Mayurbhanj)	7.7–9.8%
6.	Sukumar et al, 2004[7]	Mumbai	5.7–27.9%
7.	Gupte et al.[13]	Gujarat, Surat, Vataliya Prajapati	22%

In India, G6PD deficiency was first reported almost 40 years ago.[5] Prevalence in India varies from 0 to 27 percent in various castes, tribes and ethnic groups.[6] A recent series from Mumbai including a large number of individuals from various population groups reported overall prevalence of 10.5 percent the variation in various castes and linguistic groups being 5.7 to 27.9 percent.[6]

Table 1 shows the prevalence rates of G6PD deficiency in various reports over the last 15 years from different parts of country and in different ethnic groups.[7-13]

MALARIA HYPOTHESIS

Variation in prevalence of G6PD deficiency has led to the hypothesis that G6PD deficiency is a polymorphism that confers protection from falciparum malaria. This hypothesis has been called G6PD/malaria or simply **"malaria hypothesis"**.[1,3] Malarial parasite grows less well in red cells deficient in G6PD. Decreased parasitemia has been documented in these individuals.[14-16] A recent series by Mohanty et al. has reported good correlation between prevalence of *Plasmodium falciparum* malaria and G6PD deficiency.[17] Thakur and Verma have also observed increased prevalence of antimalarial antibodies and higher titers among those with normal G6PD levels compared to G6PD deficient persons.[8] Mohanty et al. have referred to an interesting observation that among Parsees in India who migrated from Iran approximately 1300 years ago, prevalence of G6PD deficiency is 15.7 percent which is considerably higher as compared to prevalence among Zorasstrians in Iran who belong to the same community. An explanation offered is that at the time of migration, Gujarat and Mumbai were endemic for malaria where Parsees settled.[3]

Clinical and Biochemical Variants

Based on biochemical and other characterization, 442 different variants of G6PD have been identified. Of them, 299 have been characterized with the methods recommended by WHO.[3,18] Molecular characterization is considered more important as different variants based on biochemical studies alone have been found to be same on molecular characterization. From India, 13 different biochemically characterized variants have been reported.[3] A large series with biochemical and molecular characterization reported G6PD-Mediterranean to be the most common variant in India (60.4%) followed by G6PD-Kerala-Kalyan (24.5%) and G6PD Odisha (13.3%). Frequency distribution of various biochemical variants is different in tribal and urban population. Kaeda et al. observed that G6PD-Odisha is responsible for most cases in tribal population but is not found in urban population groups where most of the G6PD-Mediterranean is the most prevalent variant. G6PD-Chatham with undetected enzyme activity and G6PD-Insuli with normal G6PD activity are very rare among Indian population groups. [3,7,11]

Clinical Features

WHO has provided a classification of G6PD deficiency which is based on residual activity of the enzyme and other characterization and clinical presentation (Table 2).[19] The patients with G6PD deficiency can present clinically in following ways: acute hemolytic anemia (AHA), neonatal jaundice (NNJ) and chronic non-spherocytic hemolytic anemia (CNSHA).

Acute Hemolytic Anemia

Children with certain variants of G6PD deficiency are clinically in a steady state till they develop anemia of sudden onset under the effect of some oxidative stress. Within 24 to 48 hours of exposure to such stress, the child suddenly becomes pale and has discoloration of urine which is described as cola colored, strong tea colored or simply brown in color. Other symptoms and clinical findings correspond to degree of anemia and hypoxia.

Table 2 Categorization of G6PD variants

Class	Clinical expression	Residual G6PD activity (% of normal)	Variants reported
I	Severe (CNSHA)	< 20%*	94
II	Mild	< 10%	114
III	Mild	10–60%	110
IV	None	100%	52
V	None	>100%	2

*Severe enzyme deficiency with CNSHA. The enzymes levels are usually less than 20% of normal. To classify in this group, the variant must be associated with CNSHA

Mild jaundice, breathlessness and tachycardia are often present. Occasionally features of frank congestive cardiac failure, backache and abdominal pain are observed.[20,21] Urinary discoloration is on account of intravascular hemolysis but hemolysis is entirely not intravascular. Depending upon degree of extravascular hemolysis, splenic enlargement may be noticed. Hemolysis occurs after exposure to stressor but does not continue with continued exposure. This is thought to be on account of older RBCs being damaged first as they have most severe deficiency of enzyme. Once the population of deficient RBC are hemolyzed, the juvenile RBC and reticulocytes withstand the stress as they have typically higher levels of enzyme.[2] Hemoglobinemia and hemoglobinuria may result in azotemia and/or acute renal failure. In one Indian series on acute renal failure in children, G6PD deficiency accounted for 6 percent cases. Azotemia occurred in 20 percent of 35 patients with G6PD deficiency in another series.[20,22]

Laboratory findings during intravascular hemolysis and AHA include moderate to severe anemia which is usually normocytic-normochromic. RBC morphology shows anisocytosis due to increased number of juvenile red cells and contracted cells. Poikilocytosis with presence of 'bite cells' (as if some portion of red cell has been bitten away) may be seen. Intense reticulocytosis is present. Plasma hemoglobin level is increased and so is unconjugated bilirubin level. Haptoglobin and other hemoglobin binding proteins are decreased.[1]

Pathogenesis of such events involves oxidation of glutathione (GSH) to GSSG. On account of G6PD deficiency, the red cells of these patients have limited capacity to regenerate GSH and the reserve gets depleted soon. Exhaustion of GSH allows oxidation of sulfhydryl group of hemoglobin (and other proteins) resulting in denaturation of hemoglobin. Coarse precipitates of hemoglobin lead to damage of red cell membrane and hemolysis. As is evident, the prerequisite for such a series of events is oxidative stress which occurs in the form of exposure to various drugs and "triggers". In fact, G6PD was first described during investigation for 'primaquin sensitivity'. Since then many drugs have been incriminated to result in intravascular hemolysis in individuals with G6PD deficiency. Table 3 lists the drugs which can cause such hemolysis.[23-25] Other than drugs, ingestion of fava beans (favism) and various infective agents have been identified as triggers. In an Indian series bacterial sepsis, malaria and hepatitis were identifiable triggers other than drugs.[20] Hepatitis results in very high levels of serum bilirubin and increased morbidity.[20,26] Mehta et al. have reported AHA following ingestion of soft drink containing ascorbate.[27]

Table 3 List of drugs known to cause hemolysis in G6PD deficient patients (In alphabetical order)

Actyl salicylic acid	Ascorbic acid	Chloramphenicol
Chloroquine	Ciprofloxacin	Colchicine
Dapsone	Diphenhydramine	Dopamine
Doxorubicin	Furazolidone	Isobutyl nitrite
Isoniazid	Menadiol sod. sulfate	Menadione
Menadione sod. bisulfate	Mepacrine	Nalidixic acid
Naphthelene	Niridazole	Nitrofurazone
Nitrofurantoin	Norfloxacin	Paracetamol
Para amino-benzoic acid	Phenacetin	Phenytoin
Phenylbutazone	Phytomenadione	Primaquine
Probenecid	Procainamide	Proguanil
Pyrimethamine	Quinidine	Quinine
Streptomycin	Sulfadiazine	Sulfadimidine
Sulfaguanadine	Sulfmethoxazole	Sulfanilamide
Trimethoprim		
New Drugs added in 2002		
Astemizole	Azatidine	Cetirizine
Chlorpheniramine	Cyproheptadine	Diphenhydramine
Loratadine	Promethazine	Terfenadine

NEONATAL JAUNDICE

Other than AHA, neonatal jaundice (NNJ) is a common manifestation of G6PD deficiency. Almost one-third of male neonates have been described to have NNJ. NNJ resulting from G6PD deficiency has worldwide distribution occurring in Mediterranean countries, Africa and Asia. In an Indian series, in 12 out of 100 neonates with jaundice, G6PD deficiency was the cause, of them[10] being G6PD Mediterranean type.[28] In another large series of 551 cases of NNJ, G6PD deficiency was the largest single identifiable cause accounting for 17.1 percent.[28] Among neonates with severe jaundice requiring exchange transfusion, G6PD deficiency has accounted for a large number of cases.[29,30] In a review of cases of kernicterus in world literature, G6PD deficiency was the cause in 13 out of 88 cases which is next in frequency to only Rh and ABO incompatibility.[31] Severe intrauterine hemolysis and hydrops fetalis have been reported following maternal ingestion of oxidative agents and hemolysis has been observed in breastfeeding neonates following maternal ingestion of fava beans.[32]

Why only some and not all neonates with G6PD deficiency develop NNJ is not easily explained. It was postulated that NNJ is associated with only certain variants of G6PD (just like CNSHA) but occurrence of cases from various parts of the world does not support this hypothesis. Similarly, correlation with residual level of enzyme activity has also not been proven.[1,30] NNJ occurring due to some other insult or trigger is another explanation offered and is supported by higher rates of exposure to naphthalene observed in neonates with NNJ compared to those without NNJ.[1] Infants with G6PD deficiency and associated mutation of uridine diphosphoglucoronate glucoronosyltransferase-1 gene promoter (UDPGT-1) have been found to be particularly susceptible to hyperbilirubinemia secondary to impaired hepatic clearance of bilirubin. UDPGT-1 is the enzyme affected in Gilbert disease.[33]

CHRONIC NONSPHEROCYTIC HEMOLYTIC ANEMIA

The term CNSHA in relation to deficiency of G6PD and other enzymes is used to describe chronic anemia with normal/near normal red cell morphology, particularly to differentiate it from hereditary spherocytosis. CNSHA develops in a minority of cases with G6PD deficiency (Class I variants). Clinical picture is quite variable. Unlike NNJ which can affect female children, CNSHA affects only male patients. Generally patients have had NNJ which might have required therapeutic intervention. The patient presents later with anemia and jaundice. Splenomegaly initially is small but later it may increase in size. Anemia is normocytic normochromic, slight macrocytosis may be observed due to reticulocytosis. Unconjugated hyperbilirubinemia, increased levels of lactate dehydrogenase and decreased levels of haptoglobin are present. As most of hemolysis is extravascular, hemoglobinemia and hemoglobinuria is not present. Continuous hemolysis in these patients is thought to result from red cell membrane damage due to oxidation of sulfhydryl group of hemoglobin resulting in its precipitation. This is supported by observation of high molecular weight aggregates in the red cell membrane of patients with CNSHA. This phenomenon is not seen G6PD deficient individuals without CNSHA. G6PD deficiency has been found to be associated with sickle cell disease and other hemoglobinopathies. Diop et al.[34] found that prevalence of G6PD deficiency was higher in sickle cell disease patients (21.6%) than in normal subjects (12.3%) ($p = 0.001$). Will this association influence the severity of sickle cell diseases is an obvious question because of the nature of the two diseases. However, no difference was found in the two groups of male sickle cell disease patients concerning number of vaso-occlusive crisis, number of transfusion, frequency of infectious episodes, number of chronic complications, disturbances on patient's activity and total index severity.[34] In an earlier report on this association from India, decreased prevalence of painful crisis was observed possibly due to poor survival of RBC with HbS due to associated G6PD deficiency leading to decreased chances of sickling of cells. Hemolytic crisis was observed to be more when sickle cell disease was associated with G6PD deficiency.[35]

Diagnosis

Various tests are available for diagnosis of G6PD deficiency.

Fluorescent spot test and dichlorophenol indophenol (DPIP) de-colorization method and quantitation of enzyme are suitable methods for routine use.

The tests are likely to be negative during episodes of AHA as the neocytes are rich in G6PD which are in abundance during AHA compared to steady state. Thus, a negative test does not exclude but a positive test will confirm the diagnosis. If negative, it is recommended to repeat the test after three months of acute episode.[3,21] G6PD genotyping can be performed using PCR but the test is not routinely available. Quantitative methods are available for estimating enzyme levels.

ELISA based method have been developed for field use. A recent article reported a good sensitivity and specificity of this test and recommended for use in resource limited settings.

Another test for field studies is NADPH fluorescence test on paper (NFP test). This test was compared with polymerase chain reaction (PCR)-based G6PD genotyping also using blood samples on filter papers. There was good agreement between the NFP test results and the PCR findings. The estimate of the sensitivity of the NFP test was 98.2 percent (95.8–99.6%) and the specificity was 97.1 percent (94.2–99.2%).[36,37]

PREVENTIVE STRATEGIES AND TREATMENT

Management issues in cases with G6PD deficiency include prevention of AHA and NNJ and treatment of acute and chronic anemia and NNJ. Prevention of NNJ is based on neonatal screening for enzyme defect and then taking precautions in cases with enzyme deficiency. Such a screening strategy has to be considered taking into account the prevalence of G6PD deficiency in the population group as is being followed in Sardinia and some of the Mediterranean countries.[1] WHO recommends screening all newborns for G6PD deficiency I population groups with prevalence rates of 3 to 5 percent or more in males. Prevention of AHA centers around avoiding the known trigger drugs in these patients.

The list in Table 3 shows that many antihistaminic drugs are incriminated in AHA in G6PD deficiency, hence it would be useful to avoid the cough and cold medicines in general which otherwise are of unproven efficacy in treating the upper respiratory infections. Some of the known offending drugs have been successfully used in certain population groups.[38]

Management of NNJ is like any other cause of unconjugated hyperbilirubinemia including phototherapy and exchange transfusion. Phototherapy in these neonates may be started at a lower level than otherwise recommended.[31,39]

A novel approach is use of heme-oxygenase inhibitor-tin-mesoporphyrin (Sn-MP) which reduces bilirubin production. It has been found to be extremely useful in preventing the development of significant hyperbilirubinemia in G6PD deficient neonates.[40-42]

Treatment of acute episode of intravascular hemolysis includes transfusion support for anemia and supportive care. Fluid therapy during such episodes is important for prevention and treatment of acute renal failure.[22,22]

Patients having CNSHA require meticulous monitoring. In most cases occasional exacerbation will require blood transfusion. During steady state, administration of folic acid is recommended as the requirement is increased due to increased red cell turnover. Very few cases will need to be started on chronic transfusion therapy like patients with thalassemia and hemoglobinopathies. Splenectomy may be required due to large size, development of hypersplenism or to decrease the transfusion requirement.[1]

Deficiency of Pyruvate Kinase and Other Enzymes of Glycolytic Pathway

As compared to G6PD deficiency, the defects of glycolytic pathway are very uncommon. Of them, pyruvate kinase deficiency is most well recognized and along with G6PD deficiency it is the most common cause of CNSHA.[43,44] In

Table 4 Deficiency of enzymes of glycolytic pathway

S. no.	*Enzyme, inheritance*	*Clinical features*	*Hematological/other laboratory findings*	*Treatment*
1.	Hexokinase deficiency, AR	22 cases reported, NNJ, anemia, splenomegaly, CNSHA, gallstones, hyperhemolytic episodes	Red cell morphology unremarkable	Transfusions Folic acid Splenectomy
2.	Glucose phosphate isomerase, AR	46 cases from 34 pedigree, 30% NNJ, hydrops, CNSHA, hyperhemolytic episodes	Very high reticulocyte count, MCV increased	Same as above
3.	Phosphofructokinase deficiency, AR	Involves red cells, muscle related symptoms predominate—exertional myopathy, easy fatiguability (Type VII Glycogen storage disease), mild hemolytic anemia	No lactate production in "ischemic arm test", muscle biopsy	Unsatisfactory
4.	Aldolase deficiency, AR	Very few cases, severe CNSHA, mental retardation	Normal red cell morphology	Undefined
5.	Triose phosphate isomerase deficiency, AR	Moderate to severe CNSHA, neonatal anemia, progressive neurological disease (unrelated to kernicterus)	Very high reticulocyte count	Transfusions, folic acid, splenectomy
6.	Glyceraldehyde-3-phosphate dehydrogenase deficiency, AR (Autosomal recessive)	Need not result in anemia, associated with other defects like hereditary spherocytosis	Nonspecific	-
7.	Phosphoglycerate kinase deficiency, X-linked recessive	CNSHA, NNJ, seizures, movement disorders, psychomotor retardation, aphasia, tetraplegia	Reticulocytosis	? Splenectomy
8.	2,3-Bisphosphoglycerate mutase deficiency, AR	Complete absence may be associated with polycythemia, neonatal onset of progressive anemia described with 50% activity		Phlebotomy for symptomatic polycythemia
9.	Enolase deficiency	Shortened red cell survival not necessary, nitrofurantoin induced hemolysis	Spherocytosis	Undefined
10.	Pyruvate kinase deficiency, AR	*See* text		
11.	Lactate dehydrogenase deficiency	Decreased levels not associated with anemia	-	-

Indian population, the deficiency of these enzymes has only sparingly been studied.[45,46] In contrast to G6PD deficiency, patients with deficiency of enzymes of glycolytic pathway usually have CNSHA with onset in neonatal period. Drug induced hemolytic anemia- is not a common problem in them. Most cases benefit from splenectomy. Pyruvate kinase deficiency is described in the following section. Rest of the conditions with their common clinical findings are listed in Table 4.

PYRUVATE KINASE DEFICIENCY

Deficiency of pyruvate kinase (PK) is the most common enzyme deficiency of glycolytic pathway with over 350 cases reported.[47]

It is transmitted as autosomal recessive trait and is seen in patients of north European descent. PK enzyme is a tetramer with four tissue specific subunits—R (RBC), L (liver), M_1—muscle and M_2 platelets and leukocytes. Genetic control is separate for RL subunit and M_1 M_2 subunits.[43,47] Prevalence of PK deficiency has varied from 0.14 to 6 percent.[38]

The hallmark of PK deficiency is CNSHA Cases with neonatal anemia and hydrops have occurred. NNJ requiring exchange transfusions have been described. Patients with CNSHA have unconjugated hyperbilirubinemia and splenomegaly which at times may become massive. Chronic leg ulcers are a complication in some individuals. Aplastic crisis due to parvovirus infection can occur. Gall stones are increasingly seen after first decade.[43,47,48]

Table 5 describes the clinical and laboratory features in a large series of 61 patients (all ages).

Red cell morphology is normocytic normochromic. Macrocytosis due to active regeneration and acanthocytosis may be observed. Reticulocyte count is increased but not proportionate to hemolysis as in other hemolytic anemias. This is on account of selective sequestration of young RBCs and reticulocytes by spleen. A paradoxical rise in reticulocytes after splenectomy therefore is a known phenomenon. On account of hepatic PK deficiency, liver enzymes are elevated and coupled with presence of hyperbilirubinemia may appear to be the clinical picture with chronic liver disease. Iron overload disproportionate to transfusions is an important observation in certain cases and has been explained on the basis of associated hemochromatosis mutations.[43, 47-50]

Treatment of PK deficiency includes transfusion support, folate supplementation, splenectomy and cholecystectomy for gallstones. Use of salicylates has resulted in hyperhemolytic crisis and should be used with caution in PK deficient patients having juvenile rheumatoid arthritis.[47]

Table 5 Clinical characteristics in pyruvate kinase deficiency

Clinical feature	*Number*
Consanguinity	4/56
Anemia	55/61
Jaundice	43/61
Neonatal jaundice	33/56
Splenomegaly	47/58
Splenectomy	18/61
Cholecystectomy	14/56
Aplastic crisis	1/61
Transfusions	18/59
Exchange transfusions	25/56
Desferrioxamine treatment	16/58

Clinical Approach to a Child Suspected to have Enzyme Deficiency

Clinical diagnosis of erythroenzymopathy requires a high index of suspicion. In cases presenting with acute intravascular hemolysis, history of exposure to trigger drugs helps in the diagnosis. Family history of jaundice, anemia, splenectomy and cholelithiasis may also point towards such a disease.[2,50] In absence of such history, the diagnosis may be difficult. Other causes like autoimmune hemolytic anemia and malaria (blackwater fever) can be excluded by appropriate tests.

Cases with chronic hemolytic anemia pose a diagnostic problem. In our country, common causes of hereditary hemolytic anemia include β-thalassemia syndrome and sickle cell disease. Both these conditions can be diagnosed by hemoglobin electrophoresis. The diagnosis of enzymopathy should be suspected in all cases with chronic hemolysis and unexplained unconjugated hyperbilirubinemia particularly if red cell morphology is unremarkable. Intense reticulocytosis supports the diagnosis of various enzymopathies (see Table 4). The diagnosis can be confirmed by appropriate enzyme assay. Unfortunately, the tests are not widely available. Demonstration of accumulation of proximal or depletion of distal intermediary compounds of glycolytic pathway supports the diagnosis.

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CHAPTER 22

Autoimmune Hemolytic Anemia

Rajiv Kumar Bansal

Immune hemolytic anemia (IHA) is the clinical condition in which IgG and/or IgM antibodies bind to RBC surface antigens and initiate RBC destruction via the complement system and the RE system.[1] Autoimmune hemolytic anemia (AIHA) refers to a collection of disorders characterized by the presence of autoantibodies that bind to the patient's own erythrocytes, leading to premature red cell destruction. The antigens targeted often have a high incidence so that both native and transfused RBCs are destroyed. In contrast to AIHA, in alloimmune hemolytic anemia exposure to allogenic RBCs leads to formation of alloantibodies which do not react with autologous RBCs. A positive direct antiglobulin test (DAT, also known as the Coombs test) is essential for diagnosis.

Specific characteristics of the autoantibodies, especially the type of antibody; its optimal binding temperature; and whether complement is fixed, influence the clinical picture. In all cases of AIHA, however, the autoantibody leads to a shortened red blood cell survival (i.e. hemolysis) and, when the rate of hemolysis exceeds the ability of the bone marrow to replace the destroyed red cells, to anemia and its attendant signs and symptoms. Subjects of all ages are affected by AIHA: from infants in the first few months of life to the elderly. Although, it occurs less frequently than in adults, AIHA in the young is not a rarity. AIHA in children presents some differences from those of adults.

Three types of AIHA can be distinguished based on their serologic properties and clinical characteristics.

Most patients with AIHA (80%) exhibit warm-reactive antibodies of the immunoglobulin IgG isotype on their red cells. Most of the remainder of patients exhibit cold-reactive autoantibodies. Two types of cold-reactive autoantibodies to RBCs are recognized: cold agglutinins and cold hemolysins. Cold agglutinins are generally of IgM isotype, whereas cold hemolysins usually are of IgG isotype. IgG warm autoantibodies bind to erythrocytes at 37°C (99°F) but fail to agglutinate the cells; cold agglutinins, almost always of the IgM isotype, clump RBCs at cold temperatures and occasionally lead to hemolysis; and the IgG Donath-Landsteiner antibody (cold hemolysins) binds to RBC membranes in the cold and activates the hemolytic complement cascade when the cells are warmed to 37°C (99°F).[2] All three can occur as an idiopathic (primary) disorder or can coexist with another disease (secondary) (Table 1).

EPIDEMIOLOGY

It is a relatively uncommon but certainly not rare disorder, with an estimated incidence of 1 to 3 cases per 100000 population per year. There is no evidence that AIHA is confined to any particular race. It is less common than immune thrombocytopenia. In teenagers and adults, AIHA is more common in women than in men. The peak incidence in pediatric patients occurs in preschool-age children.[2] Boys are 2.5 times more likely to be affected than girls.[3] In children, occurrences in patients younger than 2 years and older than 12 years are more likely to have a chronic unremitting course.[2] However, the majority of pediatric cases are acute in onset and self-limiting. Often these cases resolve within 6 months without treatment,[2] and the decision to treat is based on the degree of anemia and physiologic compromise. Some reports suggest that in childhood, secondary cases are more common than idiopathic forms. Viral and bacterial agents are frequently the only recognizable stimuli; in fact, AIHA follows viral infection or vaccination much more often

Table 1 Classification of autoimmune hemolytic anemia (AIHA)*

Warm active antibodies: Autoantibody maximally active at body temperature (37°C)
Warm autoimmune hemolytic anemia: • Primary or idiopathic • Secondary (autoimmune disorders, lymph proliferative disorders) • Drug-induced immune hemolytic anemia – Autoimmune type – Drug adsorption type – Neoantigen type – Nonimmune type (first generation cephalosporins)
Cold active antibodies: Autoantibodies optimally active at temperature < 37°C
Mediated by cold agglutinins: • Primary or idiopathic chronic cold agglutinin disease • Secondary cold agglutinin hemolytic anemia – Acute transient (infections, e.g. *Mycoplasma pneumonia* or infectious mononucleosis) – Chronic (lymphoproliferative disorders) *Mediated by cold hemolysins:* • *Primary or idiopathic:* Paroxysmal cold hemoglobinuria • *Secondary* – Donath-Landsteiner hemolytic anemia: Acute transient (various viral infections) – Chronic (syphilis)
Mixed-type (cold and warm) autoimmune hemolytic anemia
• Primary or idiopathic • Secondary (autoimmune disorders, lymphoproliferative disorders)

*Modified from Gehrs and Friedberg. Autoimmune hemolytic anemia. American Journal of Hematology. 2002;69:258-71.

in children than in adults. When it is associated with infections in the former, AIHA is usually acute and of short duration. Immunodeficiency or malignancy (especially malignancies of the lymphoreticular tissues), systemic lupus erythematosus (SLE), and other types of collagen vascular diseases are most commonly associated with immune hemolysis in children. Drugs are less commonly associated with AIHA. The reason for this finding is probably that the common inducers of immune hemolysis, such as α-methyldopa in the past, are not normally prescribed for children. When drug-induced AIHA occurs, it is usually due to immunoglobulin G (IgG) antibodies and it is associated with antibiotics such as penicillin. Biphasic hemolysin (historically related to syphilis) is, nowadays, associated with viral infections such as measles, rubella, and chickenpox.[4]

deLuca et al. reported 29 children with autoimmune hemolytic anemia (AIHA) in 1979 from Rome, Italy. Patients were divided into two groups, i.e. patients with transient AIHA (15 cases) and patients with the chronic form of the disease (14 cases).[5] The criterion for this distinction was based on the episode of increased hemolysis which was either shorter or longer than 3 months. If a relapse of AIHA occurred, the case was considered to be chronic by them. There were no patients with cold autoagglutinins or biphasic hemolysins in this study. Associated diseases were found in 27 patients and included infectious diseases, immunodeficiency, autoimmune related disorders.

Lymphoproliferative Disease and Rh-hemolytic Disease

Oliveira et al 2006 evaluated 17 patients younger than 15-years-old admitted from 1988 to 2003 in Brazil.[6] The median age at diagnosis was 10.5 months. The direct Coombs polyspecific test was positive in 13 patients and negative in four patients. Monospecific testing was performed for 14 patients. The most frequent red cell autoantibody was IgG (five patients), followed by IgM in two. Thirteen patients had severe anemia and needed blood transfusions. Underlying diseases were identified in four patients: systemic lupus erythematosus, Hodgkin's lymphoma, autoimmune hepatitis and Langerhans cell histiocytosis. The remaining patients were classified as having primary disease. The median follow-up period was 11 months (5–23 months). Three children died, two after splenectomy and one with complications of the underlying disease.

Vaglio et al. published one of the largest studies of AIHA in children in 2007.[4] A retrospective review of 100 cases of childhood AIHA (age range 6 months–16 years)

Table 2 Distribution of 100 patients with AIHA based on disease association and serologic findings (Vaglio et al)[4]

Condition	*Warm AIHA*	*Cold AIHA*	*PCH**	*Mixed AIHA*
Idiopathic AIHA	38	6	0	2
Autoimmune diseases	0	0		
Idiopathic thrombocytopenic purpura (ITP)	9			
Systemic lupus-erythematosus (SLE)	1			2
Infectious diseases	6	6	6	
Neoplasia	0	5	0	0
Hematologic disorder				
Sickle cell anemia	1	0	0	0
Thalassemia major	3	1		
Myelodysplasia	4	0		
Non-Hodgkin's lymphoma	1	3		
Chronic myelogenous leukemia	0	1		
Acute myelogenous leukemia	0	1		
Acute lymphoblastic leukemia	0	3		
Liver and kidney transplant	1	0	0	0
Total	64	26	6	4

* PCH: Paroxysmal cold hemoglobinuria.

by them, diagnosed over a 20-year-period revealed a peak incidence in the first 4 years of life (Table 2). No sex predilection was observed. The majority of patients (64%) were diagnosed with warm AIHA, whereas cold agglutinin disease and PCH accounted for 26 percent and 6 percent of children, respectively. A mixed type of AIHA was observed in four subjects. Overall, 54 percent of children had coexisting disease, including hematologic disorders, autoimmune disease, infection, and neoplasia. All cases of PCH were associated with a recent viral illness.

There are no large studies on AIHA in Indian literature. Sporadic case reports have been published from time to time. Naithani et al. 2007 published one of the largest pediatric series from India.[7] A series of 26 cases were seen by them over a period of 5 years in Delhi, India. Of the 26 patients, 11 were males and 15 females. Patients were in the age group of 2 months to 17 years (median; 11 years). Nine (35%) children had secondary AIHA in this series. Of them 5 had autoimmune conditions, 2 had Evans syndrome, 2 had SLE, 2 had JRA, 1 had nephritis, 1 had endocrinopathy and 4 had various infections.

In a study of twelve children diagnosed with autoimmune hemolytic anemia over a period of four years Gupta et al. (2008) from BHU, Varanasi found 9 had primary disease and 3 had secondary disease.[8] Tubercular infection was seen in 2 patients with secondary disease.

Drug-induced immune hemolytic anemia (DIIHA) is rare.[9] The incidence is around 1 in 1 million of the population. The number of drugs and the suggested mechanisms associated with DIIHA has changed over the last 40 years. In 1967, only 13 drugs were implicated; in 1980, 32 drugs were reported and in 2007, 125 drugs were reported.[10] Three groups of drugs predominated: 42 percent were antimicrobials; 15 percent were anti-inflammatory; 11 percent were anti-neoplastics.[10] The specific drugs mainly implicated have changed dramatically. In the 1970, the most common drug, by far, to cause DIIHA was methyldopa, which caused a true AIHA with no drug antibody involved and accounted for 67 percent of all DIIHA. High-dose intravenous penicillin accounted for 25 percent of DIIHA. When these therapies became less commonly used, the most common causative group of drugs became the cephalosporins, which, from the 1990s, account for 70 percent of the DIIHA. The drugs most frequently associated with DIIHA at this time are cefotetan (more than 50 percent cases of DIIHA), ceftriaxone, and piperacillin.

Ceftriaxone is the second most common drug to cause DIIHA.[9] Some children have dramatic HA; 50 percent are reported as fatal HA.[11,12] Analysis of 21 patients (15 children and 6 adults) showed that 40 percent of the children started hemolyzing ≤1 hr after receiving ceftriaxone. Hemoglobin levels fell to ≤ 5 g/dL in 62 percent and to

≤ 1 g/dL in 20 percent of the patients. Fatal HA occurred in 38 percent of the patients. The children have always received ceftriaxone previously, the DAT is usually positive (all have RBC-bound complement and most have IgG in addition), and ceftriaxone antibodies are detectable in the patient's serum. The HA is usually not as dramatic in adults. The fall in hemoglobin is much less and does not occur in a few hours; fatalities are less common.

Piperacillin can cause DIIHA and/or positive DATs. Although a semi-synthetic penicillin, unlike other semi-synthetic penicillins (e.g. ampicillin), it reacts differently than penicillin G. In contrast to penicillin, the *in vivo* RBC destruction can be complement-mediated; most of the DATs are positive due to RBC-bound complement and IgG.[13]

Hydrocortisone can also cause DIIHA.[14] This adds another possible explanation for poor responses to steroid therapy in some cases of AIHA, where steroid-induced DIIHA may be masked by the autoimmune process.

PATHOGENESIS

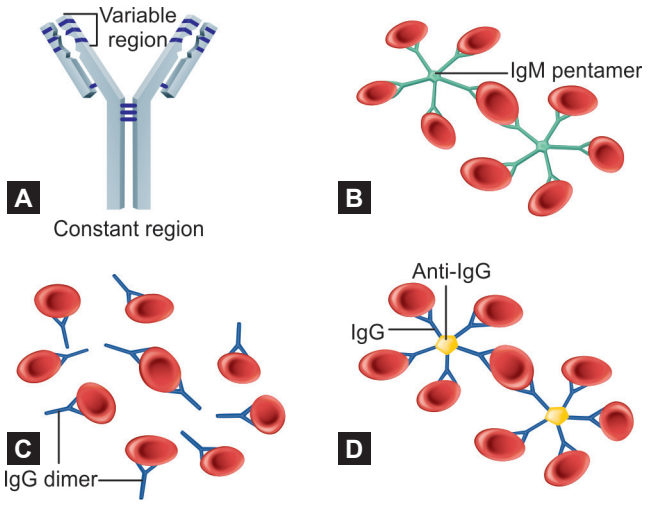
AIHA is an autoimmune disease in which there is loss of self tolerance. Self tolerance refers to a lack of responsiveness to an individual's own (self) antigens. In the case of AIHA, the antibodies are directed against self RBC antigens, leading to their enhanced clearance through F_c-receptor–mediated phagocytosis (extravascular hemolysis) or complement-mediated breakdown (intravascular hemolysis). There is some evidence that AIHA may be in large part due to self-reactive antibodies against erythrocyte band 3, an anion transporter found in erythrocyte membranes. In AIHA, autoantigenic T-cell epitopes have recently been mapped for the RhD autoantigen. The degree of hemolysis in AIHA depends on the characteristics of the bound antibody (e.g. quantity, thermal amplitude, specificity, complement fixing ability and the ability to bind tissue macrophages) and also the characteristics of target antigen (density, expression, patient age).[15,16]

The cause of autoimmune hemolytic anemia is unknown. In about one-third of cases, the autoantibodies have specificity for an antigen in the Rh system. In another third, the antibodies target proteins in membrane glycoproteins (glycophorins) of the red cell; in other cases, the antibodies have specificity for antigens in the Kell or Duffy blood group system (very rarely for ABO antigens) or for structures in the membrane that are not blood group antigens (e.g. band 3, an anchor point in the membrane for the red cell cytoskeleton). In all these cases, the patient's own erythrocytes display the relevant antigen.[17] In primary AIHA, the autoantibodies of any one patient often are specific for only a single RBC membrane protein. The narrow spectrum of autoreactivity suggests the mechanism underlying AIHA development in such patients is not secondary to a generalized defect in immune regulation. Rather, these patients may develop warm-antibody AHA through an aberrant immune response to a self-antigen or to an immunogen that mimics a self-antigen. In patients with secondary AIHA, the disease may be associated with a fundamental disturbance in the immune system.

During fetal life, developing lymphocytes that come into contact with antigen are eliminated or silenced. This effect is one of the mechanisms of immunologic tolerance of endogenous antigens. The extreme rarity of autoimmune hemolytic anemia secondary to anti-A or anti-B antibodies indicates the deletion from the immune repertoire of B cells with the capacity to produce anti-A or anti-B antibodies. Such clones are probably eliminated or inactivated early in ontogeny because the embryo can synthesize A and B substances within 5 weeks of its implantation in the uterine wall.

A population of CD4+/CD25+ T cells that express the transcription factor Foxp3 restrains immune responses against autoantigens in adults. There is evidence that a deficiency of these regulatory T cells plays a role in the pathogenesis of autoimmune hemolytic anemia.

Warm antibodies-pathogenic effects: IgG anti–red cell autoantibodies mediate the destruction of red blood cells outside the circulating blood in a process called extravascular hemolysis (Figures 1A to D). By contrast, when lytic components of the complement system enter the mechanism, destruction of red cells occurs directly within the circulating blood (intravascular hemolysis). The participation of lytic complement components in IgG-mediated autoimmune hemolytic anemia is, however, rare. IgG antibodies are relatively poor activators of the classical complement pathway, but they, especially the IgG_1 and IgG_3 antibodies are recognized readily by F_c receptors on various phagocytic cells. The IgG sensitized RBCs generally are eliminated by the phagocytes of the R-E system. Presence of complement factors C3 (C3b and iC3b) potentiates extravascular hemolysis by the R-E cells in these patients as they have receptors for these complement components. Autoantibody-coated RBCs are trapped by macrophages in the Billroth cords of the spleen and, to a lesser extent, by Kupffer cells in the liver. The process leads to generation of spherical RBCs (Spherocytes) and fragmentation and ingestion of antibody-coated RBCs. Spherical RBCs are more rigid and less deformable than normal RBCs. As such, spherical RBCs are fragmented further and eventually destroyed in future passages through the spleen. Spherocytosis is a consistent and diagnostically important hallmark of AHA, and the degree of spherocytosis correlates well with the severity of hemolysis. Direct complement-mediated hemolysis with hemoglobinuria is unusual in warm-



Figs 1A to D IgG anti-red cell autoantibodies. (A) Structure of an IgG molecule demonstrating its variable and constant regions and the heavy and light chains; (B) Agglutination of red cells by pentameric IgM antibodies, which can join the cells into a lattice; (C) Coating of red cells by IgG antibodies. The antibodies are unable to agglutinate the cells; (D) Agglutination of IgG-coated red cells by an anti-IgG antibody

antibody AHA. Cytotoxic activities of macrophages and lymphocytes also may play a role in the destruction of RBCs in warm-antibody AHA.

Warm autoantibodies are panagglutinins, i.e. they react with all the RBCs in the diagnostic panel. Of the reported specificities, Rh is by far the most common (70%), including all but the Rh_{null} erythrocytes.

The degree of anemia in AIHA depends not only on the rate of red cell destruction but also on the ability of marrow to increase erythrocyte production. With an adequate supply of nutrients and growth factors, bone marrow can overcome a hemolytic rate of about three times normal; anemia does not appear until the half-life of the red cell population drops to about 10 days (the half-life of a population of normal red cells is about 30 days as measured with ^{51}Cr-labeled red cells). A half-life of 5 or 6 days is not unusual in autoimmune hemolytic anemia. The marrow can compensate for accelerated red cell destruction by increasing the number of red cell precursors by upto 10 times the normal number (erythroid hyperplasia), accelerating the release of reticulocytes, and in some cases, allowing nucleated red cells to enter the blood.

Cold Agglutinins and Hemolysins—Pathogenic Effects

Cold-active antibodies exhibit increasing titer and RBC-binding activity as the temperature decreases toward 0°C and occur in two forms: (1) Cold agglutinin disease (CAD)-associated with IgM antibodies usually directed at the RBC I antigen, typically occurs in adult patients and may be primary or secondary to another disease process, usually infectious, and (2) Paroxysmal cold hemoglobinuria (PCH)-caused by the so-called Donath-Landsteiner antibody, an IgG hemolysin.

The great preponderance of cold agglutinin molecules are IgM antibodies. Most cold agglutinins are unable to agglutinate RBCs at temperatures higher than 30°C. The highest temperature at which these antibodies cause detectable agglutination is termed the *thermal amplitude*. Generally, patients with cold agglutinins with higher thermal amplitudes have a greater risk for cold agglutinin disease. For example, active hemolytic anemia has been observed in patients with cold agglutinins of modest titer (e.g. 1:256) and high thermal amplitudes. More than 90 percent of cold-active antibodies have the *I* antigen as their target on the RBC, and the *I* antigen is the binding site for a significant portion of the remaining 10 percent. The closely related *I/i* antigens are high-frequency carbohydrates similar to the ABO antigens. Neonatal RBCs exclusively expressing large amounts of *i* antigen, converting to exclusively *I* antigen by 18 months of age. Other uncommon but reported antigen targets include Pr. The fact that *M. pneumoniae* induces anti-*I* antibodies in the majority of patients is potentially related to the finding that sialylated *I/i* antigens serve as specific *Mycoplasma* receptors. Minor modification of this antigen may incite autoantibodies.

The pathogenicity of a cold agglutinin depends upon its ability to bind host RBCs and to activate complement. IgM sensitized RBCs generally are associated with a combination of intravascular and extravascular hemolysis. The pentameric structure of IgM enables efficient complement activation. Destruction of erythrocytes sensitized with IgM antibodies is mediated by the complement system. Complement mediates RBC destruction either directly by cytolysis or indirectly via interaction of RBC-bound activation and degradation fragments of C3 with specific receptors on reticuloendothelial cells, principally liver macrophages (Kupffer cells). Due to the presence of regulatory RBC proteins such as decay accelerating factor (DAF, CD55) and membrane inhibitor of reactive lysis (MIRL, CD59), overwhelming complement activation usually is required to produce clinically evident intravascular hemolysis. However, in most clinical situations, IgM antierythrocyte antibodies are present in sublytic quantities. Under these conditions, DAF (CD55) and MIRL (CD59) are able to prevent direct RBC lysis. More commonly, IgM sensitized RBCs undergo extravascular hemolysis. While RE cells do not have receptors for the F_c fragment of IgM antibodies, they do have receptors for the abundant RBC-bound C3b and *i*C3b

resulting from complement activation. The principal site of IgM mediated extravascular hemolysis is liver and not the spleen.

Cold agglutinins may bind to RBCs in superficial vessels of the extremities, where the temperature generally ranges between 28 and 31°C, depending upon ambient temperature.[18] Cold agglutinins of high thermal amplitude may cause RBCs to aggregate at this temperature, thereby impeding RBC flow and producing acrocyanosis. In addition, the RBC-bound cold agglutinin may activate complement via the classic pathway. Once activated complement proteins are deposited onto the RBC surface, the cold agglutinin need not remain bound to the RBCs for hemolysis to occur. Instead, the cold agglutinin may dissociate from the RBCs at the higher temperatures in the body core and again be capable of binding other RBCs at the lower temperatures in the superficial vessels. As a result, patients with cold agglutinins of high thermal amplitude tend toward a sustained hemolytic process and acrocyanosis.[19] In contrast, patients with antibodies of lower thermal amplitude require significant chilling to initiate complement-mediated injury of RBCs. This sequence may result in a burst of hemolysis with hemoglobinuria.[19] Combinations of these clinical patterns also occur.

In contrast to cold agglutinins, cold hemolysins the so called Donath and Landsteiner (D-L) antibodies, are IgG antibodies and are polyclonal. They were first described by Donath and Landsteiner in 1903 in patients with a characteristic syndrome-paroxysmal cold hemoglobinuria (PCH).[20] The Donath and Landsteiner (D-L) antibody is a hemolysin that binds to RBCs at low temperatures and fixes complement. When the RBCs are warmed, they are destroyed by complement lysis. The D-L IgG antibody is a potent hemolysin, causing significant RBC destruction even in low titers. The D-L antibody is classically described as a "biphasic" hemolysin. The antibody requires the cooler temperatures (0–4°C) to bind to the RBC, but complement-mediated lysis does not proceed until the temperature is raised (37° C). The antibody in PCH is directed against the P antigen, found on the RBCs of most individuals. The P antigen is similar to the Forssman glycolipids present in many micro-organisms. This similarity suggests that infectious agents, may elicit D-L antibodies as a result of crossreactivity.

The D-L antibody occurs in three clinical syndromes: (a) chronic PCH associated with late-stage or congenital syphilis, (b) acute transient PCH occurring after an infectious illness, and (c) chronic idiopathic PCH. An increasing proportion of Donath-Landsteiner autoantibody-mediated hemolytic anemias occurs as a single postviral episode in children, without recurrent attacks (paroxysms). The prognosis for such cases is excellent. Thus, rather than paroxysmal cold hemoglobinuria, a proposed term for this latter entity is *Donath-Landsteiner hemolytic* anemia.[21, 22]

DRUG-INDUCED IMMUNE HEMOLYTIC ANEMIA

The most common drugs associated with DIIHA and the hypotheses for the mechanisms thought to be involved have changed during the last few decades. There are two types of drug-related antibodies. Drug-independent antibodies are those antibodies that can be detected *in vitro* without adding any drug; thus, *in vitro* and *in vivo* characteristics are identical to cell red blood cell (RBC) autoantibodies. Drug-dependent antibodies are those antibodies that will only react *in vitro* in the presence of drug (e.g. bound to RBCs or added to the patient's serum in test systems to detect drug antibodies); these are antibodies directed at epitopes on the drug and/or its metabolites, or a combination of drug plus RBC membrane protein. The mechanisms involved in the serological and clinical findings are controversial. It is still unknown why or how some drugs can affect the immune system to cause RBC autoantibody formation, with or without HA.[23]

Clinical Features

Preschool children have the peak incidence of AIHA in pediatric age group. It is 2.5 times more common in boys as compared to girls. The clinical findings in WAIHA are variable. They are determined by the rate of hemolysis and the ability of the body to process breakdown products and mount a reticulocytosis. Two general clinical patterns are seen. In 70 to 80 percent patients mostly in the age group of 2 to 12 years the disease has an acute transient pattern lasting for 3 to 6 months. It is frequently preceded by an infection, usually respiratory.

Onset may be acute, with prostration, pallor, jaundice, pyrexia, and hemoglobinuria, or more gradual, with primarily fatigue, dyspnea on exertion and pallor. The spleen is usually enlarged. Hepatomegaly, and lymphadenopathy may also accompany the anemia.

Mild, chronic hemolytic anemia with exacerbations in the winter is the general rule for cold agglutinin disease. Rarely, does the hemoglobin drop below 7 g/dL. Pallor and jaundice may occur, if the rate of hemolysis is greater than the endogenous capability to metabolize bilirubin. Some patients have intermittent bursts of hemolysis associated with hemoglobinemia and hemoglobinuria on exposure to cold and may be forced to move to warmer climates to prevent attacks. Acrocyanosis can occur from agglutination of RBCs in the cooler vessels of the hands, ears, nose, and feet. Digits may become cold, stiff, painful, or numb and may turn purplish. Limbs may manifest

livedo reticularis, a mottled appearance that is readily reversible upon warming of the affected area. Only rarely does actual gangrene of digits develop. If hemolysis does occur after *Mycoplasma* infections, it typically begins when the patient is recovering from the pneumonia and titers for cold autoantibodies are at their peak. Hemolytic anemia in infectious mononucleosis develops either at the onset of symptoms or within the first 3 weeks of illness.

In children <5 years, paroxysmal cold hemoglobinuria accounts for almost 40 percent of the patients. The onset of the disease is sudden with fever (even up to 40°C), back or leg pain, and hemoglobinuria after exposure to the cold even of a few minutes. Symptoms may follow shortly or several hours later. Abdomen cramps, headache, nausea, vomiting, and diarrhea may also occur. Dark red-to-black color urine is voided after the onset and typically clears in a few hours. Rarely, it persists for a few days. The spleen may be palpable during an attack and shortly thereafter, and mild jaundice may appear. Systemic symptoms may appear without the hemoglobinuria and vice versa. Reports of Vasomotor phenomena manifest as cold urticaria, tingling of hands and feet, cyanosis, and Raynaud phenomenon are there in the literature.

An antecedent upper respiratory infection in children is usually identified in PCH. Measles, measles vaccinations, mumps, *Mycoplasma pneumoniae*, influenza A, adenovirus, varicella, cytomegalovirus, *Haemophilus influenzae*, and infectious mononucleosis have been identified as antecedent illnesses. The original chronic PCH associated with syphilis has all but disappeared.

A careful history of drug exposure should be obtained from all patients with hemolytic anemia and/or a positive DAT. In drug, induced AIHA, clinical manifestations are the same as above except there is history (taken carefully) of use of the offending drug and absence of hepatomegaly and significant lymphadenopathy. Cefotetan or quinidine (by ternary complex mechanism) has been implicated in many severe hemolytic reactions. Fatal reactions may occasionally occur. Cefotetan and ceftriaxone have been associated with fatalities. Patients with hapten/drug adsorption (e.g. penicillin) and autoimmune (e.g. alpha-methyldopa) types of drug-induced hemolytic anemia exhibit mild-to-moderate hemolysis, with insidious onset of symptoms developing over a period of days to weeks.

LABORATORY FINDINGS[24-27]

Anemia

By definition, patients with AHA present with anemia, the severity of which ranges from life-threatening to very mild. In fulminant cases, in which the RBC lifespan is less than 5 days, the anemia is severe, and erythropoiesis increases 8-10 fold. The reticulocyte count may rise, sometimes upto 40 percent. Reticulocytes may be depressed early in the course. Reticulocytopenia may be because of marrow shutdown from intervening infection, malignancy myelophthisis, parvovirus B_{19} infection, or the possibility of the autoimmune antibody being directed at antigens in great concentration on the reticulocytes themselves.

On PBF examination, polychromasia indicates reticulocytosis. Spherocytes are seen in patients with moderate-to-severe hemolytic anemia. RBC fragments, nucleated RBCs, and occasionally erythrophagocytosis by monocytes and rarely neutrophils may be seen in severe cases.

Most patients have mild leukocytosis and neutrophilia. Leukopenia and neutropenia may also occur sometimes. Patients with severe hemolytic anemia and markedly increased erythropoiesis occasionally develop folate deficiency and frank megaloblastosis with raised MCV levels.

Platelet counts typically are normal but may be low in systemic lupus erythematosus or in Evans' syndrome.

The combination of a high MCV (because of reticulocytosis), a high RDW (because of the dimorphic population of reticulocytes and spherocytes), and a high reticulocyte count points to hemolytic anemia.

Clumping from the cold agglutinins complicates both the peripheral blood smear and the calculation of the red cell counts and red cell indices in cold agglutinin disease. Disolution of the clumping upon warming indicates the presence of a cold agglutinin rather than Rouleaux formation or fibrin clumping.

Bone Marrow Examination

Not recommended routinely. Indicated if uncommon findings or in cases where lymphoma is suspected. The characteristic finding in bone marrow is erythroid hyperplasia.

Biochemical Tests Suggesting Increased Destruction of Erythrocytes

Raised serum bilirubin levels, rarely >5 mg/dL with conjugated (direct) fraction constituting less than 15 percent of the total. Increased urinary urobilinogen, hemoglobinemia and depressed or absent haptoglobin can be seen in rapid hemolysis rarely in warm AIHA but more commonly in patients with cold agglutinin disease, and characteristically in patients with paroxysmal cold hemoglobinuria and with drug-immune hemolytic anemia mediated by the ternary complex mechanism, even if extravascular. Hemoglobinuria and hemosiderinuria may be seen after severe hemolysis. Serum haptoglobin levels are low, and lactate dehydrogenase levels are elevated.

Antiglobulin (Coombs) DAT Test and Indirect Antibody Test (IAT)

Diagnosis of AIHA or drug-immune hemolytic anemia requires demonstration of immunoglobulin and/or complement bound to the patient's RBCs outer membrane by DAT, also known as the Coombs test (first described in 1945 by Robin Coombs) which is pathognomonic for this disease. It is a screening procedure. If agglutination is noted with this broad-spectrum reagent, antisera reacting selectively with IgG (the "gamma" Coombs) or with C3 (the "nongamma" Coombs) are used to define the specific pattern of RBC sensitization. Monospecific antisera to IgM or IgA also have been used in selected cases.

Nevertheless, a positive antiglobulin test requires cautious interpretation when there are no other features of autoimmune hemolytic anemia. False-positive test results are not unusual. The reported incidence of positive antiglobulin tests in normal blood donors and general populations of hospitalized patients varies widely—from 1 in 100 to 1 in 15000. Differences in the technique used in performing the test account for this variation. The most common reason for a false-positive direct antiglobulin test is low-avidity adherence of nonspecific IgG to red cells. In rare cases, however, the result is not a false-positive but a harbinger of the development of autoimmune hemolytic anemia. False-negative tests are usually due to low-affinity autoantibodies that spontaneously elute from the red cell *in vitro* or amounts of erythrocyte-coating antibodies that are below the limit of detection by the antiglobulin test. The distinction between a true-positive and a false-positive direct antiglobulin test can be made by eluting the antibody from the red cells and testing its ability to bind to normal red cells. In a false-positive reaction, the eluted antibody does not bind to normal red cells, whereas binding occurs in a true-positive test.

In >95 percent of warm AIHA cases, the DAT is positive: Series vary in their DAT results. Between 20 and 66 percent have only IgG on the surface, 24 to 63 percent have IgG and C3, 7 to 14 percent have only C3, and 1 to 4 percent are DAT-negative. Patients with SLE are particularly prone to positive tests for complement on their RBCs. IgG_1 predominates, either alone or in combination with other subclasses (Table 3).

"Free" autoantibody may be detected in the plasma or serum of these patients by the IAT. In general, patients whose RBCs are heavily coated with IgG more likely exhibit plasma autoantibody. Patients with a positive IAT as a result of a warm-reactive autoantibody should also have a positive DAT. A patient with a serum anti-RBC antibody (positive IAT) and a negative DAT probably does not have an autoimmune process but rather an alloantibody stimulated by prior transfusion.

Table 3 Results of DAT in 100 pediatric patients with different serologic type of AIHA

	AIHA			
DAT	*Warm (n = 64)*	*Cold (n = 26)*	*Mixed (n = 4)*	*PCH (n = 6)*
DAT neg	5	11		5
DAT pos	59	15	4	1
Compl (C)	5	15		1
IgG	19			
IgG + C	31		3	
IgG + IgA	1			
IgG + IgA + C	1		1	
IgG + IgA	1			
+ IgD + C IgA	1			

Vaglio et al. 2007

Patients with cold agglutinin disease have a more homogeneous DAT results than patients with warm AIHA. Since IgM antibodies are involved in this disease DAT is positive almost exclusively with anti-C3 and polyspecific reagents and negative with anti-IgG.

PCH is caused by Donath-Landsteiner antibody—a biphasic IgG antibody. It fixes complement at low temperature and ultimately dissociate at higher temperatures. As a result, DAT is positive with anti-C3, but is generally negative with anti-IgG unless performed at colder temperature. Biphasic IgG autoantibodies bind RBCs efficiently at 0–4°C and subsequently, fix complement C1 at that temperature.

D-L antibodies are potent, so even a small titers can produce hemolysis.

THERAPY[17,22, 24,28]

General principles of treatment are guided by the severity of hemolysis. Severe cases with very low hemoglobin may warrant immediate blood transfusion while mild-to-moderate cases may either need only observation or else need to modulate the immune system's production of autoantibody and destruction of antibody-coated RBCs.

Blood Transfusion in Autoimmune Hemolytic Anemia

In AIHA, the decision to transfuse does not depend on compatibility test results and, instead depends on an evaluation of the patient's need for transfusion. The indications for transfusion in patients with AIHA are not significantly different than for similarly anemic patients

without AIHA. Patients with autoimmune hemolytic anemia (AIHA) frequently have anemia of sufficient severity as to require a blood transfusion. It is impossible to find compatible blood when, as is frequently the case, the autoantibody in the patient's serum reacts with all normal red blood cells.

Because the antibody in this disease is usually a "panagglutinin," reacting with nearly all normal donor cells, compatible cross-matching is impossible. The goal in selecting blood for transfusion is to avoid administering RBC with antigens to which the patient may have alloantibodies.

A common procedure is to adsorb the panagglutinin present in the patient's serum with the patient's own RBC from which antibody has been previously eluted. Serum cleared of autoantibody can then be tested for the presence of alloantibody to donor blood groups. ABO-compatible RBC matched in this fashion are administered slowly, with watchfulness for signs of an immediate-type hemolytic transfusion reaction.

Physicians should provide as many RBCs as may be reasonable because the autoadsorption procedure is the most effective method for detecting alloantibodies in patients with warm autoantibodies. The warm autoadsorption test is not useful in patients who have been transfused recently (within about the last 3 months) because even a small percentage of transfused cells may adsorb the alloantibody during the *in vitro* adsorption procedure, thus invalidating the results. An alternative approach, which may be about as effective in avoiding the effects of alloantibodies, but which is not widely implemented in transfusion services, is to perform extensive RBC phenotyping of the patient and the donor units. Other simple tests that provide safety include routine testing of the patient's serum against a red cell panel and diluting the patient's serum before doing compatibility testing.

However, one need to remember that only a few percent of all hospitalized patients have RBC alloantibodies so that if transfusion is extremely urgent, the lesser risk may be to transfuse rather than waiting for completion of the compatibility testing. In very urgent situations, the quickest, but least reliable, techniques for detection of alloantibodies are the dilution technique and partial RBC phenotyping. Warm autoadsorption test should be performed if there is adequate time, since it is highly effective for detection and identification of alloantibodies and only requires one to three adsorptions of the patient's serum with the patient's (ZZAP-treated) RBCs. Allogeneic adsorption, which is the most time consuming, is indicated if the patient has been transfused recently or if the patient's RBCs are not available for autoadsorption.

Alleviation of signs and symptoms of anemia usually can be accomplished with relatively small quantities of RBCs, as little as 0.5 to 1 units. Transfusion should be given when indicated even before all serological tests are completed.

Compatibility testing in cold antibody AIHAs is less labor intensive. In cold agglutinin syndrome, the autoantibody does not often react upto a temperature of 37°C, whereas clinically significant RBC alloantibodies will react at this temperature. Accordingly, the compatibility test can be performed strictly at 37°C (Petz & Garratty, 2004). If the transfusion service is not able to perform testing strictly at 37°C, one or two cold autoadsorptions should be done, which will not remove a high titer cold agglutinin completely, but are likely to eliminate reactions that occur at 37°C.

GLUCOCORTICOIDS

Glucocorticoids are the main stay of treatment in warm AIHA. If the disease is mild and compensated, no treatment is needed. However, if the hemolysis is severe with significant anemia with its antecedent signs and symptoms, glucocorticoid treatment is started.

Glucocorticoids decrease the rate of hemolysis by blocking macrophage function by downregulating $F_{c\gamma}$ receptor expression and thus may suppress RBC sequestration by splenic macrophages, decreasing the production of the autoantibody, and perhaps by enhancing the elution of antibody from the RBCs. The standard of practice is administration of prednisone in a dose of 1.0 to 2.0 mg/kg/day. In some patients with severe hemolysis, doses upto 6 mg/kg/day may be required to reduce the rate of hemolysis. A response, manifested by a rise in the hematocrit and a fall in the reticulocyte count usually within 3 to 4 weeks. The duration of treatment at this dose is an unsettled question. A patient who fails to improve within this time is unlikely to respond to further treatment with prednisone. In a patient who responds, slow reduction of the dose of prednisone is essential to avoid a relapse. The dose is tapered only when the rate of hemolysis decreases significantly and then reduced to 5 to 15 mg/day. Thereafter, slow, cautious tapering over a period of at least 4 months is the rule. A rise in the reticulocyte count or a fall in the hematocrit should prompt an increase in the dose, usually to the previous level. The disease tends to remit spontaneously within a few week or month. The Coombs test result may remain positive, even after hemolysis has subsided. About 25 percent of patients treated with corticosteroids enter a stable, complete remission; half the patients require continuous, low-dose prednisone; and the remaining 25 percent respond only transiently or not at all or are unable to tolerate continuous corticosteroid treatment. There is no reliable evidence that alternate-day maintenance treatment is superior to daily treatment, but some patients tolerate this schedule better than daily prednisone.

Very high doses of intravenous methylprednisolone may be tried in cases who do not respond to oral prednisolone before other treatments are initiated or are critically ill.

SPLENECTOMY

Because the spleen is the major site of red cell destruction in autoimmune hemolytic anemia, splenectomy should be considered for patients who have not responded to corticosteroids or who have maintained a stable, but corticosteroid-dependent remission. A complete, durable remission follows splenectomy in half to two-thirds of cases. Attempts to predict responsiveness to splenectomy with measurement of splenic sequestration of ^{51}Cr-labeled erythrocytes are not reliable. However, the relapse rate following splenectomy is disappointingly high. Many patients require further glucocorticoid therapy to maintain acceptable hemoglobin levels, although often at a lower dose than required prior to splenectomy. The only way of knowing the effectiveness of splenectomy in a given patient is to perform the procedure. Laparoscopic splenectomy, a safe method of removing the organ, is now the preferred surgical technique. In most cases, a reasonable approach is to continue glucocorticoids for 1 to 2 months while waiting for a maximal response. However, if no response is noted within 3 weeks, the patient's condition deteriorates, or the anemia is very severe, splenectomy should be performed sooner.

Splenectomy removes the primary site of RBC trapping. The beneficial effect of splenectomy may be related to several factors interacting in complex fashion. The spleen is also believed to be a major producer of IgG antibodies. Continuation of hemolysis after splenectomy is partly related to persisting high levels of autoantibody, favoring RBC destruction in the liver by hepatic Kupffer cells. Splenectomy has little effect on the clearance of IgM-coated RBCs and therefore would not be indicated in the unusual patient with a warm-active IgM antibody.

Splenectomy is complicated by a heightened risk of infection with encapsulated organisms, particularly in patients younger than 2 year. Prophylaxis is indicated with appropriate vaccines (pneumococcal, meningococcal, and *Haemophilus influenzae* type B given atleast 2 weeks before splenectomy) and with oral penicillin/amoxicillin after splenectomy. The usual dose of penicillin for prophylaxis is 250 mg twice daily for 2 to 3 years after splenectomy (or at least until the age of 5). Education of the patient concerning the risk for serious infection after splenectomy is also important. Subsequent to splenectomy, patients should be given antibiotics promptly with any febrile illness preferably after sending a blood culture.

A rise in the platelet count occurs after splenectomy in almost all patients. The increase rarely exceeds 500000/μL and usually subsides within 3 to 5 months. Routine antithrombotic prophylaxis is not indicated for post-splenectomy thrombocytosis as there is a low risk for thromboembolism.

Immunosuppressive Therapy

Patients who have failed to respond to splenectomy or have relapsed after splenectomy, when splenectomy poses an unacceptable risk, and for patients who cannot tolerate steroid therapy, are the candidates for immunosuppressive therapy.

Cytotoxic and immunosuppressant drugs, such as cyclophosphamide, azathioprine and cyclosporine A, give a 40 to 60 percent response rate. However, these treatments may be associated with serious side effects, such as bone marrow suppression, nephrotoxicity and secondary malignancies, while the effectiveness of other options, such as IVIG, plasmapheresis and, danazol, is controversial.

A reasonable immunosuppressive regimen might include azathioprine (80 mg/m^2/day) or cyclophosphamide (60 mg/m^2/day), concomitantly with prednisone (40 mg/m^2/day). Prednisone may be tapered over 3 months or so, and the cytotoxic agent continued for 6 months before reducing the dose gradually. Bone marrow suppression may dictate minor dose adjustments. Rapid withdrawal has led to rebound immune response. Alternatively, high-dose cyclophosphamide (50 mg/kg/day × 4 days) has produced a complete remission in 66 percent of patients who were refractory to other therapies. Severe myelotoxicity and its attendant potential for complications are expected. Hemorrhagic cystitis, bladder fibrosis, secondary malignancies, sterility, and alopecia are some of the major side effects.

RITUXIMAB[29,30]

Rituximab is a monoclonal antibody directed against the CD20 antigen expressed on B-lymphocytes and is used for treatment of B-cell lymphoma. Its use for treatment of AHA is based on the antibody's ability to eliminate B lymphocytes, including presumably those making autoantibodies to RBCs. However, the mechanism of action is more complex than that, as the effect of rituximab can occur very early, before the autoantibodies can recede. Rituximab (375 mg/m^2 weekly for a median of 4 weeks) is effective in treating both warm AIHA and CAD, with an overall response rate ranging from 40 to 100 percent (median with 60%), and with patients of all ages responding. Moreover, many of these responses were durable, lasting >3 years in some patients. In a large prospective series,[30] 13 of 15 (87%) children with

warm-antibody AHA responded to rituximab 375 mg/m^2 weekly for 2 to 4 weeks. Twenty-three percent of the responders relapsed, but subsequent courses of rituximab induced additional remissions in this series. Thus far, few side effects have been reported, but rare reactions to the infusion have been documented. B-cell counts remain low for months after treatment, raising the risk of infections due to poor immune response. If remissions remain durable and potential side effects are less harmful than other treatments for warm AIHA, such as prolonged steroid use or splenectomy, its use may become more common.

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CHAPTER 23

Paroxysmal Nocturnal Hemoglobinuria

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Paroxysmal nocturnal hemoglobinuria (PNH) is a rare acquired clonal disorder of hematopoetic stem cells. The molecular defect in PNH is a somatic mutation in the *PIG-A* gene causing defect in glycosyl phosphatidyl inositol (GPI) anchored proteins. Deficiency of these GPI-anchored proteins (GPI-AP) on the membrane of hematopoietic cells lead to the various manifestations of PNH.

The clinical manifestations of PNH are characterized by a triad of cytopenias, intravascular hemolysis and venous thrombosis at unusual sites. PNH can be broadly classified into hypoplastic and classical type. The predominant manifestation of hypoplastic type is bone marrow failure with a small clone of PNH cells.

The classical type is characterized by:

- Intravascular hemolysis
- Thrombosis
- With or without cytopenias.

The disease is insidious in onset and causes significant morbidity due its prolonged natural course. Management of PNH depends upon proper classification, as the treatment differs in different subgroups.

MOLECULAR GENETICS OF PAROXYMAL NOCTURNAL HEMOGLOBINURIA

Glycosyl Phosphatidyl Inositol Anchor (GPI-anchor)

Although most membrane proteins traverse the lipid bilayer one or more times, certain membrane proteins (i.e. GPI-anchored proteins) adhere to the cell surface by means of a glycolipid moiety. In PNH cases, the defect is attributed to mutations in the *PIG-A* gene.

PIG-A Gene and its Significance in PNH

PIG-A is an endoplasmic reticulum membrane protein. In PNH, there is a somatic mutation in the PIG-*A* gene in the hematopoetic stem cell, resulting in a deficiency in the surface expression of all GPI anchored proteins due to defective synthesis of GPI in the endoplasmic reticulum. The defect in *PIG-A* gene is always in the somatic cells and is never identified in the germ line; thus it is always acquired.

Effect of the Defect

The mutation in *PIG-A* gene leads to little or no GPI anchor being made. This leads to lot of proteins missing on the membrane of these cells which are GPI anchored.

INCIDENCE AND NATURAL HISTORY

PNH is a rare disease whose incidence is reported to be 15/million population in United States. As a result of improved diagnostic tools, more and more patients are suspected and screened for PNH.

Indian Perspective

Koduri PR, Gowrishankar S et al. from Apollo Hospital Hyderabad, in their series of 11 patients of PNH in 1992 reported that the Indian patients were younger and showed a marked male preponderance. Severe thrombocytopenia with its hemorrhagic manifestations and infectious complications were not seen. However, thrombotic complications were common. Parab RB et al. in 1990 presented a retrospective study of 17 patients of PNH diagnosed on positive sucrose lysis. Anemia was present in all of them, whereas about 50 percent of the patients had jaundice, fever and bleeding tendency.

Agarwal MB, Mehta BC et al. also reported a study of 20 PNH patients in 1981. Neelam Verma et al. from PGI Chandigarh found that 9.6 percent and 30.7 percent of the Aplastic anemia patients were positive for PNH on flow cytometry on diagnosis and on follow up respectively. The conventional tests, however, diagnosed only 4.6 percent patients on follow up. None of the MDS patients tested positive by any of these means, on diagnosis or at follow up.

CLINICAL MANIFESTATIONS (TABLE 1)

The clinical presentation of PNH is variably overlapped by three features:

I. Intravascular hemolysis
II. Thrombosis
III. Hematopoiesis deficient.

Intravascular Hemolysis

It is one of the most important manifestations of PNH and the disease derives its name from this symptom.

Pathophysiology of Intravascular Hemolysis

The cause of the hemolysis is the deficiency of 2 GPI-AP in the RBC membrane, that is CD55 (Decay accelerating factor-DAF) and CD59 (Membrane Inhibitor of Reactive Lysis-MIRL). CD55 and CD59 protect the RBCs from complement mediated hemolysis, especially CD 59. PNH cells are divided into three types (Table 2).

Aggravating factors for hemolysis:

1. *Nocturnal hemolysis:* The nocturnal pattern of hemolysis, initially thought to be a consequence of systemic acidosis due to retention of CO_2 during sleep is now attributed to the nocturnal absorption of lipopolysaccharides (LPS), a byproduct of bacterial cell wall from the gut. LPS markedly activates complement system. LPS is normally bound by monocytes through a GPI linked protein, CD14, which is missing in PNH.
2. *Concurrent inflammation or immune reactions to infections:* Complement is also activated by concurrent inflammation or the immune reaction to infections. Most viral disorders will result in a burst

Table 1 Clinical manifestations of PNH

Due to intravascular hemolysis
• Anemia, hemoglobinuria, fatigue
• Acute/chronic renal failure, recurrent urinary tract infection
• Back pain, headache
• Abdominal pain, bloating
• Esophagospasms
• Cholelithiasis
• Erectile dysfunction
Rare
• Choledocho dyskinesia
• Acute pancreatitis
• Ischemia and ulceration of duodenum or colon
Due to thrombosis
Venous thrombosis
• Abdominal vein thrombosis
– Budd-Chiari
– Splenic vein
– Mesenteric veins
– Portal hypertension, esophageal varices, caput medusae (dilated abdominal veins)
– Renal vein thrombosis
• Cerebral vein thrombosis
– Headache
– Hemorrhagic infarct
– Retinal vein thrombosis
– Loss of vision
– Deep vein thrombosis
– Pulmonary emboli
Rare
• Cutaneous vein thrombosis
• *Pyoderma gangrenosum*
• Arterial thrombosis (less common)
• Stroke
• Myocardial infarction
Due to bone marrow failure
• Anemia, infections, bleeding
• Myelodysplastic syndrome
• Bone pain
Rare: Transformation to acute myeloid leukemia (AML)

Table 2 Types of PNH cells

Type	*CD55/CD59*	*Complement sensitivity*	*Erythrocyte survival ($t_{1/2}$)*	*Cell of origin*
I	Normal	Normal	120 days	Normal stem cell
II	10–50% of normal	3–15 times	30–60 days	Defective clone with limited ability to make GPI anchor
III	Absent	15–25 times	4–6 days	Clone unable to make GPI anchor

of hemolysis. The most serious hemolysis results from gastrointestinal inflammation, usually due to viral gastroenteritis. This may be due to increased absorption of LPS and the effect of the immune reaction against the virus.

3. Immune reactions, which otherwise cause minimal hemolysis in normal persons, can cause severe hemolysis in PNH patients', e.g. infectious mononucleosis.
   - *Immunizations and vaccinations:* Special precaution should be taken while giving repeated doses of polysaccharide containing vaccine against *Streptococcus pneumoniae.*
   - *Blood transfusion* in a PNH patient can lead to a burst of hemolysis, due to the activation of complement by immune reactions involving leukocytes or plasma proteins. This can be avoided by washing of RBCs prior to transfusion, in a susceptible patient.
   - Few reports suggest that intravenous hematinics and injection erythropoietin may aggravate hemolysis.

Clinical Effects of Intravascular Hemolysis

1. *Hemoglobinuria:* It is due to the excretion of αβ globin dimers in urine as the reabsorption capacity of proximal tubules is exceeded. The incidence of hemoglobinuria presenting anytime during the course of the disease ranges from 27 to 84 percent.
2. *Renal dysfunction:* PNH patients can develop renal complications in the form of either acute renal failure or chronic kidney damage.
   a. *Acute renal failure:* This occurs when the concentration of hemoglobin in the tubular filtrate becomes sufficiently high to impair renal function, and acute renal failure sets in. This situation is usually seen with gastrointestinal illnesses and is complicated by the inability of the patient to take sufficient amount of water orally. Although there is usually good recovery if the condition is treated with hydration and careful monitoring of blood pressure, there often is residual renal impairment.
   b. *Chronic renal damage:* Hemoglobinuria can lead to chronic damage to the kidney. It manifests in two forms.
   c. *Proximal renal tubular acidosis:* This happens when the concentration of globin dimers in the glomerular filtrate becomes so great that the resorption capacity of the proximal tubule for other molecules normally resorbed by this epithelium is impaired.
   d. *Chronic renal failure:* This is usually slowly progressive and may result in death. It is the cause of death in 8 percent of the PNH patients.
3. *Other manifestations:* The free hemoglobin in plasma diffuses in the tissues and binds to nitric oxide (endothelial derived relaxing factor) causing a local deficiency of nitric oxide in the tissues. This leads to contraction of smooth muscles leading to various clinical manifestations such as early morning substernal tightness, dysphagia (esophageal contraction), penile erectile dysfunction in males, sense of fatigue and weakness, abdominal pain, Jaundice due to indirect hyperbilirubinemia. The patients of PNH may be chronically jaundiced due to the ongoing hemolysis in the circulation. This is sometimes aggravated in acute hemolysis.

Thrombosis

Thrombosis has been reported to be a leading cause of morbidity in PNH patients.

Pathophysiology of Thrombosis

The pathophysiology of thrombosis in PNH is not clearly understood. Various mechanisms are postulated:

- Absence of CD59 on the platelets may play a role in the hypercoagulable state of PNH.
- The PNH platelets are more sensitive to aggregation by thrombin than normal platelets.
- The abnormal monocytes of PNH lack the receptor for plasminogen activator and thus are less efficient in fibrinolysis.

Thrombosis in PNH is mostly venous, and at unusual sites. Preference for these sites may be due to the locally retarded blood flow, sufficient for the formation of platelet aggregates. This also permits activation of complement on the surface of RBC that can be transferred to the neighboring endothelial cells resulting in the expression of tissue factor and initiating clotting. The common sites for thrombosis are:

Hepatic veins and veins of portal system: These are particularly affected. Hepatic and portal vein thrombosis in PNH can present in either of the forms:

- *Acute onset of thrombosis:* This results in the classic Budd-Chiari syndrome, often seen in the setting of severe hemolysis, leading to
  - Tender hepatomegaly
  - Ascites
  - Jaundice
  - Elevation of serum enzymes indicative of liver damage.
- Gradual hepatic vein thrombosis
  - Pain in the right upper quadrant
  - Hepatomegaly
  - Gradual onset of ascites
  - Signs of portal hypertension

Radiological diagnosis can be made in both instances.

- *Inferior vena cava thrombosis:*
  - Can lead to lower body anasarca
  - Renal vein thrombosis can cause renal dysfunction in the form of proteinuria and renal failure.
- *Splenic vein thrombosis:*
  - Causing massive splenic enlargement and even rupture.
- *Splanchnic vein thrombosis:*
  - Results in a syndrome of recurrent abdominal pain sometimes bowel necrosis
- *Cerebral venous thrombosis:*
  - Common in PNH patients
  - Sagittal sinus is most frequently involved
  - Patients have headache, signs of raised intracranial tension and focal neurological deficits
- *Retinal vein thrombosis:*
  - Manifests as diminution of vision.

These are the common sites of venous involvement in PNH. In addition to this rarely there can be

- *Thrombosis of dermal and epididymal veins:*
  - In the former, there is necrosis of the skin
  - The later results in a syndrome that is confused with epididymitis, orchitis or torsion of the testis
  - Thrombosis of the veins of the uterus can cause a syndrome resembling pelvic inflammatory disease.

It is postulated that the PNH granulocyte clone size is predictive of the risk of thrombosis. The overall incidence of thrombosis in patients with granulocytic clone size larger than 50 percent is 53.5 percent, as compared to an incidence of only 5.8 percent in patients with a small PNH granulocytic clone. Thrombosis as a complication predicts poor survival.

Factors associated with high-risk of thrombosis during the disease course:

- Thrombosis at diagnosis
- Age over 54 years
- Presence of infection at diagnosis.

Thrombosis in PNH mostly involves the venous system. However, there are few case reports of arterial thrombosis also.

Hematopoiesis Deficient

Many patients of PNH have a history of aplastic anemia and diminished hematopoiesis to a greater or smaller extent. Nearly 50 percent of patients of aplastic anemia have a readily detectable PNH clone, particularly during or after recovery with antithymocyte globulin therapy.

Many patients of PNH develop aplastic anemia in the final stage. PNH like cells are found in 20 percent of patients of MDS. Anemia and hemorrhage are more common in pediatric than in adult PNH patients.

Classification

PNH is a disease of varied clinical presentations. It is classified into subtypes, based on predominant clinical manifestations and laboratory features.

There are various proposed systems of classification of PNH.

Clinically PNH can be divided into two types:

1. *Classical PNH:* Patients have predominant hemolytic and thrombotic manifestations and some degree of hematopoietic deficiency.
2. *Hypoplastic PNH:* Patients have features of bone marrow failure with a PNH clone which may or may not have hemolytic or thrombotic manifestations.

Wendell P et al. divided PNH into five groups:

1. *Aplastic anemia with detectable PNH cells [AA-PNH]:* This group is characterized by bone marrow failure with detection of fewer than 5 percent PNH granulocytes in the peripheral blood by flow cytometry. These patients may not develop overt PNH symptoms in future.
2. *Aplastic anemia—PNH [AA-PNH]:* In this group the predominant syndrome is bone marrow failure, but with presence of >5 percent PNH cells which can lead to some clinical features.
3. *PNH-aplastic anemia [PNH-AA]:* In this group the predominant clinical syndrome is PNH with significant evidence of bone marrow hypoplasia including granulocytopenia and thrombocytopenia.
4. *Classic PNH [PNH]:* Syndrome of PNH without bone marrow hypoplasia.
5. *MDS-PNH or PNH-MDS:* In this group, PNH cells are present in a patient with predominant myelodysplastic hematopoiesis in the former or when the clinical syndrome is predominantly due to the abnormal PNH cells with some elements of MDS as in the later (Table 3).

WHO SHOULD BE SCREENED FOR PNH? (TABLE 4)

PNH is a rare disease with varied clinical manifestations. The International PNH interest group has suggested screening for PNH in the following patients (Table 3):

- Patients with hemoglobinuria
- Patients with Coombs-negative intravascular hemolysis (based on abnormally high serum LDH), especially patients with concurrent iron deficiency.
- Patients with venous thrombosis involving unusual sites:
  - Budd-Chiari syndrome
  - Other intra-abdominal sites (e.g. mesenteric or portal veins)
  - Cerebral veins
  - Dermal veins.

Table 3 The International PNH interest group (Parker et al. Dec. 2005) classification

Types	*Hemolysis/Thrombosis*	*Bone marrow*	*Cytogenetic abnormality*	*Flow cytometry*	*Hams/Sucrose lysis test*
Classical	Yes	Erythroid hyperplasia	Absent	Positive	Positive
PNH/SAA*-MDS**	Yes	Associated disorder (SAA, MDS, MF)	May be seen	Positive	Positive
Subclinical	No	Associated disorder (SAA*, MDS**, MF***	May be seen	Positive	Negative

*SAA: Severe aplastic anemia; **MDS: Myelodysplastic syndrome; ***MF: Myelofibrosis

Table 4 Who should be tested for PNH and how often?

Once	*Repeatedly#*
• All patients with hemoglobinuria • All patients with unexplained hemolysis (increased LDH) • All patients with abdominal and cerebral vein thrombosis • All patients with thrombocytopenia and macrocytosis or signs of hemolysis	• All patients with PNH • All patients who have aplastic anemia • All patients who have had aplastic anemia (except after bone marrow transplantation) • All patients with myelodysplastic syndrome (MDS)

#Initially once every 6 months; then annually

- Patients with aplastic anemia (screen at diagnosis and once yearly even in the absence of evidence of intravascular hemolysis).
- Patients with refractory anemia—MDS.
- Patients with episodic dysphagia or abdominal pain with evidence of intravascular hemolysis.

LABORATORY DIAGNOSIS (TABLE 5)

Laboratory workup of a patient suspected of PNH includes the essential tests to diagnose and classify PNH as well as some ancillary tests to determine the prognosis and treatment.

The various laboratory tests include:

CBC

The blood picture varies. There may be:
- Severe pancytopenia
- Bicytopenia
- Normal counts
- Virtually all patients are anemic with mild macrocytosis
- Occasionally, when urinary iron loss is considerable, the red cells may appear microcytic and hypochromic
- In addition polychromasia, normoblasts and fragmented red cells may be seen on peripheral smear
- Relative reticulocytosis.

This may be marked but the absolute reticulocyte count is often lower than that found in association with other hemolytic disorders at comparable degrees of anemia.

This discrepancy reflects underlying marrow dysfunction that is invariably a component of the disease.

Urine Hemosiderin

Due to intravascular hemolysis, there is continuous presence of hemoglobin in the glomerular filtrate in the kidney. This excess of hemoglobin is deposited in cells of the proximal convoluted tubule as hemosiderin and can be detected in urinary sediments. At least 3 samples of urine should be tested for hemosiderinuria which indicates chronic intravascular hemolysis.

Bone Marrow Examination

Morphologic analysis of the bone marrow aspirate and biopsy and cytogenetics are needed for proper classification, as PNH is often observed in association with marrow failure syndromes.
- Bone marrow cellularity may vary from hypo, normo to hypercellular.
- In classic PNH it is normo to hypercellular with erythroid hyperplasia.
- It is usually hypocellular in hypoplastic PNH.
- The presence of dysplasia other than in erythroid series and cytogenetic abnormalities (present in 20%) suggest associated hematological disorder.
- The bone marrow iron stain shows decreased iron in case of iron loss due to hemolysis and increased iron in case of transfusion dependency.

Table 5 Laboratory tests for the diagnosis of PNH

Diagnostic tests	
Traditionally	
• Ham test (acidified serum lysis) • Sucrose lysis test • Thrombin lysis test	The lysis of PNH red blood cells exposed to activated complement tests for the deficiency of CD59 and CD55 on red blood cells. The tests vary in the pathways activating complement. *Advantage:* Cheap and simple to perform. *Disadvantage:* Labor intensive, decreased sensitivity due to the short half-life of circulating PNH red blood cells.
Today	
• Flow cytometric analysis – CD59 and/or CD55 peripheral blood red cells	*Advantage:* Useful to determine the degree of GPI anchor deficiency (PNH type I, type II, type III). *Disadvantage:* Decreased sensitivity due to the short half-life of circulating PNH red blood cells.
CD59, CD24, CD16, or any other GPI-linked proteins expressed on peripheral blood granulocytes	*Advantage:* The deficiency of at least 2 linked proteins is sensitive and specific for the diagnosis of PNH. *Disadvantage:* Might be difficult to perform in severe aplastic anemia when the number of circulating granulocytes is very low.
FLAER (fluorescently labeled inactive toxin aerolysin) binding of peripheral blood granulocytes	FLAER binds the GPI anchor. *Advantage:* The lack of FLAER binding on granulocyte is sufficient for the diagnosis of PNH. *Disadvantage:* Cannot be used for the analysis of red blood cells or platelets. Might be difficult to perform in severe aplastic anemia when the number of circulating granulocytes is very low.
PIGA gene mutation analysis	Although very specific is NOT used for diagnosing PNH

Serum Iron Studies and Ferritin

Due to chronic urinary iron loss in patients with hemolytic PNH, they may have iron deficiency. In contrast, patients of aplastic anemia with a clone of PNH may have normal or sometimes even increased serum iron due to transfusion dependency.

Complement Based Assays

Principle

PNH cells are unusually susceptible to lysis by complement. This can be demonstrated *in vitro* by a variety of tests.

Acidified Serum Lysis Test (Ham Test)

The principle of this test is that patient's red cells are exposed at 37°C to the action of normal or patients own serum, suitably acidified to the optimum pH for lysis (pH 6.5–7.0). In PNH, 10 to 50 percent lysis is usually obtained.

The Ham test is relatively specific but less sensitive. The only disorder, other than PNH that may appear to give a clear cut positive test is a rare congenital dyserythropoietic anemia CDA Type II or HEMPAS. In contrast to PNH, however HEMPAS red cells undergo lysis in only a proportion (about 30%) of normal sera, do not undergo lysis in the patient's own acidified serum, sucrose lysis test is negative and the expression of GPI-AP is normal.

The standard Ham test can be negative when there are less than 5 percent PNH Type III cells or less than 20 percent PNH Type II cells.

Sucrose Lysis Test

The sucrose lysis test is based on the fact that red cells absorb complement from serum at low ionic concentrations. PNH cells, because of their greater sensitivity will undergo lysis but normal red cells do not. In PNH, lysis usually varies from 10 percent to 80 percent, but exceptionally may be as little as 5 percent. Sucrose lysis test is more sensitive and less specific than the Ham test, as red cells from some cases of leukemia or myelofibrosis may undergo a small amount of lysis, almost always less than 10 percent. In these cases the Ham test is usually negative.

GPI—Anchor-based Assays

Today these tests are the mainstay of the diagnosis of PNH.

Principle

It involves the use of monoclonal antibodies against specific GPI-anchored proteins in conjunction with flow cytometry to diagnose PNH.

Advantages

Flow cytometry offers several advantages over complement based assay for diagnosing PNH:

- It is more sensitive and specific
- Measures the size of the PNH clone
- It is less affected by blood transfusions.

RBCs are the easiest to deal with but are not the most sensitive cell type to test because hemolysis or transfusion may decrease or even eliminate the PNH clone. Assaying granulocytes is better, assuming there is not severe granulocytopenia, but not all GPI-anchored surface antigens expressed granulocytes will give identical results. The recommendation is that more than one GPI-AP must be demonstrated to be abnormal and depending upon circumstances, more than one cell line should be evaluated.

Flow cytometry can be useful to know the extent of involvement and monitoring therapy in PNH. The standard flowcytometer can detect a PNH clone of >3 percent. The best estimate of clone size is given by CD55, CD59 and CD66b in granulocytes and CD55, CD59 and CD14 in monocytes.

Aerolysin Based Assays

A fluorescently labeled aerolysin, FLAER, is now the gold standard for diagnosis of PNH. These flow cytometry test are now done in many centers.

TREATMENT OF PNH

Treatment of PNH is under evolution. It aims at:

- Supportive treatment
- Definite treatment

Supportive treatment is as follows:

Anemia: It is invariably present in PNH patients. It may be either due to hemolysis or bone marrow failure.

Anemia due to Hemolysis

If anemia is predominantly due to ongoing hemolysis, it needs to be treated. The various treatment options are:

Eculizumab

This is a recombinant humanized monoclonal antibody against the terminal complement components and is an effective therapy in PNH. It is a remarkably safe drug and has been approved for the treatment of PNH. However, there is a slightly increased risk of developing infections with encapsulated organisms' especially meningococcal infections. Thus, all patients should receive appropriate vaccinations and early therapy as required.

Effects

- Stabilization of hemoglobin levels, reduction or cessation of transfusion requirement
- Reduction of the intravascular hemolysis, cessation of hemoglobinuria
- Clinically significant improvement in the quality of life
- Significant reduction in the rate of thrombosis.

Corticosteroids

The use of corticosteroids in treating chronic hemolysis is debated because of the empiric nature of therapy and no experimental data to support the explanation in ameliorating complement mediated hemolysis. It may have a role in attenuating acute hemolytic exacerbation given in a dose of 0.25 to 1 mg/kg of prednisolone. A rapid response (within 24 hours) suggests complement inhibition either by directly inhibiting alternate pathway or dampening the inflammation.

Androgens

The mechanism of the amelioration of anemia in PNH is thought to be complement inhibition and stimulating erythropoiesis. Danazol is usually used in these cases. A starting dose of 400 mg twice a day is recommended, but a lower dose (200–400 mg/d) may be adequate to control chronic hemolysis. Benefit of danazol was attributed to reduced hemolysis rather than enhanced erythropoesis. Lack of venous thrombotic complications is an advantage of danazol over other androgens. Danazol has been shown to increase fibrinolytic activity thus offering protection against thromboembolism.

Folate

Supplemental folate (5 mg/d) is recommended in all patients to compensate for increased utilization associated with heightened erythropoiesis that is a consequence of hemolysis.

Chronic Transfusion Therapy

Transfused red cells are CD55 and CD59 positive and thus have normal survival. It also suppresses erythropoesis in addition to increasing hemoglobin. Iatrogenic hemochromatosis is delayed in PNH patients due to iron loss.

Iron Replacement Therapy

Patients of PNH often become iron deficient as a result of hemoglobinuria and hemosiderinuria. Iron replacement, both oral and parenteral causes exacerbation of hemolysis.

However, iron therapy should not be withheld because of this and the exacerbation should be treated with steroids, androgens and blood transfusion.

Splenectomy

There are only anecdotal reports of amelioration of hemolysis and improvement of cytopenias by splenectomy, but there is an increased risk of postoperative thrombosis; therefore it is not recommended in the treatment of PNH.

Treatment of Nonhemolytic Anemia

Pancytopenia with low reticulocyte count indicates anemia due to bone marrow failure. Thus treatment should aim at the underlying disease (Aplastic anemia, myelodysplastic syndrome). Hence, when immunosuppressive therapy is used in a patient of PNH, therapy is aimed at the treatment of bone marrow failure. Androgens may also be beneficial in patients with PNH who have a hypoproliferative component to their anemia.

Thrombosis: Thrombosis is the leading cause of mortality in patients of PNH. The occurrence of thrombosis has been related to the size of the granulocytic clone.

Prophylaxis: Prophylactic anticoagulation in PNH patients is a matter of debate in the International PNH Interest Group and not yet a standard recommendation.

Management of Thromboembolic Disease

Patients of PNH usually present with venous thrombosis. Any acute thromboembolic event requires anticoagulation with heparin.

Patients presenting with acute Budd-Chiari syndrome should be managed with thrombolytic therapy and/or radiological intervention.

Thrombocytopenia should not come in way of anticoagulant treatment. It is a relative but not an absolute contraindication for anticoagulation. It can be managed with repeated platelet transfusions. However this may not always be possible.

Patients with a thrombotic episode require life-long anticoagulation. Long-term anticoagulation should be reassessed in any patient who undergoes a spontaneous remission or in whom the PNH clone size falls to below 50 percent.

Stem cell transplantation is indicated in recurrent life threatening thrombotic complications.

Definitive Treatment of PNH

The definitive treatment of PNH includes stem cell transplantation and gene therapy.

Stem Cell Transplant for PNH

This is the only definite treatment for PNH. The overall survival for PNH patients who undergo transplantation using an HLA-matched sibling donor is in the range of 50 percent to 60 percent.

Indications for consideration of transplantation: International PNH Interest Group recommendations:

- Bone marrow failure
  Decision on transplantation is based on underlying marrow abnormality (e.g. aplastic anemia)
- Major complications of PNH
  - Recurrent, life-threatening thromboembolic disease
  - Refractory, transfusion-dependent hemolytic anemia. The availability of new treatment options (e.g. eculizumab) may influence the decision to recommend transplantation.

PEDIATRIC PNH

PNH can occur in the young (about 10% of patients are younger than 21) but is often misdiagnosed and mismanaged. A retrospective analysis of 26 cases underscored the many similarities between childhood and adult PNH. Signs and symptoms of hemolysis, bone marrow failure, and thrombosis dominate the clinical picture, although hemoglobinuria may be less common in young patients. A generally good response to immunosuppressive therapy may be seen. However, based on the lack of spontaneous remissions and poor long-term survival (80% at 5 years, 60% at 10 years, and only 28% at 20 years), sibling-matched stem cell transplantation is the recommended treatment of childhood PNH. A recent Dutch study confirmed the common presentation of bone marrow failure in 11 children with PNH, and reported that 5 patients eventually underwent bone marrow transplantation (BMT; 3 matched unrelated donors and 2 matched family donors), of whom 4 are alive. Mortality appears high in young patients with PNH treated with transplantation using unrelated donors although surviving cases have been reported.

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CHAPTER 24

Diagnosis and Management of Acquired Aplastic Anemia in Children

Nitin K Shah

Aplastic anemia (AA) is defined as pancytopenia caused by bone marrow failure with bone marrow hypocellularity without infiltration or fibrosis.[1,2] It is a difficult proposition to treat in resource crunched set-up lie in developing countries and carries high mortality. It accounts for 20 to 30 percent cases presenting with pancytopenia[2,3] and pediatric AA accounts for nearly 16 percent of all AA.[4] In USA and Europe, incidence of childhood AA is estimated at 2 to 6/million.[1] Incidence in India is not known exactly but is perceived as higher than in the West by Indian pediatric hematologists. A recent study from Canada has reported increased incidence in children of east and south-east Asian descent (7.3/million/year) compared to white or mixed ethnic groups (1.7/million/year).[5] In Western countries, AA has been reported with equal frequency in boys and girls. However, in Indian studies AA is reported to occur three to four times more frequently in boys compared to girls.[6,7] It can be inherited (Inherited bone marrow failure syndromes like Fanconi's anemia) or acquired which may be then primary or idiopathic; or secondary to some insults like drugs, etc. It is also classified as mild to moderate, severe and very severe depending on the severity of pancytopenia. In large series, nearly 70 percent cases have been reported to be severe/very severe and almost 70 to 80 percent of acquired aplastic anemia cases to be idiopathic.[4,8]

DIAGNOSIS OF APLASTIC ANEMIA IN CHILDREN

As in any other disease diagnosis involves detailed clinical history, head to toe clinical examination and laboratory investigations; and in that order!

Clinical Presentations

Clinical presentation will include effects of depressions of all three peripheral blood cell lines, viz anemia, neutropenia and thrombocytopenia; and effects of the cause of AA.

- It will include effects due to anemia like pallor, easy fatigability, tiredness, headache, breathlessness, puffiness of face and edema of the feet, tachycardia, tachypnea, frank cardiac failure.
- Effects due to neutropenia like fever, sepsis, oral ulcers.
- Effects due to thrombocytopenia like petichea, purpura, ecchymosis, mucosal bleeds like epistaxis, gum bleeds, gastrointestinal (GI) hemorrrhage, hematuria, intracranial bleeds, etc. One should carefully look for one of the secondary causes in any case of AA as shown in Table 1.

  One should also look for findings suggestive of inherited bone marrow failure syndromes like skeletal anomalies, mental retardation, short stature, peri-oral hyperpigmentation, nail dystrophy, renal anomalies on ultrasound and a strong family history like consanguinity, other sibs affected in family and cousins and some family members having some of the anomalies without frank AA.
- One should also look for findings like presence of lymphadenopathy, hepatosplenomegaly, bone pains and weight loss which may suggest pancytopenia due to other causes like leukemia, lymphomas, myelodysplastic syndromes, myelofibrosis, megaloblastic anemia, osteopetrosis, etc.

Laboratory Investigations

Basic tests done in a case of suspected AA include complete blood count (CBC), PS examination and corrected reticulocyte count. CC will show different

Table 1 Classification of aplastic anemia in children

- *Acquired:*
  - Viruses
    - i. EBV
    - ii. Hepatitis
    - iii. HIV
  - Immune diseases
  - Eosinophilic fascitis
  - Hypoimmunoglobulinemia
  - Thymoma
  - Pregnancy
  - Paroxysmal nocturnal hemoglobinuria (PNH)
- *Inherited:*
  - Fanconi anemia
  - Dyskeratosis congenital
  - Shwachman-Diamond syndrome
  - Reticular dysgenesis
  - Amegakaryocytic thrombocytopenia
  - Familial aplastic anemia
  - Nonhematological syndromes
  - Down syndrome
  - Dubowitz syndrome
  - Seckel syndrome

severity of pancytopenia with anemia, neutropenia and thrombocytopenia.

- Corrected retic count will be lower than 1 percent. Mean corpuscular volume (MCV) is usually high with normal differentiating it from megaloblastic anemia where MCV is high but red cell distribution width (RDW) is also high.

  The PS will confirm macrocytosis without anisocytosis, neutropenia and thrombocytopenia. Presence of some changes would suggest other diagnoses like blasts (leukemia), leukcoerythroblastic changes (osteopetrosis), etc.
- *Bone marrow aspiration and bone marrow trephine biopsy:* Diagnosis is confirmed by bone marrow aspiration as well as bone marrow trephine. On aspirate smears, one will see hypocellularity with increased fat spaces, however, this could also be due to technically dilute marrow or a hypocellular myelodysplastic syndrome (MDS). On the other hand, a hypercellular marrow does not necessarily rule out aplasia on bone marrow aspirate alone as it could have hit one of the few persisting cellular foci. Hence, a trephine biopsy, which gives a wider area to look at, is mandatory for diagnosis of aplastic anemia. A hypocellular MDS should be kept in mind, even if hypocellular marrow is found on trephine biopsy, if there are dysplastic changes present. In such cases a repeat bone marrow test is required after 2 to 3 weeks to see for evolution of changes of hypocellular MDS. When hypocellular MDS is suspected, heparinized bone marrow should be also sent for cytogenetic study.
- Lastly, one should also do stress cytogenetic study to rule out Fanconi's anemia in all cases of pediatric AA and irrespective of presence of physical anomalies as some cases of Fanconi's anemia may be phenotypically absolutely normal. It is important to rule out Fanconi's anemia in all cases of pediatric AA as the treatment for Fanconi's anemia is totally different and immunotherapy has no role in these patients. Stress cytogenetic study can be done on peripheral blood lymphoctyes. Recently, even genetic study for Fanconi's anemia is available in some centers which is useful to confirm Fanconi's anemia in rare cases where there is strong suspicion clinically but repeated stress cytogenetic studies are inconclusive.
- *Other tests that are required include:*
  - Liver function tests as a baseline before starting androgenic steroids.
  - Renal function tests before starting cyclosporine.
  - Serum lactate dehydrogenase (LDH) and uric acid which, if high may suggest leukemia as diagnosis.
  - HbF levels which, if high may suggest Fanconi's anemia.
  - One should also look for secondary causes like HIV/HCV/HBsAg/Parvovirus as aplastic anemia can follow one of these viral infections.
  - PNH study by flow cytometry for CD 55 and CD 59 cells or HAMs test or sucrose lysis test, if flow cytrometry is not available.

RISK STRATIFICATION OF APLASTIC ANEMIA IN CHILDREN

International Aplastic Anemia Study Group takes in to consideration three peripheral blood criteria, i.e. absolute neutrophil count (ANC) below 0.5×10^9/L, platelet count below 20×10^9/L and corrected retic count below 1 percent (or absolute retic count below 20×10^9 /L); and two bone marrow criteria, i.e. bone marrow biopsy showing severe hypocellularity < 25 percent; or moderate hypocellularity (25–50%) with hematopoietic cells representing less than 30 percent of residual cells.[9] Based on these AA is classified as:

- Severe aplastic anemia (SAA) when any 2 of the 3 peripheral blood criteria and either marrow criterion are present.
- If ANC is below 0.2×10^9/L, then it is labeled as very severe aplastic anemia (vSAA).
- All other cases are classified as mild-moderate AA.

It is important to classify AA as inherited vs. acquired and acquired AA as severe, very severe or mild-moderate

as the outcome and treatment needed are different for different severity. Most inherited causes need stem cell transplant (except TAR which normally resolves by 1 year and Diamond-Blackfan syndrome which has 25 percent chances of spontaneous regression) whereas many mild-moderate and some severe AA may resolve with immunosuppressive therapy or sometimes even with supportive care alone; and many severe and all very severe AA will need stem cell transplant.[10,11]

The number of days taken for ANC to improve with G-CSF and non-response to any specific therapy at 6 months have also been proven to be risk factors in one study.[12] Certain cytogenetic changes at diagnosis like presence of telomerase gene mutations leading to short telomeres make the prognosis unfavorable.[14] Presence of cytogenetic abnormalities after diagnosis also alter prognosis like trisomy 8, chromosome 13 abnormalities, chromosome Y deletion, etc. suggest favorable outcome, whereas monosomy 7 and complex abnormalities depict an unfavorable outcome.[13]

Supportive Care

Supportive care is the main stay besides definitive therapy, and for many ill affording patients it is the only treatment available. It includes use of blood components to control anemia and thrombocytopenia, management of infections, psychosocial support and financial help.

Transfusion Support

- *Packed red blood cell:* Target is to keep hemoglobin in near physiological range of about > 9 gm% so as to improve quality of life. One should do complete antigen phenotype before first transfusion so as to use a particular rare blood group donor should there be sensitization after multiple transfusions. It is mandatory to use leukodepletion by using white blood cell (WBC) filters with each transfusion so as to avoid HLA sensitization as well as prevent febrile transfusion reactions. It is preferable to use irradiated blood products to prevent transfusion associated graft versus host disease, especially in post-transplant period. One should also always avoid relative donors for any transfusion, especially if the patient is for a potential transplant candidate. Cytomegalovirus (CMV) negative donor is desirable but not practicable as most of the donors in India are CME positive. In presence of bleeding and infection, packed red blood cells (PRBCs) are used more liberally.
- *Platelets:* Platelet transfusion is reserved for patient with mucosal bleeds. It is not required for only skin bleeds, howsoever grotesque they may look. It is also not required prophylactically unless platelets counts are less than 500 to 10,000/cumm in a non-sick child, <20,000/cumm in a sick febrile child or patient is undergoing some surgical procedure as shown in Table 2. Platelets may be liberally used in presence of sepsis and DIC with platelets < 20,000/cumm as there is a lot of consumption of platelets in these conditions. For minor mucosal bleeds (except hematuria) one can use platelets sparing drugs like tranexamic acid in the dose of 75 mg/kg/day in 3 divided doses orally. Similarly, menorrhagia may be a problem in adolescent girl who can benefit with hormonal replacement therapy.[14,15]

Table 2 Indications of using prophylactic platelets in AA

Prophylactic platelets (without bleeding)
• < 500–10000/cumm in a non-sick child
• *< 20000/cumm in a sick child with:*
– Severe mucositis
– DIC
– Platelet likely to fall < 10,000/cumm before next evaluation
– Associated coagulopathy/anticoagulation
• Before surgery
– Bone marrow aspiration/biopsy can be without platelet support
– Lumbar puncture < 30,000/cumm
– Other surgeries < 50,000/cumm
– Surgery at critical sites like CNS, eyes < 100,000/cumm
• < 50,000/cumm with acute bleeding, massive hemorrhage, head trauma, multiple trauma

Care of Infections

Infections are the most common problems and cause of death in untreated severe and very severe AA patients, and are difficult to eradicate in spite of effective antimicrobials. Infections are also related to use of immune suppressive therapy as well as in post-transplant period. Bacterial infections are the most common infections in AA patients followed by fungal, parasitic and of course viral infections which are otherwise also so common in children in general. Gram negative sepsis is more common than gram positive sepsis in India. Recently several hospitals are facing extended spectrum beta lactamase (ESBL) producing gram-negative organisms as well as methicillin resistant *Staphylococcus aureus* sepsis in neutropenic patients. Partly, this is because of haphazard use of antimicrobials in general. Initial choice of antimicrobials in a patient with suspected sepsis in AA patients will depend on the recent local experience with the type of microorganisms grown and treatment failure with a particular antimicrobial. Initially, broadspectrum antimicrobials that will cover

gram negative as well as gram positive organisms will be 1st line choice like third generation cephalosporins like ceftriaxone plus aminoglycoside like amikacin. In case, ESBL producing organisms are common in a local set-up, one may have to start cefaperzone-sulbactum or piperacillin-tazobactum plus amikacin as the 1st line therapy. Drugs will be the changes based on cultures and antimicrobial sensitivity. However if there is no growth and patient is not responding, one will have to add vancomycine to cover for MRSA on day 3 to 5. If patient still fails to respond or deteriorates, one may have to switch to carbapenums like meropenum with vancomycine. Lastly, in desperate ELBS producers which are carbapenum resistant one will have to add IV colistin. Anti-fungals will be added either empirically on day 5 to 7 when patient does not respond to second line antibiotics or if culture grows a specific fungus.[16] Recently several hospitals are faced with problems of fluconazole resistant candida species sepsis or increasing trends of infection with *Aspergillus*, especially if some construction work is going on in near vicinity.[17] There is no role of prophylactic antimicrobials or antifungals as it will only add to the problem of drug resistance. Lastly unlike in patients with leukemia, there is no role of giving *Pneumocystis carinii* pneumonia (PCP) prophylaxis to AA patients, and sulfa drugs will be contraindicated in patients with AA.

Prevention of Infections

Various measures are required to prevent infections which include chlorhexidine mouth washes after every major meals, chlorhexidine bath, betadine application to groins and axillae after bath, sterile or well-cooked diet, avoidance of contaminated, uncooked and open or overnight left over food, hand sanitization by care taker before handling patients, cleaning well fruits and eating only well preserved or fully skin covered fruits after peeling, avoiding going to crowds, etc.

General Measures

- No drugs should be administered per rectally due to fear of infection and bleeding. Avoid contact games and injuries
- Avoid altogether brushing or brush with soft tooth brush, especially if patient is thrombocytopenic.
- Psychological support to the patient and family members is of utmost importance like in any other chronic life-threatening illnesses stressing the chronic nature of disease and slow response to treatment.[14,15]
- All vaccinations should be avoided during the active disease as live vaccines can lead to vaccine induced infection and killed may not be efficacious. OPV should be avoided even in siblings and inactivated poliovirus vaccine (IPV) could be used in to stead. Live vaccines are especially contraindicated in transplant patients.
- Intramuscular injections are contraindicated in patients with severe thrombocytopenia due to fear of bleeding.
- IV access may be difficult in patients due repeated infections and use of peripheral lines, hence central lines or a port may be a better option especially for patients on immune suppressive therapy or transplant patients as they will need prolonged supportive care.

SPECIFIC THERAPY IN APLASTIC ANEMIA

Options available: There are several options available to offer a cure to patients with AA. Several mild-moderately severe AA patients may need supportive care alone or with immune suppressive therapy (IST). Severe and very severe AA patients will need either immune suppressive therapy or stem cell transplant (SCT). Many patients in India cannot afford IST or SCT and would receive only supportive care that too haphazardly leading to high mortality. Such patients are often treated with corticosteroids, androgenic steroids alone. There are also anecdotal reports of use of danazol, cyclophosphamide, splenectomy, etc. which are sort of given up now with the advent effective IST and SCT.

Immunosuppressive Therapy for Aplastic Anemia

Immunosuppressive therapy (IST) currently comprises use of anti-thymocyte globulin (ATG) and cyclosporine (CsA) as standard first line therapy along with short course low dose steroids. Clinical experience and laboratory data suggests that the mechanism leading to bone marrow failure is probably secondary to activated cytotoxic lymphocytes, which produce T-helper type 1 (Th1) cytokines including interleukin-2, interferon gamma and tumor necrosis factor. These cytokines in turn induce apoptosis of the hematopoietic stem cells. Immuno-suppressive agents exert their action by inhibiting T cell activation and dose dependent lympholytic activity as well as stimulation of hematopoietic growth factors.

Indications

Children tolerate hematopoietic stem cell transplantation (HSCT) exceedingly well with excellent outcome making it as the modality of choice for all severe and very severe aplastic anemia cases. However, a large chunk of children with SAA/vSAA in developing countries cannot afford HSCT or do not have a matched donor ; for them IST is the only alternative. For non-severe aplastic anemia cases IST

is the first modality of choice as it is associated with less risk than HSCT.

Eligibility

All patients should have a stress cytogenetics test done to rule out Fanconi anemia as IST is of no use in patients with Fanconi anemia. Patient should be relatively well and free of serious infections. Central venous access is required as ATG can cause peripheral venous sclerosis. Platelet and packed red cell transfusion should be given to keep platelets above 20,000/cumm and Hb above 7 g/dL before and during ATG course. Post-ATG transfuse packed red cells to maintain Hb >7 g/dL. It is desirable to use leukodepleted blood products. Even use of irradiated blood products is desirable post IST as there is profound immune suppression following IST. Immunosuppressive therapy should be instituted with facilities for resuscitation and intensive life support under care of a qualified medical team familiar with this treatment.

ATG/ALG

- Horse and Rabbit ATG is available in India containing 100 to 250 mg in 5 mL vial. ALG is used in the dose of 40 mg/kg/day for 5 to 10 days, and ATG is used in the dose of 15 mg/kg/day for 5 to 10 days, however one should follow manufacturer's instructions. Chances of serum sickness go up when given for more than 7 to 10 days. Lyophilized powder is reconstituted in normal saline shaking it vigorously, avoiding plastic bottles as ATG tends to stick to the sides, looking for visible contamination, discoloration or precipitation, and given over 8 hours under close monitoring after obtaining informed written consent. Reconstituted solution should be used as early as possible. Premedication in form of paracetamol, chlorpheniramine and hydrocortisone or dexamethasone is given prior to starting ATG/ALG infusion daily to prevent allergic reactions. First vial should be administered slowly. Look for toxicities like allergic reactions, anaphylaxis, urticaria, etc.
- Abandon ATG/ALG infusion if patient develops anaphylaxis as suggested by development of reactions like hypotension, dyspnea, poor peripheral circulation, etc. and immediately start standard measures of resuscitation.[1,18]
- Patient may develop serum sickness by 10 to 14 days after ATG infusion presenting with fever, rash, arthralgia and arthritis. Hence observe the patient in hospital for 2 weeks after ATG course for the same. Steroids in form of prednisolone in dose of 1 mg/kg/day in 3 divided doses should be given along with ATG infusion and continued for 2 weeks and then tapered over next one week to prevent serum sickness. One has to use IV hydrocortisone or dexamethasone to treat serum sickness should it occur and then switched to oral course thereafter. Requirements of PRBc, platelets may go up after IST course and even infections may increase post-IST.

Cyclosporine (CsA)

Oral cyclosporine is given in the dose of 5 to 10 mg/kg per day in 2 divided doses and the dose is adjusted by keeping trough levels at 100 to 150 ng/mL by testing the levels 15 days after starting the dose and any increments done thereafter. CsA is usually started on day 21 after stopping prednisolone, as combined administration often leads to hypertension. Common side effects of CsA include hirsuitism and gum hypertrophy. Toxicities include hypertension, liver function abnormality, and renal toxicity; hence monitor liver function tests and renal functions, and blood pressure weekly. CsA is continued for a minimum 3 to 6 months before any response can be seen. The oral liquid is highly concentrated and comes as 100 mg/mL. Hence a small child may need fraction of an milliliter as the dose and patient should be taught how to measure tiny doses using 1 mL syringe.

Response to IST (ATG Plus CsA)

It takes 3 to 6 months for response to be seen, hence, one should not stop therapy before 6 months. Patient is assessed after 3 to 4 months and periodically thereafter for response as well as toxicities.

Response is defined as complete when patient maintains hemoglobin levels normal for age, ANC above 1500/cumm and platelets > 150,000/cumm on two occasions 1 month apart without any transfusion support. In case patient where the counts improve but is still transfusion dependent to maintain normal counts it is taken as partial response.

If patient satisfies all criteria of AA and is completely transfusion dependent for PRBCs and platelets it is taken as failure of first course of IST in which case either HSCT should be considered or if that is not feasible, a second course of IST can be given using a different brand of ATG/ALG than what was used in the first course. When giving second course patient should be closely monitored for anaphylaxis as there might have occurred sensitization with first course. Chances of response to first course are 40 to 50 percent and that with a second course is around 60 percent in moderate cases.

Other Drugs

Various drugs like corticosteroids including high dose methylprednisolone, androgenic steroids, cyclophosphamide, danazol, stanozolol, etc. have been in past with anecdotal response.

Nandrolone enanthate is used in a dose of 2 to 5 mg/kg/day of injectable form once in 10 days and continued till response is evident. Efficacy is doubtful and the outcome may be in fact adverse due to side effects like masculanization, stunted growth, hepatotoxicity, Ca liver, etc. Though these are currently not the ideal choice of treatment, they can be tried in those children with AA who cannot afford HSCT or IST.

Corticosteroid stimulates erythropoiesis. It also stabilizes capillary membrane and decreases bleeding. They are useful to counteract side effects of androgenic steroids on growing epiphysis and the serum sickness of immunotherapy. Oral prednisolone is used in the dose of 0.5 to 1 mg/kg/day and tapered to a minimum effective dose. High dose IV methylprednisolone was popular in Europe in past and probably effective in patients treated within few weeks of diagnosis, this therapy is reserved for occasional patients due to tremendous toxicity. It is used in the dose of 15 to 20 mg/kg/day for 5 days followed by tapering doses over next 15 days. Side effects include hypertension, fluid electrolyte imbalance, infections, suppression of neuroendocrinal axis, psychosis, avascular necrosis of head of femur, etc.[19]

Danazol 100 mg BD/TDS for girls, stanozolol 10 mg BD for boys have shown some response in children with non-severe AA.

HEMATOPOIETIC STEM CELL TRANSPLANTATION IN APLASTIC ANEMIA

In young patients hematopoietic stem cell transplantation (HCST) is the best modality of therapy as it is quite safe at that age, has almost 80 to 90 percent cure rates and except for initial period of profound neutropenia related infections, and graft-versus-host disease (GVHD), there are not many long term complications.[7] Most of the patients are free from transfusion support and medications within 1 year or so. This is in contrast to IST which has lesser cure rates, more toxicities, slower response, need for prolonged support with blood products and quite a cost.[20] However HSCT needs a HLA matched donor and less than 40 percent of the patients will be lucky to find such a matched sibling donor from the family. This is a big problem now with smaller family size. Unrelated matched donor is difficult to get in India and is prohibitively expensive to get from the Western registry. Besides, HSCT itself is quite expensive and not many centers offer such a therapy at an affordable price in public health set-up. As such very few centers have invested for HSCT even in private set-up and hence the patient has to travel long distance to get HSCT done outside major metro cities. In elderly patients HSCT has its own complications rates especially due to GVHD and success rates are less with higher mortality making IST as the first choice especially for > 40 years of age.[21]

Ideal is HLA matched sibling or family donor. Partial matched family donor or unrelated matched donor are less desirable as complications like GVHD and graft rejection are higher in this settings. Donor need not be ABO compatible, but should be healthy. Best experience is with bone marrow or peripheral blood stem cells as the source of HSCT. Umbilical cord blood stem cells are nor preferred. For peripheral blood stem cells donor is given G-CSF for 4 to 5 days and then subjected to apheresis to collect enough stem cells from peripheral blood.

Most protocols would use non-myeloablative conditioning based on combination of ATG, cyclophosphamide and fludarabine.[22-25] Engraftment would occur normally in 10 to 15 days and till then patient will need effective supportive care. All aseptic precautions should be taken to avoid infections during the critical neutropenic period that includes chlorhexidine bath, chlorhexidine mouth washes, sterile drinking water, sterile diet or well cooked food, avoiding of crowds, sterile cloths or at least clean ironed cloths. And hand sanitizer for the contacts. Only irradiated blood products should be used to avoid transfusion associated GVHD which has very high fatality should it occur. Once engraftment is well established and patient has no infection, patient can go home and follow-up on outdoor basis for further management and usually is kept under follow-up for 3 to 6 months.

COMPLICATIONS AFTER SUCCESSFUL ENGRAFTMENT

- Graft-versus-host disease (GVHD) and rarely graft rejection are two main complications of HSCT.[22] Acute GVHD can occur up to 100 days post-transplant and will characterize with skin rash, diarrhea and liver disease with jaundice and sometimes fever. Treatment includes immune suppression with steroids and cyclosporine. Chronic GVHD develops after 100 days and is characterized by scleroderma with skin rash, sicca complex, hepatic dysfunction, and sclerosing bronchiolitis. Management includes again immunosuppressive agents. Most patients develop tolerance after 1 year or two and are able to stop immune suppression thereafter. Other complications include viral infections like CMV, EBV, HSV; fungal infections, bacterial infections with encapsulated organisms, etc.

- *Immunization:* Revaccination with routine childhood vaccines against polio, diphtheria, tetanus, pertussis, Hib, and pneumococcal vaccines is carried out at 1 year after successful HSCT and when the patient is off all immune suppression. Typically 2 doses of Tdap, IPV, Hib, hepatitis B, and PCV13 are given at one month interval followed by 3rd dose after 6 months. These children also receive age appropriate doses of inactivated influenza vaccine which is then continued annually. In India they will also receive hepatitis A 2 doses at 6 months interval and Vi typhoid vaccine one dose to be repeated every 3 to 5 years. Live vaccines like MMR and Varicella are given as 2 doses 8 to 12 weeks apart starting at 2 years after HSCT.

ROLE OF PEDIATRICIAN IN SHARED CARE

Before HSCT primary pediatrician should encourage the patient to take proper treatment, treat infections vigorously, discuss options of IST and HSCT with parents, refer the patient to appropriate referral center for further management, avoid unnecessary transfusions, and avoid any transfusion from family members. After discharge primary pediatrician can continue to follow-up patient for minor ailments and carry out immunization as advised.

FOLLOW-UP AND PROGNOSIS

Patient, if followed clinically for regression or reappearance of symptoms that may suggest response or graft failure like pallor, bleeding, fever. They are followed for complication like skin rash, diarrhea, jaundice, skin changes, dryness of mucosal surfaces which may suggest acute or chronic GVHD. If patient is on immune suppression like steroids or cyclosporine, patient is monitored for side effects like hypertension, renal toxicities, hyperglycemia, etc. In a sick child, follow-up is on a daily basis and in a well child, it could be once in 2 to 3 weeks. Laboratory tests are also done periodically including CBC, LFT, RFT depending presence of complications. CBC are usually done weekly initially and then monthly once patient shows successful engraftment.[26,27]

LONG-TERM COMPLICATIONS

Patient may develop long-term complications like relapse of disease, leukemia, PNH, etc. Check bone marrow aspiration and bone marrow biopsy are done after 6 to 12 months.

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CHAPTER 25

Inherited Bone Marrow Failure Syndromes

Revathi Raj

Inherited bone marrow failure syndromes (IBMFSs) are rare genetic disorders characterized by defective production of red cells, white cells and platelets. This results in a single cell line failure or pancytopenia depending on the gene mutation inherited.

Based on the cell lines affected IBMFS can be classified as Table 1.

Table 1 Classification of inherited bone marrow failure syndrome

Disorder	*Cell line affected*	*Gene mutation*	*Mode of inheritance*	*Chromosome affected*
Fanconi anemia	Pancytopenia	FANC A to G	Autosomal recessive	Several 16, 9,13,3,6,11
Dyskeratosis congenita	Pancytopenia	DKC1/TERC	X linked or autosomal recessive	Xq28, 3 q26
Pearson syndrome	Pancytopenia	Mitochondrial DNA	Maternal	Maternal
Reticular dysgenesis	Pancytopenia	Unknown	Unknown	Unknown
Congenital amegakaryocytic thrombocytopenia	Pancytopenia	cmpl	Autosomal recessive	1p34
Diamond-Blackfan anemia	Anemia	RPS19	Autosomal dominant	19q
Congenital dyserythropoietic anemia	Anemia	CDAN1	Autosomal recessive	15q, 20q
Congenital sideroblastic anemia	Anemia	ALAS2	X-linked recessive	Xp11
Kostmann syndrome	Neutropenia	ELA2	Autosomal dominant	19p
Shwachman-Diamond syndrome	Neutropenia	SBDS	Autosomal recessive	7q11
Thrombocytopenia absent radii	Thrombocytopenia	Unknown	Autosomal recessive	Unknown

Fanconi Anemia

Children with Fanconi anemia (FA) present with distinct dysmorphic features and can be diagnosed even at birth before the onset of cytopenias. REFAIN—Registry for Fanconi anemia in India has followed up over 150 children with FA over 15 years. The main somatic features noticed in the Indian FA registry are growth retardation, hyperpigmentation, microphthalmia, *café-au-lait* spots, renal anomalies like horse shoe kidney, cardiac anomalies like atrial or ventricular septal defects, cryptorchidism and radial ray defect with hypoplastic thumb. Hypopigmented spots appeared in the palms with the onset of pancytopenia.

Fanconi anemia results from multiple defects in FANC proteins and the incidence is high in South India due

to high consanguinity. FANC proteins help in monoubiquitination that helps DNA repair. Mutations in FANC proteins thus result in defective DNA repair and increased chromosomal fragility which is the hallmark of FA. Gene complementation studies have helped in antenatal diagnosis and prevention of new births and this is possible in our country through the FA registry.

The children present with cytopenia from the age of 3 years. Peripheral blood testing to assess chromosome breakage with mitomycin C or diepoxybutane helps confirm the diagnosis. Bone marrow aspiration and karyotyping are required as the presence of an abnormal clone such as monosomy 7 heralds the onset of acute myeloid leukemia. The children initially respond to androgenic steroids such as stanzolol or oxymetholone. Careful monitoring of liver enzymes is required during drug therapy.

Hematopoietic stem cell transplantation offers the sole chance of cure. Outcomes are better when children are referred early as multiple transfusions increase the chance of graft rejection and graft-versus-host disease. Transplantation is done using low dose conditioning as the children are extremely sensitive to chemotherapy. Overall survival after transplantation for FA approaches 90 percent survival with sibling donors and 60 percent with matched unrelated cord blood transplantation. All children need long-term surveillance for malignancies, especially head and neck cancers.

Dyskeratosis Congenita

Dyskeratosis congenita can present at any age from preschool to late thirties with pancytopenia. There are characteristic nail changes with dystrophy and a bald tongue with skin hyperpigmentation especially around the neck. These children are particularly prone to pulmonary fibrosis and transplantation carries a higher risk of mortality due to lung complications. Mutations in the DKC gene or TERC genes are pathognomic of this condition. Cancer predisposition is high as in other marrow failure syndromes.

Pearson Syndrome

Pearson syndrome is a mitochondrial cytopathy that causes failure to thrive, pancreatic insufficiency and pancytopenia. Bone marrow aspiration shows characteristic changes with vacuolation in the marrow precursor cells. Death in infancy results from infections and the role of transplantation is not clearly defined.

Reticular Dysgenesis

This is a severe defect in the lymphohematopoietic system with features of severe combined immune deficiency and marrow failure.

Congenital Amegakaryocytic Thrombocytopenia (CAMT)

Defects in thrombopoiesis ultimately result in pancytopenia due to marrow failure. Children can also present with developmental delay and cardiac defects such as ASD/VSD. Treatment is with hematopoietic stem cell transplantation although rejection rates are high. Cancer predisposition is also noted in CAMT.

Diamond-Blackfan Anemia

Pure red cell aplasia can present at birth or later in life. The children show dysmorphic features such as craniofacial anomalies and thumb anomalies often in association with deafness and growth retardation. The majority of children respond to prednisolone starting at 2 mg/kg/day and then tapered over 8 to 12 weeks. Steroid dose is kept at a minimum required to sustain hemoglobin levels. Prednisolone dependent or refractory children need to be treated as per guidelines for thalassemia major with transfusion and oral chelation. Transplantation helps achieve cure and children need long-term follow-up for screening for malignancies.

Kostmann Syndrome

Severe mutations in the ELA2 gene causes Kostmann syndrome whilst milder mutations result in cyclical neutropenia. Management consists of aggressive treatment of infections and GCSF at a dose between 5 and 20 mcg/kg/day or more is needed to keep the neutrophil count above 500. Clonal evolution and transformation to myelodysplasia or acute myeloid leukemia is known to occur after the second decade.

Thrombocytopenia Absent Radii (TAR)

Most children show spontaneous regression of thrombocytopenia following the first birthday. Orthopedic procedures to correct hand anomalies are best postponed till there is platelet recovery above 75,000. Children with TAR have the best outcome of all the inherited marrow failure syndromes.

Key Points in Management of Children with Cytopenias

- Always suspect and screen for inherited marrow failure syndrome even when single cell line is involved as the disease may progress to pancytopenia
- Avoid marrow suppressive drugs for treatment of infections
- Preserve DNA for future counseling and guiding families through subsequent pregnancy

- Early referral to a transplant center results in improved outcomes
- Careful screening of sibling donors prior to transplantation is important as they may be affected asymptomatic individuals
- There is an increased risk of malignancies in all forms of IBMFS and cancer surveillance is an important aspect of follow-up
- Test for Fanconi anemia before ATG is given as part of immunosuppressive therapy for aplastic anemia.

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CHAPTER 26

Benign Disorders of Neutrophils

Bharat R Agarwal

This chapter describes benign disorders of neutrophils (Malignant disorders are described elsewhere). Disorders of neutrophils can be quantitative (Neutropenia and neutrophilia) or qualitative (Cytoplasmic or nuclear abnormalities). All these various conditions are considered in the following sections as follows:

- Neutrophilia
- Neutropenia
- Neutrophils: cytoplasmic anomalies
- Neutrophils: nuclear anomalies.

NEUTROPHILIA

Except for the first weeks of life, the upper limit of normal is approximately 7.5×10^9/L. Rarely, artifactually high counts may be obtained by automated particle counters:

- Precipitation of cryoprotein on cooling. Error revealed by examination of stained film. Examine fluid preparation by subdued light or phase contrast for crystals.
- Incomplete lysis of erythrocytes. Examination of stained film will reveal error.

Neutrophilia (Table 1) has little value in specific diagnosis, with some exceptions considered below.

Infection

As a general rule, neutrophilia is more likely in bacterial than in viral infection. However, neutrophilia is not infrequent in the early stages of viral infection and neutropenia may occur in severe bacterial infection. The neutrophil count (and other 'routine' characteristic) is of value in assessing the likelihood of appendicitis in children admitted to surgical wards with abdominal pain (Table 2). All characteristics examined in this study were useful in discrimination, i.e. they were reasonably common in, and reasonably specific for, appendicitis. The most useful and easily obtained is the neutrophil count – a value of 15×10^9/L is a useful cut-off. The difference in frequency of atypical ('viral') lymphocytes is an indication of the high frequency of viral infection in non-surgical abdominal pain.

Substantial neutrophilia ($< 50 \times 10^9$/L) is not infrequent in bacterial infection, especially bacterial pneumonia, empyema, bacterial meningitis, septicemia, urinary tract infection and bacterial endocarditis, and is characteristic of the rare genetic deficiency of integrin adhesion molecules on neutrophils and lymphocytes (delayed separation of umbilical cord, severe bacterial infections, periodontitis, gingivitis, poor pus formation).

Sweet's Syndrome (Acute Febrile Neutrophilic Dermatosis)

A rare syndrome of unknown cause, characterized by high neutrophilia, raised, tender, erythematous plaques and bullae in the skin, spiking fevers and often arthralgia, myalgia and headache. It may occur without underlying disease or in association with leukemia, usually myeloid (in remission and not in a neutropenic phase). Diagnosis requires skin biopsy (dermal infiltration with mature neutrophils, edema, vesiculation). Without treatment, resolves spontaneously in weeks; no response to antibiotics, but prompt relief from steroids.

Familial Neutrophilia

A familial, lifelong, substantial (to 62×10^9/L) neutrophilia of segmented and, to a lesser degree, stab forms. Neutrophils are normal in morphology and function. NAP may

Table 1 Benign neutrophilia

- Artifact
- Infection[1]
- Inflammation
  - Kawasaki
  - Inflammatory bowel disease
    - i. Crohn's
    - ii. Ulcerative colitis
  - Rheumatoid arthritis
  - SLE
  - Histiocytosis
- Tissue necrosis (e.g. hepatic)[1]
- Leukemoid reactions[1,2]
  - Viral infection (may mimic e.g. AML M2, M3, M4)
  - TAR syndrome
  - Randall's syndrome
  - Disseminated tuberculosis
- Nonhemopoietic malignancy
- Postsplenectomy
- Drugs
  - Steroid
  - Ranitidine
  - Leukocyte stimulating factors[1]
    - i. Colony stimulating factors
    - ii. Interleukins
  - Lithium
- Stress
  - Vomiting
  - Convulsions
  - T Hypoxia, near-drowning
  - Acidosis–diabetic, other
  - Postoperative
- Sweet's syndrome
- Familial[1]

[1] Count often exceeds 50 x 10^9/L
[2] Nonleukemic shifts to left, including myeloblasts

Note: Placement of some disorders, e.g. Kawasaki, histiocytosis is tentative.

be increased. Associated features include increased incidence of chromatid breaks in blood chromosomes, Gaucher-like cells in marrow and spleen, thickening of skull bones (widening of diploë) and hepatosplenomegaly. Regarded as inherited, autosomal dominant.

QUALITATIVE CHANGES IN LEUKOCYTES IN INFECTION

Appraisal of leukocyte numbers and morphology usually gives some indication of the likelihood of infection, its broad nature (bacterial vs viral) and occasionally the precise cause. Changes in morphology (Table 3) are considered here.

Toxic Change in Neutrophils

Toxic change is characterized by various combinations of the abnormalities listed in Table 3. When an apparently toxic change occurs in isolation, e.g. shift to left or heavy granulation, suspicion should be raised of mimicry (Table 4).

Toxic change in neutrophils, in combination with other abnormalities in the film, is a useful guide in distinction of bacterial from viral infection (Table 5). However, toxic change per se is not specific for infection (Table 8). A quantification of toxic change has been devised (Table 6); this may be combined with other leukocyte changes and the platelet count to produce a score for risk of sepsis in the neonate, in whom (more so than in older children) infection is likely to produce rapid deterioration (Table 7). However, C reactive protein levels on 2 successive days appear to be more useful than morphologic assessment (if readings can be obtained rapidly); sepsis is likely if values are raised on day 1 and/or day 2, and can be confidently excluded if values are normal on both days.

Each feature has a score of 1. A total score of ≥ 3 is strong evidence for, and ≤ 2 is strong evidence against sepsis. If no mature neutrophils in film, score 2 rather than 1 for abnormal total count.

Phagocytosis by Neutrophils

- *Of cells (leukocytes, erythroblasts, erythrocytes):* Phagocytic neutrophils are less conspicuous than phagocytic monocyte, which accompany them (see below).
- *Of organisms:* Careful and systematic examination of the film with a low to medium power objective (×10–×25) for 5-10 minutes will detect organisms in a significant proportion of cases of septicemia, especially those with severe effects such as circulatory shut-down. On the other hand, blood from an indwelling venous line may contain a profusion of organisms (from colonization) with surprisingly mild accompanying symptoms.

Optimal parts of the film for search are edges and tails. Infected leukocytes are more numerous in films of the first drop of blood from the previously unmanipulated earlobe (because of trapping in capillary bed), and in films of buffy coat. Densely-staining organisms (e.g. cocci) are more readily visible than the pale-staining organisms (Gram-negative rods).

Criteria for acceptability of organisms in films as genuine evidence of septicemia are summarized in Table 9A; descriptions are given in Table 9B.

Table 2 Hematology of appendicitis (n=50)[1] vs nonsurgical abdominal pain (n=50)[2]

		Sensitivity[3]	*Specificity*[4]
Total leukocytes, x 10^9/L	>15.0	29/50	43/50
	> 20.0	15/50	48/50
	> 25.0	7/50	50/50
	> 30.0	1/50	50/50
Neutrophils, x 10^9/L	>15.0	25/50	48/50
	> 20.0	7/50	49/50
	> 25.0	3/50	50/50
	> 30.0	1/50	50/50
Neutrophils, shift to left, %	>10.0	17/49	37/50
	> 20.0	11/49	50/50
	> 30.0	4/49	50/50
Atypical lymphocytes: 0 in 200 leukocytes		13/50	48/50
ESR, mm in 1 h	> 30.0	5/31	38/38

[1] Histologically proven
[2] Admitted to surgical wards with abdominal pain – 46 with various diagnoses discharged without laparotomy. 4 with histologically normal appendix removed
[3] Sensitivity = proportion of patients with positive test
[4] Specificity = proportion of controls with negative test

Table 3 Qualitative changes in leukocytes in infection

- Neutrophils
  - Shift to left
  - Toxic granulation
  - Degranulation
  - Döhle bodies
  - Vacuolation
  - Pelgeroid change
  - Chromomeres
  - Gigantism
  - Phagocytosis of:
    i. Leukocytes
    ii. Erythroblasts/cytes
    iii. Organisms
  - Cell death, fragmentation
  - Agglutination
  - NAP usually ↓ in bacterial infection, ↑ in viral
- Lymphocytes
  - Cell death, fragmentation
- Monocytes/macrophages
  - Vacuolation, enlargement, activation, Döhle-like bodies
  - Phagocytosis of:
    i. Leukocytes
    ii. Erythroblasts/cytes
    iii. Organisms
  - Chromomeres
  - Cell death, fragmentation
  - Transformation to 'histiocytes'
  - Infection-associated hemophagocytosis (marrow, blood)
- Giant cells with intranuclear inclusions (marrow, CMW infection)

Cell Death

Death (apoptosis) of leukocytes is common in infection. Because anticoagulation and storage also cause damage, cell death is most reliably assessed in films made directly from vein, finger or ear.

The process affects leukocytes in general. Whole cells as well as fragments may be observed. Though phenomenon may be noted in non-infective conditions such as malignancy and SLE, in childhood it almost always is a result of infection and may be striking in viral infections such as infectious mononucleosis, measles and neonatal herpes in viral infection death may affect atypical lymphocytes as well as normal leukocytes.

Possible mechanisms include immune effect, interleukin-2 starvation as a result of cell hyperactivity and invasion by virus. Dead cells are a stimulus to phagocytosis by monocytes/macrophages and neutrophils.

TRANSIENT NEUTROPENIA

Neutropenia is occasionally spurious.

- Clotting in sample (likely to depress counts of all cell types rather than neutrophils only)
- MPO deficiency (neutropenia wrongly identified by counters, e.g. Hemalog D, which recognize neutrophils cytochemically)
- Aggregation, in some cases EDTA-induced
- Cell fragility (smudging) in chylomicronemia.

Neutropenia in childhood (Table 10) is most commonly due to infection, especially viral. Neutropenia is usually

Table 4 Differential diagnosis of toxic changes in neutrophils

Changes	*Differential diagnosis*
Toxic granulation	Alder granulation
Degranulation	Genetic disorder Myeloid leukemias
Döhle bodies	Döhle-like bodies in May-Hegglin, Fechtner and Sebastian syndromes
Vacuolation	Anticoagulant/storage artifact Jordans anomaly
Giant neutrophils Pseudo-Pelgerization Cell death	Hereditary giant neutrophils Anticoagulant/storage artifact

Table 5 Common changes in leukocytes in bacterial and viral infection

	Bacterial infection	*Viral infection*
Neutrophilia	Common	Uncommon
Neutropenia	In severe infection	Common
Toxic change in neutrophils	Common, often severe	Slight; marked in some infections, e.g. measles
Atypical lymphocytes	Small numbers or absent	Increased
Organisms in leukocytes	Sometimes	0 (zero)

Table 6 Quantitation of toxic change in neutrophils[1]

	% cells affected	*Score*
Vacuolation	0	0
Döhle bodies	<25	+
	25–50	2+
	51–75	3+
	>75	4+
Toxic granulation	Normal granulation	0
	Slight toxic granulation	+
	Approx 50% cells affected	2+
	Toxic granules most cells	3+
	gross; nucleus obscured by toxic granules	4+

[1]For use with (Table 7)

Table 7 Hematologic scoring system for sepsis in the neonate

	Abnormality
Total neutrophil count	↑ or ↓
Immature/total neutrophil ratio	↑
Immature/mature neutrophil ratio	≥ 0.3
Immature neutrophil count	↑
Total leukocyte count	↓ or ↑
	(≤ 5.0 x 10^9/L or ≥ 25.0, 30.0 or 21.0 at birth, 12-24 h and day 2 onward respectively
Toxic change in neutrophils	≥ 3 + for vacuolation, toxic granulation or Döhle bodies
Platelets	≤ 150 x 10^9/L

Table 8 Toxic changes in neutrophils. Some causes other than infection

- Collagen disease
  - Rheumatoid arthritis
  - SLE
- Other vasculitides
  - Kawasaki
  - Stevens-Johnson
- Inflammatory bowel disease
  - Crohn's
  - Ulcerative colitis
- Near-drowning[1]
- Heat stroke
- Tissue necrosis
  - Hepatic
- Necrotizing enterocolitis[2]
  - Burns[1]
- Drugs
  - Colony-stimulating factors
  - Cytotoxics

[1] Added infection common
[2] Septicemia from normal gut flora a significant component

not severe (>0.2 x 10^9/L) and usually begins to recover within 5 days, rarely not for 2-3 weeks. Destruction may be due to direct effect or immune mechanism. Morphologic evidence in blood films of antibody/opsonic effect includes agglutination and phagocytosis of neutrophils by monocytes or rarely neutrophils. The marrow appears normal or shows 'maturation arrest' at the myelocyte/stab stage or, rarely, destruction. In a high proportion the organism is not identified (or sought). Recovery of count after treatment with antiviral agents, e.g. ganciclovir, suggests a viral cause.

Mechanisms for neutropenia in sepsis include destruction by endotoxin or phagocytosed organisms and agglutination by activated complement components, e.g. 5a. The marrow may be depleted of granulocytes,

Table 9A Organisms in blood films: acceptability as genuine evidence of septicemia

At least a proportion intracellular; appearances must be typical. Intracellular organisms are unequivocal evidence of septicemia only in films of capillary blood or films of venous blood made directly from needle (skin organisms may rarely be phagocytosed by leukocytes in venous samples in the interval before films are made)

Disregard

- Inclusions in lymphocytes
- Infected skin squames, epidermal cells
- *Granules in basophils:* More variable in size and shape than cocci, often hollow, no characteristic grouping, no capsule, paler than most cocci, no or minimal staining with Gram toxic granules.

Differential diagnosis

- *Granules in basophils:* More variable in size and shape than cocci, often hollow, no characteristic grouping, no capsule, paler than most cocci, no or minimal staining with Gram toxic granules
- Coarse azurophil granules
- Nuclear appendages
- Chromomeres (detached, pyknotic nuclear fragments)
- Phagocytosed nuclear fragments

Table 9B Characteristics of bacteria in Romanowsky-stained blood films[1]

N. meningitides	Plump cocci, a proportion in pairs, with some flattening of opposed faces, intense blue-black. *N. gonorrhoeae* indistinguishable in morphology—rare in childhood and clinical features different. Gram negative
S. pneumoniae	Fine cocci, some slightly elongated, most in pairs, with clear space (capsule) around; intense, almost black staining. Infection may be heavy postsplenectomy
S. aureus	Coarse cocci, usually in small groups, but may be single, in Pairs or short chains (to about 4), redbrown to violet or almost black, no capsule
β-hemolytic streptococci	Fine cocci in groups or chains, intense violet to almost black
Coliforms	Small to large rods, gray to gray-pink, *Klebsiella* often encapsulated. Organisms usually not profuse in individual cells and infected cells usually sparse
H. influenzae	Small rods or coccobacilli, often encapsulated, usually not profuse
Clostridia	Large rods, often square ended; gray to gray-pink, no capsule

[1] Gram and PAS stains may be useful on spare films if infected cells plentiful.

Table 10 Neutropenia transient

- Infection
  - Viral (including HIV)
  - Bacterial
    - i. Pyogenic infection *Staphylococcus*, *Streptococcus*, *Coliforms*, *Meningococcus*, *Haemophilus*, if severe
    - ii. Brucella, typhoid, paratyphoid[1]
  - Tularemia[1]

Malaria, occasional cases[1]

- Burns
- Hemodialysis
  - Drugs

[1]Neutropenia may be chronic

especially in neonate. Transient neutropenia associated with burns and hemodialysis is attributed to agglutination by activated C5.

NEUTROPENIA, CHRONIC (MORE THAN 3 MONTHS)

Less frequent than transient neutropenia. An empiric classification has been given (Tables 11 to 14), as in many cases the mechanism is unknown. As suggested schema for initial investigation is given in Table 11A.

Cyclic Neutropenia

Periodicity of infections, especially upper respiratory and oral, should suggest the diagnosis. The cycling period is usually 19–21 days; neutropenia lasts for 3–6 days, with complete absence for 1–3 of these. In neutropenic phases, patients feel unwell from fever and infection, especially staphylococcal with streptococcal. Oral infection may be associated with cervical lymphadenopathy. Monocytes are usually increased in neutropenic phases, but infection is nevertheless a problem, as monocytes are less efficient than neutrophils in tracking and destroying bacteria. Monocytes, lymphocytes, eosinophils and platelets also show some cycling (usually from normal to above normal), while reticulocytes cycle above to below normal. An increase in large granular lymphocytes is often noted in cases of adult onset.

In neutropenic phases the marrow shows 'maturation arrest' at the myelocyte stage. Increase in mature forms and paucity of precursors preceded by some days increase in neutrophils in blood.

In some cases oscillations tend to dampen over the years and evolve to chronic neutropenia. The condition does not appear to predispose to leukemia, though cyclic neutropenia may rarely be a harbinger of ALL or myelodysplasia.

In most cases, especially those of adult onset, inheritance cannot be discerned. In familial cases (about one

Table 11A Chronic neutropenia: initial assessment

- Clinical history, age at onset, physical examination
- Blood values
- Blood film
- Neutrophil count, once or twice weekly for 6 weeks
- Bone marrow
  - Aspirate
    - Morphology
    - Karyotype
  - Trephine biopsy
  - Electron microscopy
- Serum
  - Immunoglobulins
  - Complement components
  - Autoimmune screen
  - B_{12}, folate
  - Viral serology
  - Antigranulocyte antibodies
- Lymphocyte subsets
- *As appropriate:* Tests for disorders in Tables 12–14

Table 11B Chronic isolated neutropenia in childhood – inherited[1]

- No other anomalies
  - Cyclic
  - Kostmann
  - Lazy leukocyte syndrome
  - *With abnormal marrow neutrophils:* Myelokathexis and others
- As part of genetic syndrome
  - With visible somatic anomalies (Table 12)
  - Without visible somatic anomalies (Table 13)

[1]Neutropenia usually congenital

Table 12 Genetic syndromes with visible somatic anomalies which may be associated with neutropenia

Shwachman	Exocrine pancreatic insufficiency, metaphyseal dyschondroplasia, growth retardation.
Fanconi	Abnormal pigmentation short stature, thumb and radius anomalies, abnormal head/face, hypogonadism.
Dyskeratosis congenita skin	Reticular hyperpigmentation, depigmentation, atrophy; hair loss, nail dystrophy, leukoplakia, dental dystrophy.
Chediak-Higashi	Partial oculocutaneous albinism, bacterial infections, esp. *S. aureus*, gingivitis and periodontitis, cranial and peripheral neuropathies, hepatosplenomegaly.
Cartilage-hair hypoplasia	Short-limbed dwarfism, fine hair, infections, esp. varicella
Cohen	Nonprogressive psychomotor retardation, microcephaly, short stature, delayed puberty, hypotonia, joint hypermobility, peculiar faces and teeth, myopia, narrow hands and feet, not infection-prone
Hernandez	Dystrophy of nails and hair, mild mental retardation
-	Microcephaly, psychomotor retardation, retinitis pigmentosa, marrow normal by light microscopy

third), transmission is autosomal dominant with variable penetrance.

The defect appears to reside in the stem cell/committed progenitor cell, as it is transmissible by transplantation in humans and can be cured in affected animals (Collie dogs) by transplantation of normal marrow.

Differential Diagnosis

A degree of cycling may be noted in neutropenias such as Shwachman's syndrome and monosomy 7 MPD. Cycling, however, does not have the predictability of true cyclic neutropenia.

Kostmann's Syndrome (Infantile Genetic Agranulocytosis)

Neutrophils are persistently lower than 0.3×10^9/L; bacterial infections are frequent and severe and are the usual cause of death. There is often a 'compensatory' monocytosis which, even if striking (in a personal case to 14.6×10^9/L), assists little in ameliorating infection because of the inefficiency of monocytes in handling infection. The marrow is cellular usually with maturation arrest at the promyelocyte/myelocyte stage, or in some cases normal maturation. Electron microscopy shows dysgranulopoiesis in some cases.

A deficiency in responsiveness to granulocyte colony stimulating factor (GCSF) is likely, which can however be overridden *in vivo* by administration of GCSF. Evolution to (myeloid) leukemia may occur. Inheritance is autosomal recessive.

Lazy Leukocyte Syndrome

A rare, chronic neutropenia ($<0.2 \times 10^9$/L) with morphologically normal marrow and severe curtailment of chemotaxis and random mobility of neutrophils. Neutropenia shows no response to intravenous adrenaline and subnormal response to *Pneumococcus polysaccharide*. An abnormality of surface configuration may make the cell rigid. A similar disorder may occur as a temporary (< 12 months) phenomenon.

Table 13 Genetic syndromes without visible somatic anomalies which may be associated with neutropenia

Neutropenia with immunodeficiency[1] X-linked agammaglobulinemia dysgammaglobulinemia type	O IgG, IgA, n to ↑ IgM
-	Hyper-IgA, eosinophilia, defective neutrophil chemotaxis, T, B cell function
Reticular dysgenesis	Hypogammaglobulinemia, lymphopenia (imperceptible tonsils, impalpable lymph nodes, no thymus on X-ray). Marrow depleted of granulocytes. Lymphocytes. AR mild variant described
-	Lymphopenia, T cell deficit, partial Pelgerization, benign course, X-linked recessive
Organic acidemias: – Isovaleric – Propionic – Methylmalonic	Recurrent metabolic acidosis, often with vomiting, variable mental retardation, odor of 'sweaty feet' in isovaleric acidemia; thrombocytopenia/pancytopenia in acidotic episodes of isovaleric acidemia
Glycogen storage 1b	Hypoglycemia, convulsions, failure to thrive, infections, bleeding, hepatomegaly

Abbreviations: AR: Autosomal recessive; n: Normal.
[1]Neutropenia may be a result rather than a cause of infection, as neutropenia appears to be infrequent in those treated with immunoglobulin

Table 14 Chronic isolated neutropenia in childhood–acquired

- Alloimmune
- Autoimmune[1]
- Infection
  - Virus
  - Other[2]
  - Brucellosis
  - Typhoid, paratyphoid
  - Malaria
  - Leishmaniasis
- Marrow pathology[2]
  - Leukemia
    - Monosomy 7 MPD
    - Pre-ALL
  - Neuroblastoma
  - Hypoplasia
  - Myelosclerosis
  - Osteopetrosis
- Deficiency of hematinics[2]
  - Folate, B^{12}
  - Copper
- Drugs
  - Immune
  - Nonimmune
- Sequestration
  - Spleen[2]
  - Marrow macrophages

[1] In neonates passive transfer may occur from a mother with autoimmune disease
[2] Usually with other cytopenias

Neutropenias with Abnormal Marrow Neutrophils

Myelokathexis (Kathexis = Retention)

A rare neutropenia (chronic, non-cycling) associated with, and attributed to, abnormality of segmented neutrophils in marrow which are abundant but show signs of degeneracy (nuclear pyknosis, hypersegmentation with slender connecting filaments, cytoplasmic vacuolation). Neutrophils show variable impairment of function (decreased NAP, impaired dye exclusion, mobility and phagocytosis). MPO is normal. Count increases in infection and after injection of GCSF. Possible variants include familial occurrence (father, daughter) with hypogammaglobulinemia, and association with growth retardation and dysmorphism.

Neutropenia with Gigantism and Multinuclearity of Marrow Neutrophils

Marrow promyelocytes contain up to 4 nuclei, and segmented neutrophils up to 16. Some cells are hypogranular and others hypergranular. Cells are severely deficient in lactoferrin (specific granules). The abnormalities impair survival in marrow and are attributed to aberration of centrioles. Karyotype is normal.

Blood neutrophils by contrast are normal. Increase is inconstant in infection and minimal after adrenaline and dexamethasone.

Neutropenia with Large, Binucleate, Tetraploid Neutrophils and Monocytes in Marrow

The proportion of binucleate cells increases with maturity (metamyelocytes 42%, segmented 100%). Other leukocytes are normal. Neutropenia is attributed to impaired egress of abnormal cells from marrow. Binucleate cells are rare (< 1%) in blood.

In a personally observed case, neutropenia was associated with abnormal granule structure and pancreatic fibrosis.

Neutropenia as Part of a Genetic Syndrome (Tables 12 and 13)

Neutropenia in these disorders is due to a variety of mechanisms.

- Precursor cell deficiency, e.g. Fanconi, Shwachman, cartilage-hair hypoplasia
- Intramedullary destruction, e.g. Chediak-Higashi (abnormal myeloid precursors in marrow, increased serum muramidase in absence of monocytosis or decreased survival of blood neutrophils)
- Suppression of myeloid maturation, e.g. by organic acids and glycine in organic acidemias.

Antibody-induced Neutropenias

Antibodies to neutrophil-specific antigens are an important cause of neutropenia (Minchinton & Waters 1984). Antigens shared with other cells types (e.g. HLA) do not appear to be significant in neutropenia. Serum may be tested against a panel of neutrophils of known phenotype or against films of marrow aspirate. Serum should be tested fresh, but if delay in transport to a reference lab is likely, blood should be taken into citrate-phosphate-dextrose-EDTA solution. Serum or plasma should be heated before application; to remove complement with interferes with antibody binding. Marrow films should be fresh, or stored at -30°C till and fixed with paraformaldehyde at time of testing. Antibody is demonstrable on more mature stages (metamyelocyte onward) in mild to moderate neutropenias, and on myelocytes and promyelocytes as well, in severe neutropenias.

Alloimmune Neutropenias

It is two important types in childhood:

1. **Alloimmune neutropenia of infancy:** Estimates of incidence vary from 1/200 to 3 percent of neonates. Neutropenia is severe (0-0.5 × 10^9/L, often with a 'compensatory' monocytosis. In infected infants antibody should be sought if neutropenia is excessive for the infection (neutropenia is unlikely unless bacterial infection is severe).

   Antibodies (IgG) are directed against one of the normal cell-specific antigens (well represented on cord neutrophils), most commonly NA1, NA2, NB1, NC1 and 9a. Rarely, the mother has no NA specific neutrophil antigens (NA null, CD16 negative) and as a consequence reacts to any NA (NA1, NA2) antigens on fetal neutrophils. Immunization is usual with the first child.

   Because of paucity of cells, realistic testing can be done only for antibody in the mother's serum which reacts with the father's but not with her own neutrophils (Table 15).

   Marrow examination excludes the unlikely occurrence of leukemia as the cause of isolated neutropenia. Cellularity is variable. Stages from metamyelocyte onward may be normally represented, deficient or absent. 'Maturation arrest' is due to destruction of mature forms and not to suppression of maturation.

   Neutropenia persists for 3 weeks to 3 months depending on rate of catabolism of antibody. Mortality (bacterial septicemia) is about 5 percent.
2. **Autoimmune neutropenias (Table 16):** The most common in childhood is autoimmune neutropenia of infancy.

Table 15 Alloimmune neonatal neutropenia : diagnosis

Combination	*Result*
Maternal serum + paternal granulocytes	pos
Maternal serum + maternal granulocytes[1]	neg
Baby serum[2] + paternal granulocytes	pos
Baby serum[2] + baby neutrophils[3]	pos
Maternal serum + baby neutrophils[3]	pos

[1] To exclude maternal autoimmune neutropenia, e.g. SLE
[2] Collected while neutropenia
[3] Collected when numbers become normal

Table 16 Autoimmune neutropenias of childhood

Combination
• Autoimmune neutropenia of infancy ('chronic benign')
• Viral infection
• Autoimmune disease
– SLE
– Feity
• Passive transfer from mother with autoimmune disease, e.g. SLE
• With autoimmune hemolysis and thrombocytopenia (Evans syndrome)
• Bone marrow transplantation
• Drugs (some)
• T lymphocytosis with neutropenia

Autoimmune Neutropenia of Infancy (Chronic Benign)

Neutropenia is severe (0-0.5 × 10^9/L); however there is no excess of infections compared with normal children of the same age. The count increases with infection and urticaria,

especially in the recovery period; there is little or no response to adrenalin (draws cells from marginating pool), variable response to steroid (enhances release from marrow), and good response to intravenous immunoglobulin (usually temporary occasionally permanent, effect attributed to Fc receptor blockade and decreased synthesis of antibody). 'Compensatory' monocytosis is common, so that usually total leukocyte count is within normal limits.

Marrow examination is recommended to exclude the rare possibility of leukemia as a cause of isolated neutropenia. Myeloid hyperplasia is usual; mature forms (bands and segmented) are normally represented or deficient ('maturation arrest').

Median age at detection is approximately 8 months (range 3-30). Blood counts shortly after birth have been normal. Girls are more often affected than boys (1.5/L).

The antibody is IgG, with some IgM in occasional cases. Specificity is usually for NA1 or NA2; in some cases the target antigen cannot be identified. Evidence of parvovirus infection (PCR on marrow cells, serology) was obtained in a majority of cases; the occurrence of neutropenia rather than the usual erythroblastopenia may be due to altered immune response, the antibody recognizing myeloid cells as well as virus.

Neutropenia is self-limiting with a median duration of 30 months (range 6-60), 95% recovering by 4 years. There are no known long-term or other effects.

Viral Infection

Antineutrophil antibodies have been detected in some viral infections, e.g. infectious mononucleosis, HIV and parvovirus infection. Neutropenia, however, occurs in only a minority of those with antibody. The target antigen is not clear—it does not appear to be one of the known polymorphous, neutrophil-specific antigens, NA1, NA2, etc. Neutropenia is only occasionally (< 1%) severe and prolonged enough in itself to predispose to bacterial infection.

Autoimmune Disease

- *SLE:* Antineutrophil antibodies are detectable in about 50% of patients. The antibody does not have specificity for known polymorphous neutrophil-specific antigens and is distinct from the anti-DNA present in most cases.

  Neonatal lupus syndrome is a risk if the mother has SLE (not necessarily symptomatic in the pregnancy). Major manifestations are cutaneous lupus (not manifest at birth but becoming so within 2 months) and complete heart block (at birth), usually one or the other, occasionally (< 10%) both. Skin lesions resolve usually within 6 months, but the heart block almost always is permanent. Some infants have, singly or in combination, neutropenia, thrombocytopenia or autoimmune hemolysis.
- *Felty's syndrome (rheumatoid arthritis, splenomegaly and neutropenia):* Rare in childhood. The neutropenia is of complex causation:
  - Neutrophil antibodies demonstrable in most cases
  - Hypersplenism, most patients showing sustained improvement in count after splenectomy
  - Inadequate compensatory marrow production.

Evan's Syndrome

Autoimmune thrombocytopenia and hemolysis is without detectable underlying cause such as viral infection or SLE. Autoimmune neutropenia also occurs in some cases. Antibodies to the various cell lines are different.

Marrow Transplantation

Antibodies to neutrophils (and platelets) occur frequently after marrow transplant (allogeneic or autologous). Antibodies post-allogeneic transplant can be shown by immunoglobulin allotyping to be of donor origin, i.e. antibody against engrafted cells is autoimmune, whether allogeneic or autologous.

Drug-immune Neutropenia (Table 17)

Rare in childhood.

- In most cases the condition is drug-specific; in contrast to drug-specific hemolysis, antibody will not simply react with pretreated granulocytes, but only when serum, drug and neutrophils are incubated together.
- True autoantibody, comparable to methyl dopa immune hemolysis; though autoimmune, antibody cannot be detected in serum after withdrawal of the drug.

Usually antibody is active against both mature and immature cells, in a minority against only precursor or only mature cells. The nature of the target antigen/s is for the most part unknown; the specific neutrophil series (NA1, NA2, etc.) is not involved. For quinine, at least it is a membrane glycoprotein. Antibodies from different drugs occasionally cross-react (e.g. quinine, quinidine).

Neutropenia affects only a minuscule proportion of those exposed, and is unpredictable in occurrence and course. Usually onset is abrupt, 1-5 weeks after start of treatment, sooner (or immediately) after re-exposure. Neutropenia lasts usually for 1-4 weeks after withdrawal of the drug, occasionally as briefly as 1 day. It may be severe enough to cause serious infection.

Marrow may be grossly depleted of neutrophils in general or show 'maturation arrest' at the myelocyte/metamyelocyte stage, with or without hyperplasia of precursor cells.

Table 17 Pediatric drugs which may be associated with immune neutropenia

- Antiarrhythmic
  - Aprinidine HCl
  - Flecainide acetate
  - Procainamide
  - Quinidine
- Antibiotics
  - Penicillin and derivatives
    - Ampicillin
    - Amoxycillin
    - Dicloxacillin
    - Nafcillin
    - Oxacillin
  - Cephalosporins
    - Cephradine
    - Cefotaxime
    - Ceftazidime
    - Cefuroxime
- Sulphonamides
  - Sulphamethoxazole
  - Sulphathiazole
  - Sulphafurazole
  - Sulphapyridine
- Antimalarial
  - Amodiaquine
  - Chloroquine
  - Quinine
- Analgesic/anti-inflammatory
  - Amidopyrine
  - Aminosalicylic acid
  - Diclofenac
  - Ibuprofen
  - Propyphenazone
- Antithyroid
  - Propylthiouracil[1,2]
  - Carbimazole
  - Methimazole
- Other
  - Phenytoin
  - Chloral hydrate
  - Gold thiomalate
  - Levamisole

[1] Neutropenia may not occur till months or years after exposure
[2] True autoimmune in some cases

In some cases antibody is generated against platelets as well as neutrophils (different antibodies), and rarely against other cells, e.g. erythrocytes and T lymphocytes in a hemolytic-uremic-like syndrome attributed to complement-mediated activation and adhesion of neutrophils to endothelium.

T-Lymphocytosis with Neutropenia

Rare in childhood. Neutropenia (antibody demonstrable in some cases) with substantial is increase in normal mature lymphocytes (CD8 suppressor/cytotoxic; large granular lymphocytes in some cases). Lymphocytosis may first manifest or become more obvious after splenectomy (for other reasons) and may affect marrow. Karyotype normal. It may be associated with polyclonal hyperimmunoglobulinemia. Blood is otherwise normal, no anemia or thrombocytopenia. Chronic (years) is with little effect on health. Spleen may be moderately enlarged. Evidence of EBV infection is found in some cases.

Marrow Infiltration/Replacement (Table 14)

Neutropenia is rarely the only finding. In monosomy 7 MPD, however, neutropenia, with or without macrocytosis, may be a prodrome over many years to overt disease. Rarely, neutropenia is a prodrome to ALL.

Deficiency of Hematinics

- Folate, B_{12} deficiency
- Copper deficiency.

A rare cause. Neutropenia is an important and early characteristic, usually severe ($<0.5 \times 10^9$/L); marrow usually shows vacuolation of precursor cells and 'maturation arrest' at myelocyte/metamyelocyte stage. Anemia is usually severe (to about 4.5 g/dL) and macrocytic, with megaloblastosis, vacuolated erythroblasts and ringed sideroblasts (10-15% of cells) in marrow. The genesis of these changes obscure; they do not occur in the best-known copper deficiency in man (Menkes kinky hair syndrome). Possible mechanisms include defective synthesis of cytochrome oxidase and ascorbic acid oxidase; which keep copper in the reduced state. Treatment with copper produces rapid and striking response.

Deficiency is most likely to occur in infants with prolonged diarrhea and malnutrition, and in those on prolonged total parenteral nutrition without copper supplementation. Deficiency may, however, occur without obvious cause in infants who are thriving.

Drugs and Neutropenia

Selective granulocytopenia as an idiosyncratic, unpredictable effect of drugs is rare in childhood, though it is possible with a large variety of drugs. Mechanisms include personal idiosyncrasies in pharmacokinetics, sensitivity of myeloid precursors and immune response. Drugs with potential for immune destruction are listed in Table 17. The same drug may produce granulocytopenia by different

mechanisms in different patients and possibly even at different times in one patient.

Neutropenia due to Sequestration

- Hypersplenism (e.g. biliary atresia, liver cirrhosis, cavernous transformation of portal vein).
- Hyperphagocytosis of band and segmented forms by marrow macrophages is a rare cause of isolated neutropenia; e.g. hemophagocytosis, in which macrophages contain a variety of inclusions. There is no evidence of neutrophil antibody, no or slight increase in serum muramidase, no response to adrenalin (marginating pool) but good response to hydrocortisone (marrow reserve).

GRANULOCYTES: CYTOPLASMIC ANOMALIES (TABLE 18)

Qualitative changes in leukocytes in infection are described in Tables 3 to 9.

Alder Anomaly (Table 19)

Coarse, densely-staining granulation in neutrophils, anomalous staining of eosinophil granules (violet, green or gray-black, may resemble basophil granules), and unusually coarse or densely staining granules in basophils, monocytes and mast cells. Precursor cells in marrow are also affected. To be distinguished from toxic granulation (finer, often associated with other signs of toxicity, temporary).

Table 18 Anomalies of granulocyte cytoplasm

- Alder anomaly
- Sparse coarse azurophilic granules
- Vacuolation
- Vacuolation of granulocyte precursors and erythroblasts
- Döhle-like bodies
- Neutrophil specific granule deficiency
- Eosinophil specific granule deficiency
- Peroxidase deficiency in neutrophils
- Giant granulation in granulocytes and monocytes
- Amorphous rounded 'gray' bodies
- Hemosiderin
- Bilirubin

Table 19 Alder granulation

- MPS VI (Maroteaux-Lamy)[1]
- MPS VII (Sly)
- Multiple sulphatase deficiency
- Infantile free sialic acid storage disease[2]
- Asymptomatic[3]

[1]Granules metachromatic and birefringent
[2]Partially developed Alder granulation
[3]Existence doubted

Sparse, Coarse Azurophil Granules

A minority of neutrophils contains a light sprinkling of coarse granules. Rare granules may be metachromatic. Other granulocytes are normal.

Characteristic of MPS IV (Morquio) type A (early onset). *Main features:* Normal intelligence, gross and distinctive skeletal change, mild facial coarsening and corneal clouding, risk of spinal cord compression from odontoid hypoplasia; valvular heart disease. Onset 1-3½ year's survival beyond 30 years is unusual autosomal recessive. Definitive diagnosis is by galactose-6-sulphatase (arylsulphatase A) assay in leukocytes or cultured fibroblasts. Milder forms occur, with no excess of mucopolysaccharide in urine and longer survival.

Vacuolation

Vacuolation is uncommon as a genetic anomaly. More common causes are toxic states (infection, inflammation and cytotoxics) and artifact of anticoagulation.

Vacuoles of Neutral Lipid (Jordan's Anomaly)

Vacuoles are ORO and Sudan III positive, stain red with Nile blue sulphate and occur in most to all neutrophils, eosinophils, basophils and monocytes and in a proportion of plasma cells, but not in other hemopoietic cells. Absent from myeloblasts and increase with cell maturity from promyelocyte onward.

Ichthyosis and Neutral Lipid Storage Disease

Triglyceride droplets also in other tissues, e.g. muscle, liver. *Main features:* Ichthyosis, myopathy, ataxia, sensorineural deafness, cataracts, liver dysfunction, variable mental retardation, evident at birth, autosomal recessive.

Carnitine Deficiency

A heterogeneous and incompletely characterized group of disorders is in which carnitine deficiency may be genetic or acquired (e.g. renal Fanconi syndrome, hemodialysis, total; parenteral nutrition). The defect/s in some of the genetic deficiencies is unidentified and the traditional distinction between 'muscle' and 'systemic' deficiency

may be artificial. In some there is a defect in carnitine transport across mitochondrial membranes, in others a defect in carnitine synthesis. Jordan's anomaly is associated with muscle carnitine deficiency (skeletal and cardiac), less so with systemic deficiency.

Wolman's Disease

Lipid vacuoles are inconsistent and infrequent.

Neonatal Hemochromatosis

Neutrophils with ORO positive vacuoles may occur in neonatal hemochromatosis. In infants vacuolation may be associated with nuclear pyknosis/cell death, and vacuolated cells contained (between the vacuoles) fine Perls positive granulation (finer than in adults with hemochromatosis).

Other

ORO positive neutrophils may occur transiently in biliary atresia and following GCSF treatment.

Pearson's Marrow-Pancreas Syndrome

Vacuolation of granulocyte and erythroid precursors, ringed sideroblastosis, increased storage hemosiderin, transfusion-dependent macrocytic anemia, reticulocytopenia; variable neutropenia, thrombocytopenia and splenic atrophy. It may evolve to marrow aplasia or leukemia. A deletion of mitochondrial DNA has been identified with possible maternal inheritance.

It presents in infancy with growth failure and refractory steatorrhea (pancreatic fibrosis). About half die before the age of 3 years, others improve with age.

Differential diagnosis is from other marrow-pancreas syndromes and from hereditary sideroblastic anemia.

- *Shwachman syndrome:* No vacuolation or sideroblastosis; marrow hypoplasia earlier and more prominent than in Pearson's.
- *Hereditary sideroblastic anemia:* A proportion of erythrocytes are microcytic. No vacuolation or pancreatic insufficiency.
- *Atypical cystic fibrosis with marrow hypoplasia:* No vacuolation or sideroblastosis.

Döhle-like Bodies

These are usually more sharply defined and larger than Döhle bodies, are not accompanied by toxic changes and are permanent.

May-Hegglin Anomaly

Inclusions usually easily seen, but may be inconspicuous in some cases; occur in granulocytes and monocytes but not lymphocytes; pyroninophilic (reaction abolished by ribonuclease), with distinctive ultrastructure of 7-10 nm filaments oriented in parallel in long axis. It is associated with enlarged platelets and, in about one quarter, mild thrombocytopenia autosomal dominant.

Fechtner's Syndrome (Alport's Syndrome Variant)

Dohle-like inclusions, giant platelets, nephritis (microscopic hematuria to renal failure), sensorineural deafness and congenital blue-spotted ('cerulean') cataracts. The Döhle-like bodies occur in most neutrophils and some eosinophils, are smaller and less intensely staining than in May–Hegglin and consist of segments of rough endoplastic reticulum and ribosome cluster's but no filaments.

Platelets are large, moderate to severe thrombocytopenia common autosomal dominant.

Sebastian Platelet Syndrome

It is similar to Fechtner's syndrome but without the clinical abnormalities.

Neutrophil Specific Granule Deficiency

Specific granule deficiency is usually acquired (myeloid leukemias, burns, infection, normal neonate). The genetic deficiency is never severe and is rare.

Main features:

- In stained films cells appear poorly granulated and washed out (MPO-deficient cells appear normal).
- NAP decreased or absent. NAP is not a constituent of specific granules but plasma membrane linked, deficiency being attributed to an anomaly common to both plasma membrane and specific granules.
- Granules ultrastructure consists only of the enveloping vesicle.
- Deficiency of specific granule components, e.g. lactoferrin, TCII, can be shown by biochemical or immunologic methods. Deficiency of TCII in serum may be associated.
- Deficiency of defensins (normal component of subpopulation of azurophil granules) accompanies the specific granules defect.
- Pelgeroid changes—bilobed nuclei of uneven size; micronuclei in some cells.

- Cells are defective in chemotaxis (specific granules produce chemotactic receptors) and in bactericidal capacity (deficiency of lactoferrin, defensins).
- Neutropenia due to intramedullary destruction may occur.
- Manifests as pyogenic infections, which may be indolent.
- Probably autosomal recessive.

Peroxidase Deficiency and Monocytes

The peroxidase in neutrophils and monocytes (MPO) differs from that in eosinophils and is under different genetic control. In neutrophils MPO is localized to azurophil (primary) granules.

- Acquired deficiency occurs in myeloid leukemias (M2, M3 and M4 especially) and myelodysplasias. Usually a proportion of neutrophils is completely lacking in enzyme. The gene for MPO lies in 17q in the vicinity of the breakpoint for the t(15;17) of AML M3.
- Activity is diminished by some drugs—sulphonamides, antithyroid drugs, phenothiazines, ascorbic acid.
- MPO deficiency is the most common inherited disorder of neutrophils (complete deficiency about 1/4000, partial about 1/2000. The partial deficiency affects all or almost all neutrophils, though not necessarily to the same degree. Identification is by standard cytochemical methods (MPO, SBB) or, for partial deficiency especially, automated flow cytometry using 4-chloro-1-naphthol as substrate (e.g. Hemalog D counter). MPO-deficient bloods will be wrongly identified as neutropenic by automated counters which identify neutrophils cytochemically. EPO is normal.

Morphology of neutrophils and monocytes in stained films is normal.

Surprisingly, bactericidal capacity of MPO—deficient granulocytes is only mildly diminished; killing of fungi (candida, aspergillus) however is severely affected. Patients have little susceptibility to bacterial infection, but there is a risk of disseminated candidiasis if MPO deficiency is associated with diabetes mellitus. Mode of inheritance is uncertain. Simple Mendelian genetics are unlikely and polygenic inheritance has been proposed.

Giant Granulation in Granulocytes and Monocytes

Chediak-Higashi Syndrome

An unidentified membrane abnormality results in fusion of azurophil and specific granules to form giant granules. These are positive for MPO, SBB AchPh and CAE (constituents of azurophil granules) and contain lactoferrin (specific granules). Normal specific granules are sparse and azurophil granules absent. Defects in chemotaxis, mobilization and degranulation are attributed to mechanical impediment imposed by granule size. These defects, together with neutropenia and deficient natural killer cell activity, contribute to susceptibility to infection.

Neutrophil precursors (marrow) contain large, MPO positive, pink to purple staining inclusions, often within vacuoles. Neutropenia and increase in serum muramidase are attributed to intramedullary destruction of precursor cells. Eosinophil precursors may contain giant, densely staining azurophil granules.

A minority of platelets may contain large granules. Bleeding is due to deficiency of dense bodies and, in the accelerated phase, thrombocytopenia.

The main clinical features are partial oculocutaneous albinism, cranial and peripheral neuropathy (muscle weakness, ataxia, sensory loss, nystagmus), infection (especially *S. aureus*) and bleeding. In the accelerated phase (usually preterminal, first or second decade), organ enlargement and pancytopenia are due to lymphohistiocytic proliferation and hemophagocytosis.

Autosomal recessive. Heterozygotes may show giant granulation in occasional leukocytes, but this is an unreliable test for the carrier state.

Pseudo-Chediak-Higashi Granulation

Giant granulation in granulocytes, but not lymphocytes, may occur in myeloid leukemias. Abnormal granules are formed by fusion of azurophil granules and may contain Auer-like microcrystals, which differ from true Auer rods in periodicity of ultrastructure.

Gray-staining Bodies

Amorphous, rounded, gray-staining bodies have been noted in:

- Granulocytes of the three types, monocytes and mast cells in an infant with livedo reticularis of the skin and extrahepatic biliary atresia. Leukocyte morphology in both parents was normal. Inclusions negative by routine cytochemical procedures. The anomaly is not a result of the biliary atresia.
- Eosinophils and basophils only, as a dominantly inherited, apparently asymptomatic anomaly. Inclusions absent from other hemopoietic cells, including mast cells; mildly positive with some cytochemical procedures (e.g. PAS), distinctive in ultrastructure. (Charcot-Leyden crystals, which may also occur in eosinophils, are not latticed).

- Granulocytes (all types), monocytes, lymphocytes and plasma cells, from birth in an infant with hepatosplenomegaly, spherocytic hemolysis, thrombocytopenia, neutropenia and infections. These features disappeared and the child is apparently well at the age of 2 years. Inclusions negative with routine cytochemistries. Electron microscopy showed ribosomal type composition without identifiable organelles.
- In a (possibly) similar case, inclusions identified as collections of actin microfilaments occurred in all hemopoietic cells, but mainly granulocytes in a 13-month-old boy with transfusion-dependent anemia, splenomegaly, gray skin discoloration and intermittent neutropenia and thrombocytopenia. Clinical abnormalities resolved spontaneously at about 18 months but inclusions have persisted.

Other Inclusions

Neutrophils and/or monocytes may contain hemosiderin (brown-yellow staining, often refractile, and bilirubin (yellow-green to green black).

GRANULOCYTES: NUCLEAR ANOMALIES (TABLE 20)

Pelger-Huet Anomaly

- Pelgerization is most commonly acquired, occurring especially in myeloid leukemias, myelodysplasias, bilineage ALL, toxic states and colchicine poisoning.
- The inherited anomaly may be heterozygous or homozygous and affect all or only some (5-20%) cells. In the common, heterozygous state, neutrophil nuclei have rod, dumb-bell, peanut or pince-nez shapes, and eosinophil nuclei > 2 lobes; abnormality is not readily recognizable in basophils. In the rare homozygote, most cells have rounded nuclei.

Inheritance is autosomal dominant, with some rare exceptions (following). The common, heterozygous state is not convincingly linked to any clinical illness. Associations have however been suggested with:

i. Muscular dystrophy
ii. A syndrome of episodic fever and abdominal pain (thought to be autosomal recessive because of an intermediate degree of pelgerization in one parent available for study
iii. A syndrome of leukopenia and infections, probably X-linked.

The homozygous state appears to be usually lethal *in utero*. Pelgerization is conspicuous in inherited neutrophil specific granule deficiency.

Table 20 Abnormalities of granulocyte nuclei

• Pelger-Huet and like anomalies
• Excessive tags
• Hypersegmentation of neutrophil nuclei
• Hypersegmentation of eosinophil nuclei
• Chromomeres
• Toxic states

Excessive Tags

Short projections, with head as wide as or slightly wider than the attachment stalk, to be distinguished from drumsticks (larger mass of dense chromatin, attached by short filament) and clubs (larger than drumstick, longer filament). The chromatin may be coarse and lumpy and separation of lobes indistinct.

Occurrence of 2 or more tags in > 15% of neutrophils is characteristic of trisomy 13, whether isolated or as part of triploidy. Hereditary persistence of nuclear appendages is descried as an autosomal dominant, asymptomatic defect, but its status as a genuine, independent anomaly is uncertain.

Hypersegmentation of Neutrophil Nuclei (Table 21)

The normal mean lobe count, after the neonatal period is 2.8 (2.5–3.1). Only the last 2 in Table 21 are considered here. Others are discussed elsewhere.

- Hereditary constitutional hypersegmentation—mean lobe count approximately 4 but cells normal in size. Asymptomatic, autosomal dominant.
- Hereditary giant neutrophils (macropolysytes)—5-15% of neutrophils abnormally large, with 6-10 lobes (probably tetraploid. Normally up to 2 cells per 1000, slightly more in a variety of illnesses, including cytotoxic exposure. Asymptomatic, autosomal dominant, but only the heterozygous state is known.

Table 21 Hypersegmentation of neutrophil nuclei

• Toxic states
• Megaloblastosis[1]
• Triploidy
• Myelokathexis
• Neutropenia and hypogammaglobulinemia with abundant
• Abnormal neutrophils in marrow
• Lightsey anomaly[2]
• Iron deficiency (uncommon)
• Hereditary constitutional hypersegmentation
• Hereditary giant neutrophils (macropolycytes)

[1] Benign or as a part of myeloid leukemia/myeloidysplasia
[2] Neutropenia with gigantism and multinuclearity of marrow neutrophils

Nuclear Changes in Toxic States

- Infection, treatment with cytotoxics
- Heat stroke, hyperpyrexia 'Botryoid' nuclei (shrinkage, pyknosis, clustering of lobes), often with fragmentation and chromomere formation, may occur in heat stroke and hyperpyrexia. Changes disappear within 24 hours after removal from injury.
- Colchicine poisoning

Extrusions, chromatin damage and Pelgerization may be noted.

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SECTION 4

Bleeding Disorders

CHAPTERS OUTLINE

CHAPTER 27

Approach to a Bleeding Child

Raj Warrier, MR Lokeshwar, Aman Chauhan

Hemostasis is maintained by many processes in the circulating blood and the blood vessels designed to stop hemorrhage. These functions are delicately balanced so that we will neither clot nor bleed to death. Clotting reactions are initiated in response to injury to vessels and leads to formation of a fibrin platelet plug that stops the blood loss but ultimately results in elimination of the clot, repair of any damage to the vessels and normal blood flow through the affected site.

Hemostasis involves a complex interplay between the vessel wall, platelets, and coagulation factors.

The main three components of hemostasis are:

1. Vascular and extra vascular factors
2. Platelets
3. Plasma factors—coagulation factors, fibrinolytic factors, natural inhibitors of coagulation and fibrinolysis.

Successful management of an acute bleeding episode in a child depends on:

- Detailed history and clinical examination leading to appropriate diagnostic tests.
- Ordering an array of available diagnostic tests without a clinical diagnosis is neither economically viable nor therapeutically ideal.
- Prompt implementation of therapeutic measures.

HISTORY

- Significance of bleeding
- Nature and site of bleeding
- Local vs generalized causes
- Acquired or hereditary disorder
- Vascular, platelet or factor deficiency
- Is it due to a local cause?

Local Versus Systemic

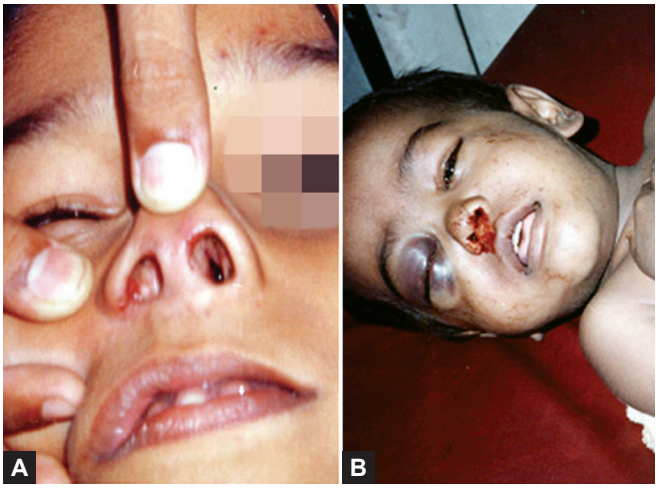
Local cause should be suspected when:

- Bleeding even if recurrent from the same site, e.g. recurrent epistaxis from one nostril may be due to excoriation of a superficial vessel in the Kisselbach triangle.
- Nose picking, polyps, foreign bodies (Fig. 1A) or rarely vascular anomalies may cause frequent nose bleeds.
- Profuse prolonged, bilateral nose bleeds that occur spontaneously without any trauma and are difficult to stop are suggestive of a coagulation defect. Rarely an angiofibroma or Rendu-Osler-Weber (Hereditary hemorrhagic telangiectasia) may cause profuse epistaxis (Figs 1A and B).
- Generalized petechiae, purpura, bruising, hematuria especially if associated with past history of bleeding from dental extractions or circumcision should arise suspicion of a bleeding disorder.

IS THE DEFECT INHERITED OR ACQUIRED?

Inherited Disorders

- Inherited disorders usually present in infancy and early childhood.
- Family history of bleeding disorder.
- Inherited disorers in milder forms may not be seen in early infancy and may present later in life with bleeding following injury or during surgery—mild hemophilia, von Willebrand disease.
- The development of bleeding later in childhood usually indicates an acquired disorder.



Figs 1A and B (A) Persistent recurrent bleeding from left nostril due to foreign body; (B) Bleeding from both nostrils and over the skin (DIC) (*Courtesy:* MR Lokeshwar)

Acquired Disorders

- Usually present later in life. Immune thrombocytopenic purpura (ITP) may however present during early childhood, i.e. 3 to 5 years of age.
- Have a negative family history.
- Underlying medical disorder that may affect hemostasis.
  - Hepatic disorders, malabsorption syndrome, may be associated with vit. K dependent coagulation factors.
  - *Renal disease:* Uremia can interfere with platelet function.
  - Low-molecular-weight coagulation proteins (factors IX and XI) are lost through the kidney in children with nephrotic syndrome.
  - Cyanotic congenital heart disease with polycythemia may have thrombocytopenia and hypofibrinogenemia with risk of bleeding and or thrombosis.
- *Infections:* Meningococcemia with DIC
- A detailed menstrual history should be obtained when applicable. The prevalence of bleeding disorders in women with menorrhagia is as high 20 percent conversely; menorrhagia is a common initial symptom in women with VWD and has been reported to occur more than 90 percent of patients.
- Past surgical procedures, serious injuries, fractures and tooth extractions without any abnormal bleeding is good evidence against the presence of a congenital hemorrhagic disorder.
- *Medications:* Aspirin and other nonsteroidal anti-inflammatory agents affect platelet aggregation. Prolonged use of antibiotics can lead to decreased levels of vitamin K deficiency leading to decreased production of liver dependent factors. Medication can also exacerbate bleeding in those with existing coagulation defects—use of aspirin in patient with hemophilia or low platelets.

INDICATIONS FOR EVALUATION

Investigations for bleeding disorders are done when there is:

- Recent bout of bleeding—unusual, spontaneous, prolonged, or delayed bleeding. Postsurgical and traumatic bleeding that is unexpected, prolonged or disproportionate to extent of injury.
- Family history of bleeding
- Abnormal coagulation test results obtained as a part of preoperative evaluation—preparation for surgery or invasive procedures
- Systemic diseases known to be associated with bleeding disorders, e.g. liver disorder, renal disorder, DIC and sepsis, etc.

Is the Bleeding due to Vascular, Platelet or a Coagulation Abnormality or a Combination of these?

Vascular disorder, thrombocytopenia or functional platelet disorders:

- Usually in the form of subcutaneous and mucus membrane bleeds like petechiae, purpura (Fig. 2C), ecchymoses, epistaxis and subconjunctival hemorrhage (Fig. 2B)
- Mucous membrane bleeding and menorrhagia can occur in von Willebrand disease also.

Factor deficiency:

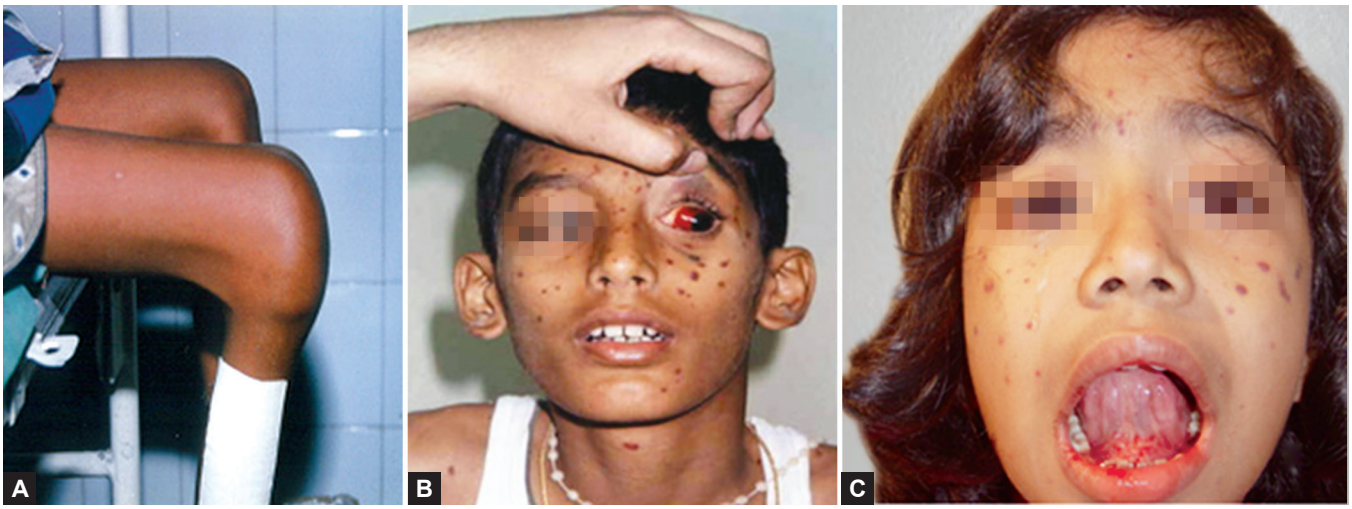
- *Hematomas (Fig. 3):* Intramuscular, soft tissue bleeding
- Hemarthrosis, retroperitoneal bleeds
- Post-traumatic bleeds are often delayed, some times hours after the injury.

Mucous membrane bleeding (epistaxis, excessive menorrhagia, bleeding from gums) is often the consequence of a problem with primary hemostasis. Namely a platelet disorder or von Willebrand disease (VWD).

- Hereditary hemorrhagic telangiectasia may also be manifested as mucosal bleeding
- Umbilical stump bleeding is typically seen with factor XIII deficiency, but it may also occur with deficiencies of prothrombin, factor X, and fibrinogen
- The immediate history often provides useful clues to diagnosis. A sick child with fever, shock, mucocutaneous purpura frequently has intravascular coagulation (DIC) (Fig. 6) associated with bacterial infection.

Family History of Bleeding

History of unusual bleeding in family members is present in hemophilia, von Willebrand and platelet function



Figs 2A to C (A) Bleeding in the knee joint; (B) Subconjunctival hemorrhage; (C) Purpura (*Courtesy:* MR Lokeshwar)

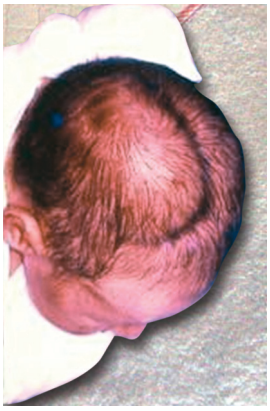


Fig. 3 Cephalhematoma in bleeding disorder (*Courtesy:* MR Lokeshwar)

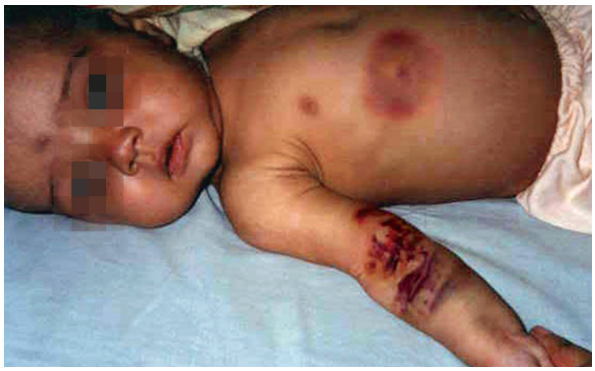


Fig. 4 Hemorrhagic disease of newborn (*Courtesy:* MR Lokeshwar)

disorders. Approximately a third of infants and young children with newly diagnosed hemophilia have a negative family history.

Proper pedigree chart, covering at least 2 to 3 generations also should take note of members who have expired, especially due to bleeding.

X-linked Recessive Pattern

Maternal brothers, cousins, uncles and maternal grandfather may be affected with X-linked transmission while the females remain asymptomatic carriers.

Bleeding disorders which have a sex-linked recessive inheritance are:

- Hemophilia A (factor VIII deficiency)
- Hemophilia B (factor IX deficiency)
- Wiscott-Aldrich's syndrome.

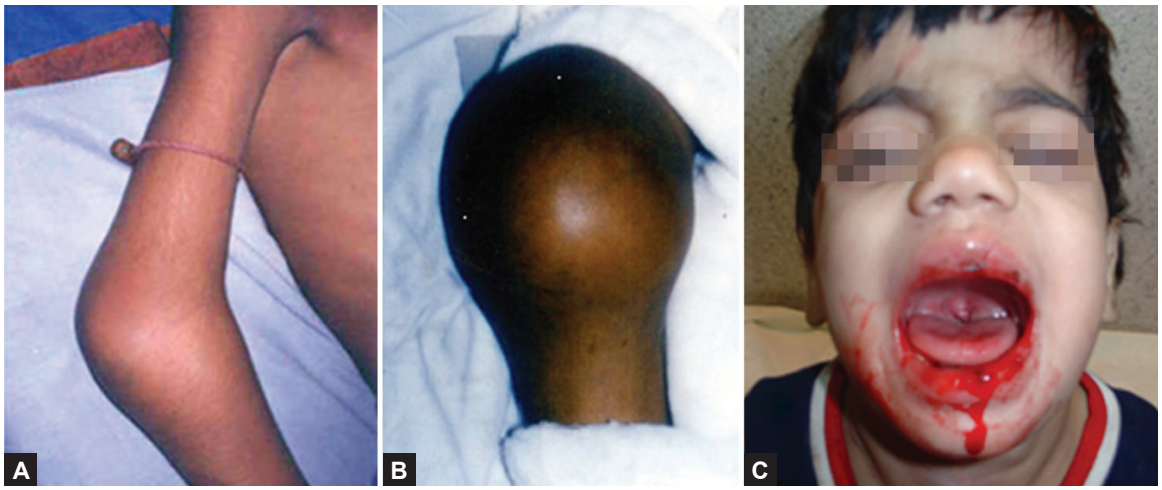
Autosomal recessive disorders: In autosomal recessive disorders, the parents of affected person are heterozygote and hence have a 50 percent plasma concentration of the relevant clotting factors. History of consanguinity should be asked for. This genetic pattern is typical of disorders of factor II, V, VII, X, XI, XII, XIII, prekallikrein and high molecular weight kininogens.

However, lack of plasma factor XII, prekallikrein or high molecular weight kininogens do not usually cause any clinically significant bleeding.

Autosomal Dominant Pattern of Inheritance

- Von Willebrand disease
- Qualitative platelet defects
- Dysfibrinogenemia
- Hereditary hemorrhagic telangiectasia.

This type of inheritance may show a variable penetrance and expressivity. Many members in different generations of the family may be affected.



Figs 5A to C (A and B) Bleeding in the joints in hemophilia; (C) Glanzmann's thrombasthenia (*Courtesy:* MR Lokeshwar)

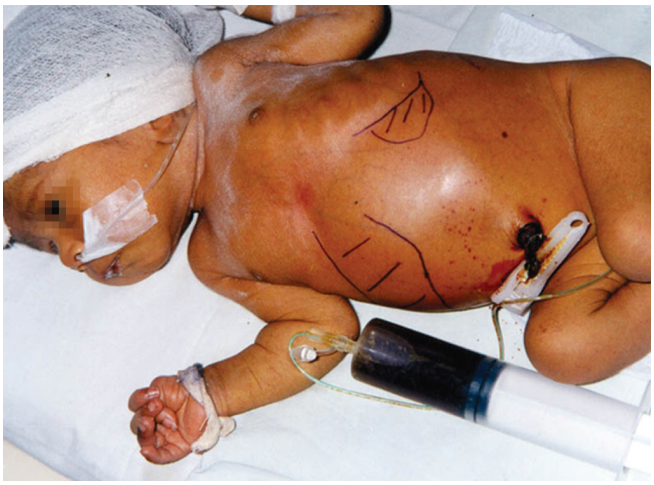


Fig. 6 Intravascular coagulation (DIC) (*Courtesy:* MR Lokeshwar)

- Negative family history does not rule out the possibility of inherited bleeding disorders.
- Family history might be negative, if the coagulation defect is mild.
- Spontaneous mutation, as is seen in 20 percent of patients with hemophilia A
- Exsanguinating bleeding is uncommon due to bleeding disorders and is more likely to be due to injury to major vessels.

History and physical examination can often help you make a specific diagnosis and point one to the right diagnostic tests.

- A male toddler who has just started crawling exhibits extensive bruising and or joint bleeding—diagnosis hemophilia A or B (Figs 2A, 5A and B)
- A girl who has had severe menorrhagia with frequent nose bleeds—possible VWD
- A well-looking child covered with petechiae often has immune thrombocytopenia (Figs 2C and 9)
- Hematuria with bruising localized to the gluteal region, ankles, and feet—Henoch-Schönlein purpura (Figs 10A and B).

ASSOCIATED UNDERLYING DISORDERS

Certain characteristic hemostatic defects are associated with specific clinical conditions.

- Liver diseases with factor II, VII, IX and X deficiency and fibrinolysis due to decreased clearance of activators and hypercoagulable state because of antithrombin III and protein C deficiency.
- Malabsorption states may be associated with multiple factor deficiencies, i.e. Vitamin K dependent factors.
- Acute promyelocytic leukemia is known to be associated with DIC due to increased cellular procoagulant activities.
- Myeloproliferative disorder may have platelet defects, thrombocytopenia and thrombocythemia.
- Amyloidosis may be associated with factor X deficiency and capillary fragility.
- Systemic lupus erythematosis—antibody to acidic phospholipase, autoantibodies to coagulation proteins and glycoproteins may be present, resulting in lupus inhibitors, factor deficiency, thrombocytopenia and thrombocytopathy.

CERTAIN SYNDROMES KNOWN TO BE ASSOCIATED WITH BLEEDING DISORDERS

- Hereditary hemorrhagic telangiectasia is associated with characteristic telangiectatic lesions in the mucus membrane and skin and may manifest with

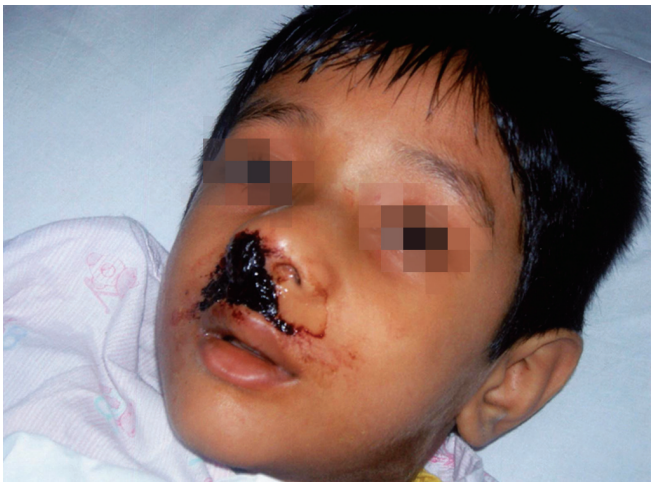


Fig. 7 von Willebrand disease (*Courtesy:* MR Lokeshwar)

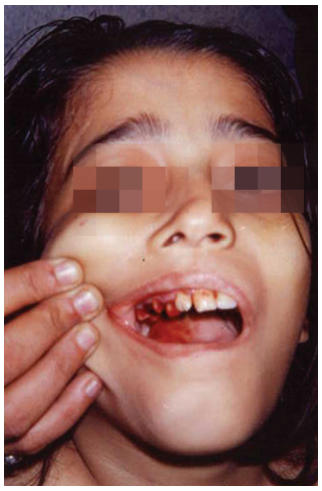


Fig. 8 Qualitative platelet defects (Glanzmann's thrombasthenia) (*Courtesy:* MR Lokeshwar)

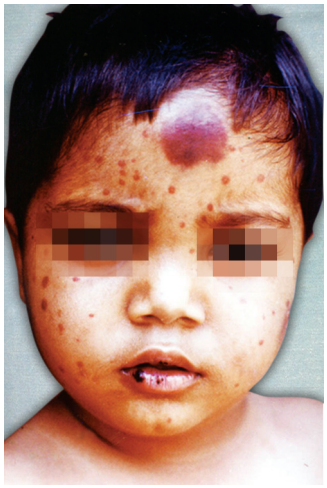
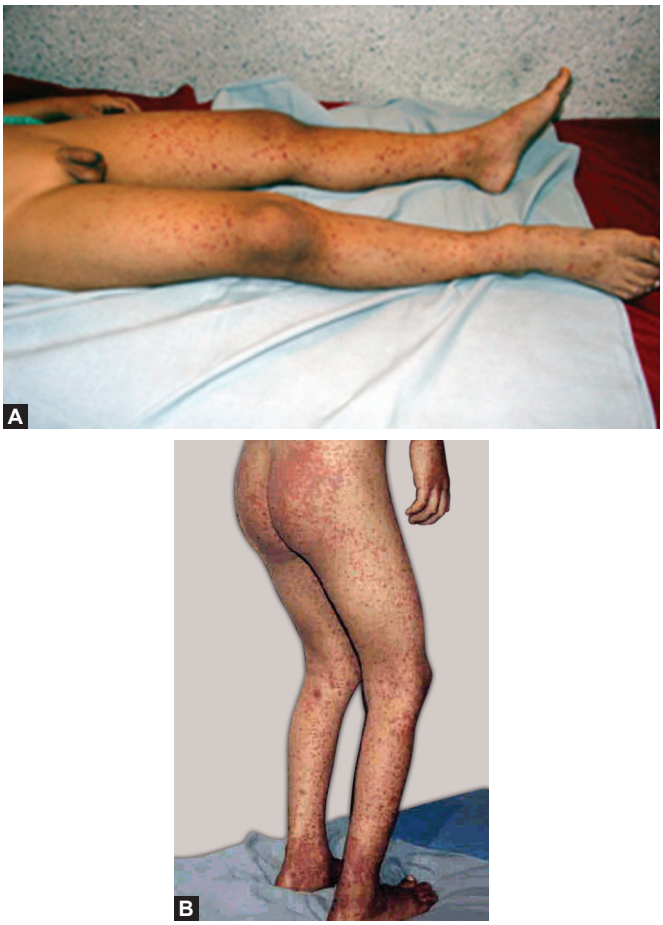


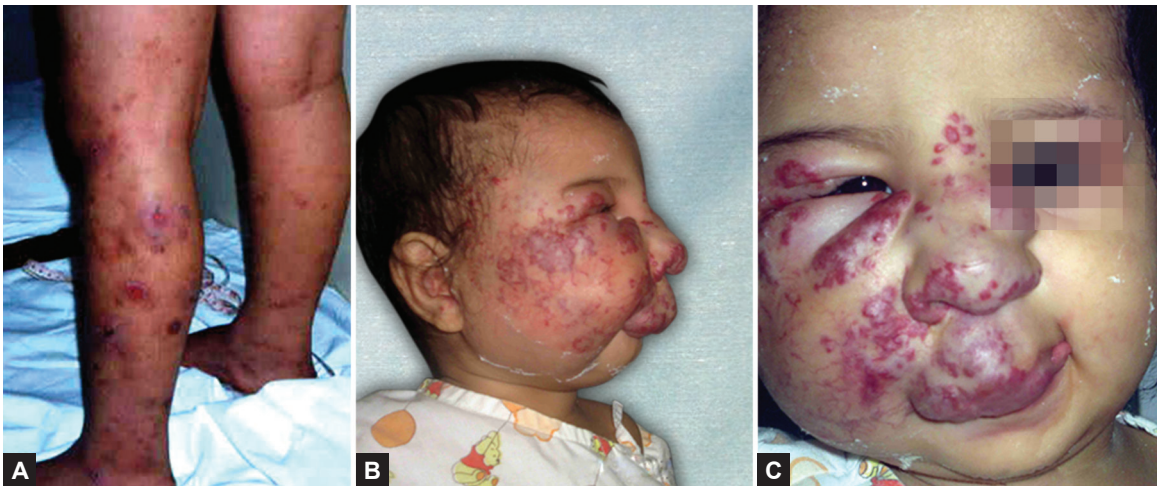
Fig. 9 Immune thrombocytopenic purpura (*Courtesy:* MR Lokeshwar)



Figs 10A and B Henoch-Schönlein purpura (*Courtesy:* MR Lokeshwar)

epistaxis, melena and bleeding per rectum. Presence of telangiectasia in the mucous membrane of nose, bulbar conjunctiva, tongue, lips and tips of fingers is the hallmark of diagnosis.

- Keloids may be seen in children with afibrinogenemia and factor XIII deficiency.
- Cigarette paper scar, hyperextensible joints suggest Ehler-Danlos syndrome.
- Presence of syndactyly with history of bleeding episode is known to be due to factor V deficiency.
- Wiskott-Aldrich syndrome (Fig. 11A) is associated with thrombocytopenia, recurrent infection, otitis media, and eczema.
- Children with albinism may have qualitative functional defects of platelets.
- Thrombocytopenia with absent radius (TAR syndrome) is easy to diagnose because of skeletal anomaly.
- Kasabach-Merritt syndrome is characterized by giant hemangioma associated with evidence of clinical and subclinical DIC and thrombocytopenia (Figs 11B and C).



Figs 11A to C (A) Wiskott-Aldrich syndrome; (B and C) Kasabach-Merritt syndrome (*Courtesy:* MR Lokeshwar)

Hemarthrosis (spontaneous), bleeding in the muscles without significant trauma points towards inherited coagulation disorders.

LABORATORY ASSESSMENT (TABLE 1)

Sample Collection and Technique

- A properly drawn blood sample is crucial for interpretation of the results of coagulation test.
- For coagulation assays blood should be obtained by clean venipuncture without air bubbles and without contamination by tissue fluids
- Drawing of samples from catheter often results in 'sample contamination or intravenous fluids and spuriously abnormal values
- Improper sample collection is one of the most common reasons for abnormal coagulation test
- Samples should be tested within 2 hours of collection if maintained at room temperature or within 4 hours if kept cold
- Plasma samples must be frozen if not tested within this time frame. When they are to be analyzed, frozen samples should be rapidly thawed at 37°C and tested immediately
- A panel of screening tests should be ordered, including a complete blood count with evaluation of platelet number, morphology-smear examination, PT, APTT, PFA (Platelet function analysis).

Though a thorough history and clinical evaluation helps in suspecting the nature and type of bleeding disorder, laboratory investigations are required to make a specific diagnosis, they can be conveniently divided into screening tests and special tests.

Table 1 Screening laboratory tests in hemostatic disorders

Platelet	*BT*	*PT*	*APTT*	*Interpretation*
↓	↑	Normal	Normal	Thrombocytopenia
↓	↑	↑	↑	DIC
↓	↑	Normal	↑	Type 2A vWD
Normal	↑	Normal	↑	vWD
Normal	↑	Normal	Normal	Platelet Dysfunction
Normal	Normal	↑	Normal	Factor VII deficiency
Normal	Normal	Normal	↑	XII, XI, IX, VIII
Normal	Normal	↑	↑	Liver disease, Vit K def, Combined def
Normal	Normal	Normal	Normal	XIII def, vascular

- Screening tests are done after evaluating the nature and clinical circumstances of bleeding and prior to surgery if indicated
- Specific tests need to be done to confirm the diagnosis after the history, physical examination and screening test point to a possible diagnosis.

Screening Tests

These are the tests for the initial assessment for bleeding tendency and include:

- CBC and PS examination
- Platelet count
- Bleeding time/PFA
- Clot retraction
- Prothrombin time/INR
- APTT.

CBC can reveal involvement of other cell lines in cases suspected to have leukemia, aplastic anemia, etc.

Proper Smear Examination also helps in Evaluating Extent of Thrombocytopenia if Present

- Presence of clumps of platelets rules out platelet deficiency and absence of platelets indicates severe thrombocytopenia usually less than 10,000–20,000/cumm
- Presence of platelets but not in clumps—indicates absence of aggregation, suggesting platelet functional disorder
- Large platelets simulating size of the lymphocytes suggest possibility of Bernard-Soulier syndrome
- Large platelets also indicate younger platelets as seen in regenerative type of thrombocytopenia where there is peripheral destruction of platelets
- Large platelets are also seen in the hereditary giant platelet syndromes
- Small platelets are characteristic Wiskott-Aldrich syndrome.

Platelet Count

- It is a simple first step in evaluating the cellular aspect of hemostasis
- However, manual count is not reliable and not reproducible and hence platelet count should be done on particle cell counter or using phase contrast microscope
- A spuriously low automated platelet count (pseudo thrombocytopenia) may result from ethylene diamine tetra acetic acid (EDTA) anticoagulant plus an IgG or 1gM platelet antibody, platelet cold agglutinins, or platelet clumping from a partially clotted sample. The normal platelet count (for all ages) ranges from 150,000 to 450,000/uL
- In the setting of thrombocytopenia increased or decreased platelet size may suggest platelet turnover or decreased production, respectively
- If platelet type of bleeding (petechiae, purpura, mucosal bleeding) is seen with normal platelet count or marginally low platelet count, then platelet functional disorders should be kept in mind.
- Electronic particle counters also provide a mean platelet volume and (size) distribution.

Bleeding Time

The bleeding time (BT) is a measure of the interaction of platelets with the blood vessel wall. This test evaluates primary hemostatic stage. It has major drawbacks and has been discarded in children by most hematologists.

The BT is an approximate measure of the relationship between platelet number and function.

The most widely used method is the modified ivy BT performed with a template. The BT is performed with a tourniquet maintained at 40 mm Hg and placement of the template device on an area of the forearm (Just below the elbow), blade makes a linear cut 1 to 2 mm deep.

With a stop watch and filter paper, the blood coming from the cut is gently blotted away while taking care to not touch the filter paper to the cut (which would remove the fragile platelet plug). A normal BT is 3 to 9 minutes with normally functioning platelets. The BT is an approximate measure of the relationship between platelet number and function.

Prolongation of bleeding time usually occurs at platelet count of < 50,000/cumm to 100,000/uL. At counts below 10,000/cumm, bleeding time is usually prolonged and is often 15 minutes or longer and hence BT should not be performed when platelets are low.

The BT can also be prolonged with congenital and acquired platelet defects.

Prolonged Bleeding Time with Nearly Normal Platelet Count

Qualitative Platelet Disorders (Table 2)

- Glanzmann's thrombasthenia (Figs 5C and 8)
- Bernard-Soulier syndrome
- Storage pool disorder
- Wiskott-Aldrich syndrome
- Acquired defects, uremia and cyanotic congenital heart disease
- Vasculitis (e.g. Henoch-Schönlein purpura)
- Connective tissue disorders such as Ehlers-Danlos syndrome
- Drugs like aspirin and nonsteroidal anti-inflammatory agents
- Von Willebrand disease (Fig. 7).

Bleeding time has been replaced by the platelet function analyzer (PFA) which is a rapid and automated form of platelet function assessment.

Clot retraction: This is not any more a commonly ordered test as a screening or diagnostic test any more.

Retraction and exudation of the serum after one hour is observed in the clotting tube. Normally, 50 percent exudation at the end of one hour of the original blood volume is taken as normal retraction.

Prothrombin Time

Normal (reference) range varies depending on the laboratory (its instrumentation and the lot of thromboplastin), but it is generally 10 to 11 seconds.

- PT measures extrinsic clotting system and the common pathway, the activities of factors I (fibrinogen), II

Table 2 Platelet aggregation response

Condition	*Platelet*		*Aggregation with*					*Comment/ Further tests*
	Count	*Size*	*ADP*	*Col*	*Ri*	*AA*	*A23187*	
Thrombasthenia	N	N	0	0	1	0	0	IIb/IIa expression
Bernard-Soulier syndrome	Low	Large	N	N	O	N	N	Gp1b expression
Storage pool defect (δ)	N	N	1	R	1	1/0	R	ATP: ADP pools
Cyclo-oxygenase deficiency	N	N	1/N	R	N	R	R	Responds to endoperoxide
Thromboxane synthetase deficiency	N	N	1/N	R	N	R/0	N	
Aspirin ingestion NSAID and retest	N	N	1	R	N	R/0	N/R	Stop aspirin
Ehlers-Danlos syndrome	N	N	N	N	N	N	N	
von Willebrand disease	N	N	N	N	0/R	N	N	Assay vWF:Ag and RiCoF

(prothrombin), V, VII, and X. PT is prolonged with deficiencies of plasma factor VII, X, V, II and fibrinogen and inhibitors of these factors

- Prolongation of the PT beyond the reference range is not generally seen until the functional level of one of these factors is less than 30 percent or until fibrinogen is less than 100 mg/dL
- A prolongation of PT with normal PTT indicates factor VII deficiency OR early in the course of anticoagulant therapy, Vitamin K deficiency (Fig. 4) or liver diseases
- The PT is also used to monitor the effect of coumarin-type anticoagulants.

Activated Partial Thromboplastin Time

Activated partial thromboplastin time (APTT) is an excellent screening test for determining abnormality of intrinsic and common pathway.

- It should be noted that the sensitivity and reproducibility of the APTT are highly dependent on the specific reagents used (particularly the activator in the partial thromboplastin reagent)
- With most APTT reagents, the APTT will not he prolonged until the amount of factor VIII is less than 35 percent (0.35 U/mL).

The reference range will generally be approximately 20 to 35 seconds for children and adults but longer (30 to 54 seconds) in term infants (and often even longer in premature infants).

The APTT measures factors I (fibrinogen), II (prothrombin), V, VIII, IX, X, XI, and XII; prekallikrein; and high-molecular-weight kininogen.

Activated Partial Thromboplastin Time is Prolonged

- During deficiency or abnormalities of high molecular weight kininogen, prekallikren, factor XII and XI, IX, VIII, X, V, II and fibrinogen
- By inhibitors of blood coagulation such as lupus inhibitors, heparin, fibrin/fibrinogen degradation product
- Deficiency of any of the latter three factors (prekallikrein; and high-molecular-weight kininogen) can result in a markedly prolonged APTT in the absence of clinically significant bleeding
- Isolated prolongation of the APTT in a patient with clinical bleeding is likely to result from a deficiency of factor VIII, IX, or XI.

Mixing study in prolonged PTT: The presumption is that addition of normal plasma to a 50 percent mix should correct the PTT if it is due to factor deficiency. Among hospitalized infants or children, unintentional contamination of patient samples with heparin is a common cause of an unexpected prolongation of the APTT

that does not correct on mixing. Failure to correct after a mix of 50 percent of normal plasma is indicative of the presence of inhibitors or antibody that indiscriminately (not specific against any factor) suppresses fibrin formation in patients and normal plasma. Lupus anticoagulant (a misnomer as it is not necessarily associated with SLE) is a very common cause of isolated prolonged PTT in children with adenotonsillar hypertrophy and infections.

Thrombin Clotting Time

The thrombin clotting time (TCT or TT) measures the thrombin-induced conversion of fibrinogen to fibrin and is performed by adding bovine thrombin to the patient's citrated plasma and recording the clotting time.

An extremely prolonged TT usually indicates a heparin effect. Reptilase, a snake venom protease, clots fibrinogen in the presence of heparin and thus can be used to identify heparin as the cause of a prolonged TT. Thus, in the presence of heparin the TT is prolonged, whereas the reptilase time is normal.

Thrombin Clotting Time is Abnormal in Patients with

- Hypofibrinogenemia whether acquired or congenital or
- Dysfibrinogenemia
- In presence of inhibitors like heparin, myeloma proteins and fibrin degradation products which block either thrombin cleavage of fibrinopeptide or fibrin monomer polymerization.
- A normal or minimally low platelet count with prolonged BT or abnormal PFA and poor clot retraction indicates the possibility of platelet functional disorders
- *Platelet aggregation studies:* Low and high concentration of ADP, epinephrine, collagen and Ristocetin are used for aggregation studies. First phase of aggregation is induced by low concentration of ADP and by direct effect of certain agents notably epinephrine and thrombin. Second phase is mediated by thromboxane A_2 and endogenous ADP released in response to numerous pharmacological and naturally occurring substance like ADP itself. Absence of first phase of aggregation—unresponsiveness to ADP in any concentration is characteristic of Glanzmann's disease. Deficiency of platelet fibrinogen and specific glycoproteins GP-II and GP-III confirm the diagnosis
- von Willebrand disease has characteristic lack of aggregation with ristocetin alone
- The classical laboratory findings in Bernard-Souiler syndrome are—prolonged bleeding time, thrombocytopenia, very large platelets on peripheral smear, deficient platelet adhesion and normal platelet aggregation with ADP, epinephrine and collagen but absence of platelet aggregation with ristocetin but with normal levels of von Willebrand factor.

In Glanzmann's thrombasthenia, the patient's platelets will agglutinate normally with ristocetin but not at all with the addition of adenosine diphosphate, epinephrine, collagen, or arachidonic acid. In Bernard-Soulier syndrome, platelet agglutination will occur normally on addition of each of these agonists except ristocetin.

Bleeding disorders not associated with any abnormalities in screening tests are:

- Factor XIII deficiency
- Alpha 2 antiplasmin deficiency, amyloidosis (may or may not be associated with factor X deficiency)
- Vascular disorders like hemorrhagic telangiectasia (Rendu-Osler-Weber syndrome)
- Scurvy—prolonged BT
- Ehlers'-Danlos syndrome—prolonged BT
- Henoch-Schönlein's purpura—prolonged BT
- Mild factor deficiencies—factor assay
- Battered baby syndrome (Figs 12A and B).

Special Confirmatory Tests

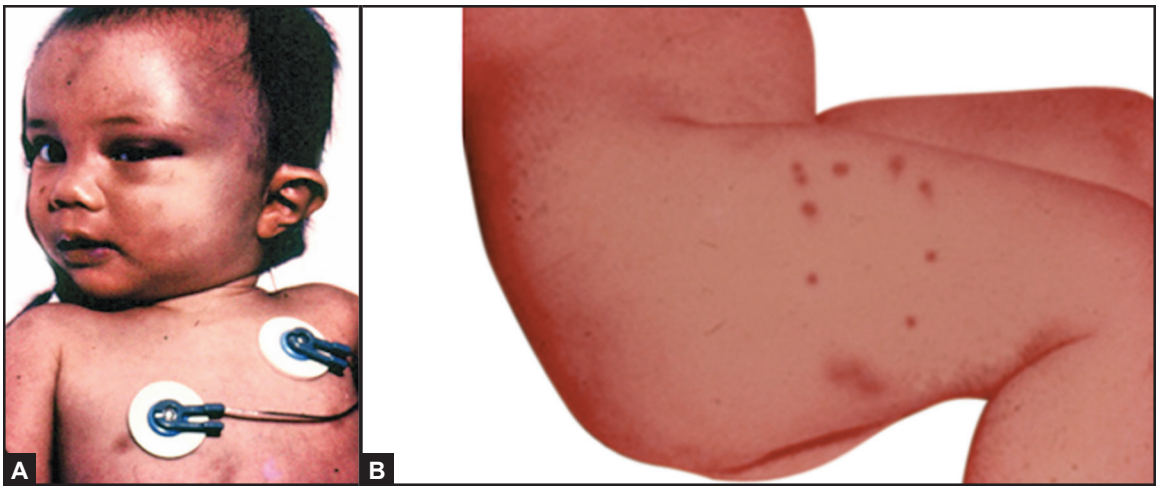
- *Specific coagulation factors:* Each of the coagulation factors of the intrinsic pathway (prekallikrein, high-molecular–weight kininogen, and factors VIII, IX, XL, and XII) can be measured by one stage, APTT based method
- FDPs are usually measured in serum samples because these degradation products consist predominantly of nonclottable derivatives that remain in solution after clot
- The D-dimer assay will identify only cross-linked FDPs (indicating that fibrin has formed intravascular, has been cross-linked, and has then been cleaved into D-dimers by plasmin
- Euglobulin clot lysis time.

The euglobulin clot lysis time (ECLT) is a screening test for excessive fibrinolysis. The normal ECLT is 60 to 300 minutes. It is shortened in conditions characterized by increased fibrinolysis (e.g. antiplasmin deficiency, plasminogen activator inhibitor 1 deficiency or systemic fibrinolysis).

Lupus anticoagulant: Abnormal mixing study followed by dilute russel vipor venom test (DRVVT) and platelet neutralization procedure (PNP) and for confirmation.

Solitary thrombocytopenia may be due to either:

- Reduced production or increased destruction of the platelets
- Idiopathic thrombocytopenic purpura is characterized by acute onset of isolated thrombocytopenia in otherwise healthy children. Other cell lines are normal and the smear reveals normal RBC, white cells with low number of platelets and occasional macrothrombocytes



Figs 12A and B Battered baby syndrome—hematoma over the forehead and punch mark over the thigh (*Courtesy:* Raj Warrier)

- Thrombocytopenia due to decreased production may be either due to involvement of only megakaryocytes as seen in TAR syndrome or in amegakaryocytic thrombocytopenic purpura and is characterized by thrombocytopenia and platelet type of bleeding.

CONCLUSION

Detailed history, thorough clinical examination and screening tests usually give sufficient information to decide, whether bleeding is due to local causes or a generalized bleeding disorder.

Depending upon type and nature of the bleeding disorder, further tests such as factor assay, aggregation tests, etc. have to be carried out to confirm the diagnosis before planning therapy.

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CHAPTER 28

Diagnosis and Management of Hemophilia Patients

Farah Jijina

Hemophilia is an X-linked hereditary bleeding disorder. Hemophilia A is the most common of these, with an annual incidence of 1/5000 male births worldwide. Hemarthrosis is the most common, most painful, physically, economically, and psychologically debilitating manifestation of hemophilia. It occurs in 90 percent of severe hemophiliacs. Prompt replacement therapy with the required factor remains the mainstay of treatment of a bleed.

The dose and choice of the product are influenced by the severity of the disease, the site and severity of the bleeding, inhibitor antibody status and the clinical scenario. However, the adjuvant role of antifibrinolytic agents and expert physical therapy in the treatment cannot be undermined in an economically burdened society like ours. It is possible to carry out any procedure on a hemophilic patient provided adequate factor is given. Outcome is directly related to the intensity of treatment and the level of compliance. Improvements translate into decrease absence from school or work, fewer bleeds and days spent in the hospital, increased personal and professional productivity, improved overall performance status and a healthier self image. Carrier testing and prenatal diagnosis can be offered to women who are interested in children bearing.

INTRODUCTION

The most prevalent of the hereditary disorders of coagulation are:

Types of hemophilia	
Hemophilia A	Factor VIII deficiency
Hemophilia B	Factor IX deficiency
von Willebrand disease	von Willebrand factor deficiency
Hemophilia C	Factor XI deficiency

CLINICAL FEATURES

- Characteristically deep/internal bleeding
- Usually spontaneous or following minimal trauma
- Occurs in frequent unpredictable episodes
- Delayed bleeding due to failure of secondary hemostasis.

Grades of Severity

Severe	Factor levels < 1%	Spontaneous bleeds
Moderate	Factor levels 2–5%	Bleeds following minor trauma or after procedures
Mild	Factor levels 6–40%	Usually do not bleed except following major trauma or surgery

Diagnosis

When to suspect?
If any of the following are present:
• Recurrent spontaneous bleeds
• Bleeding following trauma out of proportion to the injury
• Positive family history

Investigations

- A patient who is clinically suspected to have hemophilia should be subjected to coagulation tests.
- In hemophilia, the APTT is prolonged and corrects on addition of normal pooled plasma. The PT and TT are characteristically normal.
- However, in case of combined deficiencies for example, combined factor V and VIII deficiency, the PT will also be prolonged.
- Factor assay is then done to confirm the diagnosis and document the severity of the disease.
- One of the problems, we face in our country is the lack of adequate laboratory facilities at all places to carry out all the tests. Therefore a clinician suspecting a bleeding disorder should refer the patient to a center that specializes in carrying out these investigations to ensure an early accurate diagnosis. Now, most of the major cities have these diagnostic facilities.
- Once the patient is accurately diagnosed, further treatment can be given by the local treating physician with some guidance from a specialist center. The patient may then be referred to a higher center only for complicated problems. A wrong diagnosis at the beginning would result in the patient being given inappropriate treatment and the development of complications.

Treatment of Hemophilia

In the Western world today, it is possible for a child with hemophilia receiving adequate treatment to live a near normal life. An accurate diagnosis is quickly established, the family is educated on the management, and the child is put appropriate factor therapy. With this type of treatment most children with hemophilia (apart from the small number who develop inhibitors) can go to school, enjoy sports, and expect to have minimal or no joint bleeding. This is however expensive and not feasible for us. Thus we need to have our own strategies to treat our patients at lower costs.

GENERAL PRINCIPLES

Before we discuss the definitive therapy of hemophilia there are certain general precautions, *do's and dont's* which the treating physician, the parents of the patient and the patient himself can follow. All patients and their relatives need to be educated about the disease, precautions and preventive strategies for bleeding.

To Avoid

- IM injections
- All contact sports
- Aspirin and other nonsteroidal anti-inflammatory drugs (NSAIDs); all drugs that affect platelet function.

To Do

- Early factor correction; prompt treatment of a bleed. This prevents subsequent damage and deformity.
- Ice application at the site of bleed.
- All procedures to be done under appropriate factor cover, including dental procedures.
- Getting all vaccinations is recommended, including hepatitis A and B; these need not be given intramuscular but can be given subcutaneously.
- Maintaining a healthy body weight is important to avoid extra stress on joints. Thus mothers need to be educated on a proper diet for these children.
- Regular supervised physical therapy and exercises should be taught to children from an early age. This helps to develop strong muscles and thereby prevent bleeding into the joints. This is one of the most important preventive measures that the child and his parents can do.
- Very often the local physicians and dentists are not aware of the problem or the relatives tend to hide the problem and the patient comes to us with complications of an avoidable bleed. Thus, it is important to educate the parents and the patient.
- It is also worthwhile to inform the principal and teachers of the school, regarding the problems faced by these children.
- This ensures immediate institution of measures to control the bleed when it occurs.
- It should be highlighted to the caretakers that no form of physical punishment be given to the child. We have had many children coming to us with muscle bleeds following physical punishment in school. This is highly unfortunate and all efforts should be made to prevent it.

Definitive Treatment

Prompt replacement therapy with the required factor remains the mainstay of treatment of a bleed.

The dose of factor replacement (in units) is calculated as follows:

- *Hemophilia A:*
  - Each unit of factor VIII infused/kg body weight is assumed to yield a 2 percent rise in plasma factor VIII complains of fever since 1 day back levels.

  - Units of factor VIII to be given = (Desired level – patients level) × weight in kg/2. This dose is given stat and is followed by half the dose at 12 hourly intervals as required, as the biological half life of factor VIII is 8 to 12 hours
  - In case cryoprecipitate is used, the number of bags to be given is calculated according to the amount of factor VIII in each bag of cryoprecipitate. This information is available with the respective blood bank.
- *Hemophilia B:*
  - Each unit of factor IX infused/kg body weight, is assumed to yield a 1 percent rise in plasma factor IX levels
  - Units of factor IX to be given = (Desired level – patients level) × weight in kg
  - This dose is given stat and is followed by half the dose at 24 hours intervals as required, as the biological half life of factor IX is 18 to 24 hours.

HEMOPHILIC ARTHROPATHY

Hemarthrosis is the most common, most painful and most physically, economically and psychologically debilitating manifestation of hemophilia, occurring in 90 percent of severely affected patients.

In fact most of the physical, psychosocial and financial problems in a severe hemophilia patient are caused by the effects of recurrent hemarthrosis and chronic arthritis. This hemorrhage may occur spontaneously or as a result of trauma.

Pathophysiology

There is a complex relationship between recurrent bleeding, synovitis and the development of arthritis in a patient with hemophilia.

Bleeding into the joint originates from the richly vascular synovial plexus, developing spontaneously or as a result of imperceptible/trivial trauma. Following the development of the hemarthrosis, the synovial space is distended with blood; the joint becomes swollen, hot, tense leading to muscular spasm and restriction of movement. This hemorrhage will ignite an inflammatory response with the release of kinins and macrophage interleukin-1 (IL-1).

Absorbance of the intra-articular blood is usually incomplete, as the synoviocytes can absorb only a limited amount of iron and their capacity is reached easily. The excess clot formed is, therefore, unlikely to be totally removed by the fibrinolytic system and organization of the remaining clot leads to development of fibrous adhesions. The retained blood produces a chronic inflammation and hyperemia of the synovial membrane and the joint may remain swollen, painful and tender even in the absence of bleeding.

Acute hemarthrosis invariably recurs from time to time. The first episodes are generally relatively mild and the joint may regain normal function. However, with each recurrence, the synovium becomes invariably more thickened and vascular. Synovial folds and frond like villi form, which become trapped and crushed by joint action, leading to further bleeding and synovial enlargement.

There is a simultaneous weakening of the periarticular supporting structures which leads to joint instability and further predisposes the joint to recurrent bleeding.

Thus a vicious cycle of bleeding—synovitis—bleeding sets in, because of the profusion and fragility of the vessels within the tissue.

Ultimately, there is a gradual conversion of the synovium from friable hyperemic tissue to fibrotic scar tissue.

This is followed by subchondral and synovial ischemia which results in progressive loss of hyaline cartilage. As the joint cartilage progressively degrades, deterioration of joint function occurs leading to limited and painful movements.

Finally, the stage of chronic hemophilic arthropathy is reached. The cartilage becomes pitted and destroyed. There is loss of joint space; bony necrosis and cyst formation may occur. This may lead to complete destruction of the joint with fibrous or bony ankylosis.

Over a period of time, osteoporosis may develop as a result of disuse and immobilization of the joint.

Clinical Features

Hemarthrosis occurs in 90 percent of patients with severe hemophilia. Though any joint may be affected, weight bearing joints are more prone to bleed. The knee joint is the most commonly involved and the most often permanently crippled.

The joints affected in order of frequency are knee > elbow > ankle > shoulder > wrist > hip. The spine is rarely involved.

The first episode of hemarthrosis usually occurs when the child begins to walk or crawl. There is often a "target joint", one which is more prone to repetitive bleeding. The onset of bleed is often heralded by an "aura", which may be a feeling of vague warmth/tingling sensation/a sense of mild restlessness/or anxiety.

A hemophilic patient may present in various ways:

Acute Bleed in a Relatively Normal Joint

The earliest definitive symptom is excruciating pain in the affected joint. It is swollen, warm and tender, with restriction of movement and muscle spasm.

Subacute or Chronic Synovitis

In these patients, in spite of no active bleed, the joint appears swollen, tender and inflamed with a boggy synovium and some restrictions of movement (Figs 1A and B).

Chronic Hemophilic Arthropathy

If untreated, symptoms of chronic arthropathy typically develop by the second or third decade. In chronically damaged joints due to the thickening of the articular capsule, external evidence of bleeding may not show.
These patients may present with pain in the joint due to:

- The degenerative arthritis
- Referred pain due to bleeding in adjoining structures
- Actual bleeding in the joint, which may not be apparent clinically

It is also important to keep in mind that HIV positive hemophiliacs are prone to develop septic arthritis, the features of which may mimic an acute bleed.

Practical Approach to Management of Hemophilia Patients in India

Optimal treatment of acute hemarthrosis involves:

- Factor replacement
- Relief of pain
- Rest
- Supervised rehabilitation

- *Factor replacement:* Early factor replacement is the mainstay of treatment
- Prompt replacement.

Prompt Replacement

- It reduces duration of bleed
- It reduces joint damage, absenteeism
- It reduces overall amount of factor required and cost.

Doses: 25 to 30 percent correction, with factor VIII is given IV, 12 hourly. Though these are standard textbook recommended doses, one can generally use less. Often 10 u/kg can be given, and repeated if required.

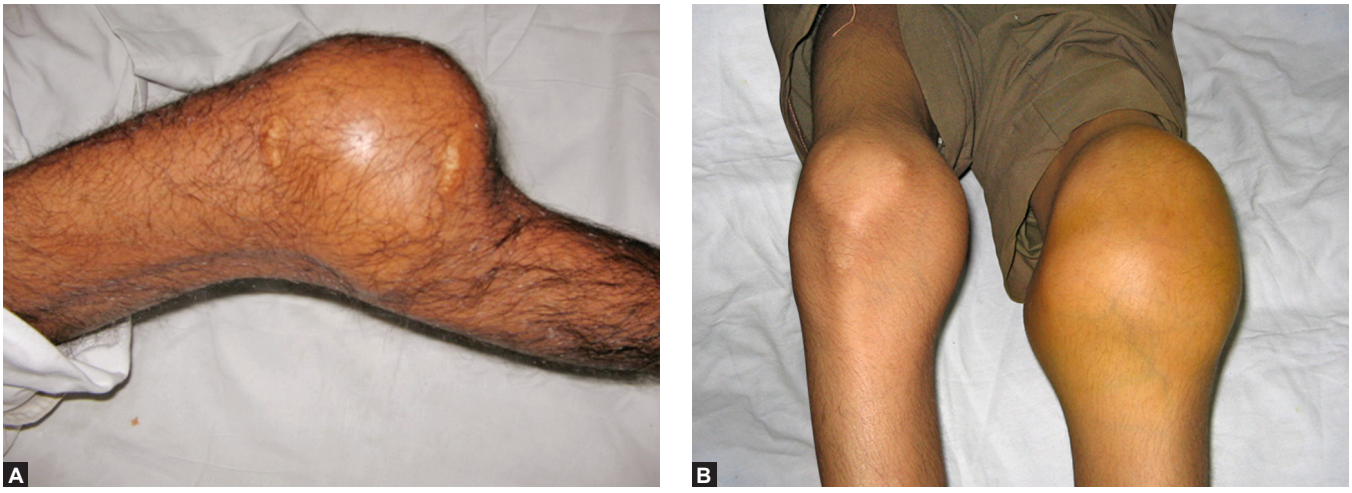
The duration is decided on the basis of the patients' symptoms. Once the pain subsides and there is no increase in the swelling, further factor need not be given. Thus most patients may require 1 to 3 doses only. It is important to note that the swelling itself may take a while to subside and in situations where there is a shortage of factor and finances are tight, one need not continue with the factor.

Home Therapy Programs

Wherever possible it is advantageous for the patient or a family member to be taught to administer the factor.

Advantages

- Improved preservation of joint function, as no time is wasted in getting the factor



Figs 1A and B Severe hemophilia showing chronic synovitis of the knee joint

- Decreased hospital care and cost
- Decreased absenteeism from school and work
- Patient is more active, mobile and independent
- Self-reliance improves the patients' confidence and family interaction.

Relief of Pain

- Analgesics
- Ice application

All patients should be given analgesics to relieve the pain. The drugs which can be safely used in these patients are dextropropoxyphene (proxyvon), paracetamol (crocin), opiods and Cox 2 inhibitors. Nonsteroidal anti-inflammatory drugs (NSAIDs) which affect platelet function should be avoided.

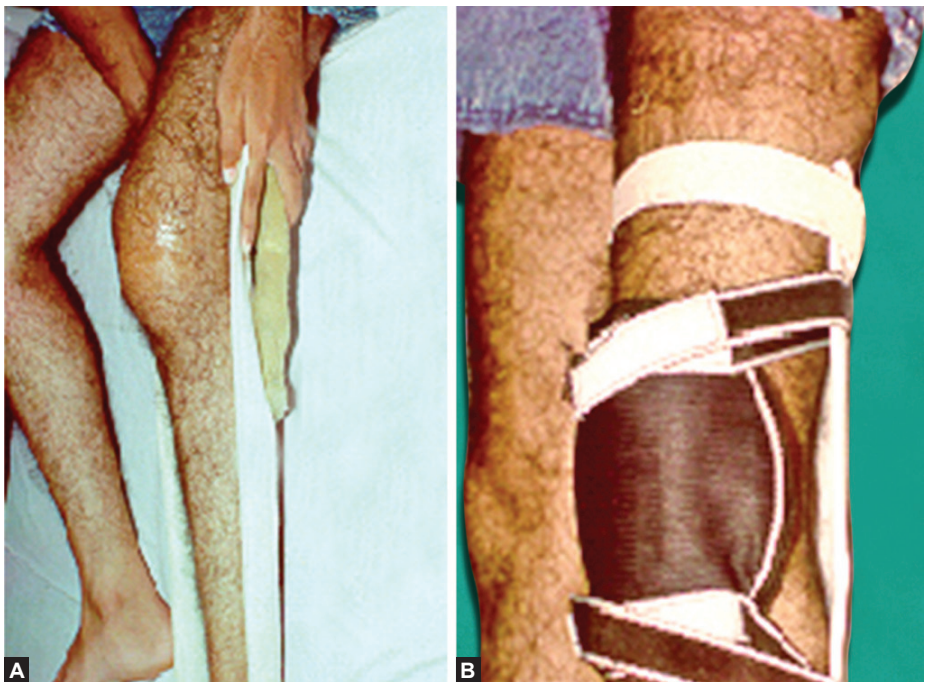
We usually recommend that the patient applies crushed ice or an icepack to the joint, over a wet towel intermittently for periods of 5 minutes to achieve a 10 to 15°C lowering of temperature in the deeper tissues. The efficacy of this however, is not definitely proven.

Physical and Rehabilitative Therapy

- Early physical therapy should be instituted as soon as the acute hemarthrosis subsides, i.e. once the pain subsides. Exercises should be increased gradually.
- However, energetic physiotherapy should be done only in conjunction with small doses of factor replacement
- Orthopedic devices—splints/braces/traction/corrective footwear—may be used as per the patients needs (Figs 2 to 4).

Prophylactic Therapy

- Prophylaxis is the treatment by intravenous injection of factor concentrate to prevent anticipated bleeding.
- The goal of prophylactic therapy is to convert a severe hemophilia patient to a moderate hemophilic by maintaining trough levels of factor above 1 percent and thereby preventing spontaneous bleeds.
- This prevents bleeding and joint destruction and helps to preserve normal musculoskeletal function.
- In patients with repeated bleeding, particularly into target joints, short-term prophylaxis for 4 to 8 weeks can be used to interrupt the bleeding cycle. This may be combined with intensive physiotherapy or synoviorthesis.
- Prophylactic administration of clotting factor concentrates is advisable prior to engaging in activities with higher risk of injury.
- Prophylaxis is best given in the morning to cover periods of activity.



Figs 2A and B Severe hemophilia A with chronic synovitis undergoing dual force stretching and exercise. The patient recovered completely in two weeks time

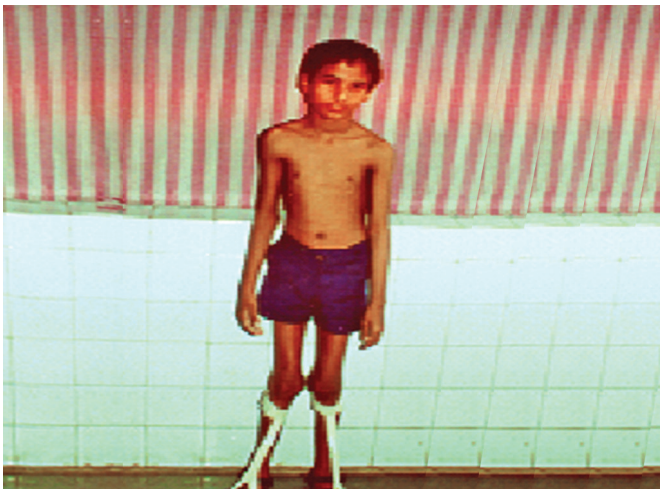
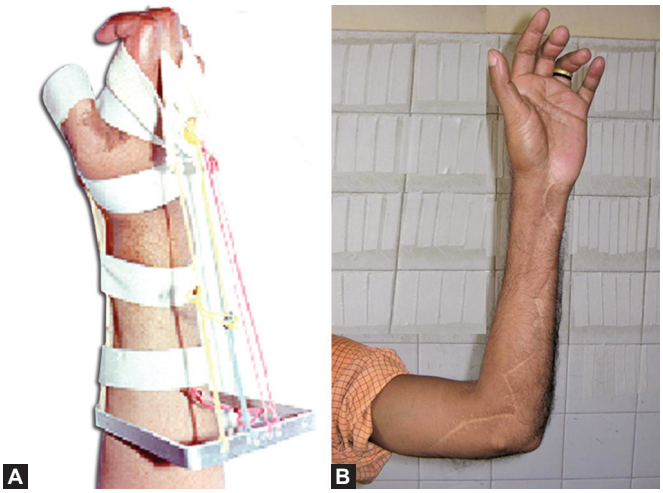


Fig. 3 Customized device for footdrop. This patient also developed this drop as a result of compartment syndrome



Figs 4A and B Customized traction system for Volkmann's ischemic contracture in a patient of severe hemophilia A. The patient is now fully functionally and is an active member of the society

Primary Prophylaxis

Regular continuous treatment initiated in the absence of documented osteochondral joint disease, determined by physical examination and/or imaging studies, and started before the second clinically evident large joint bleed and age 3 years

Secondary Prophylaxis

Regular continuous treatment started after 2 or more bleeds into large joints and before the onset of joint disease documented by physical examination and imaging studies.

Tertiary Prophylaxis

Regular continuous treatment started after the onset of joint disease documented by physical examination and plain radiographs of the affected joints.

Intermittent (Periodic) Prophylaxis

Treatment given to prevent bleeding for periods not exceeding 45 weeks in a year.

Continuous is defined as the intent of treating for 52 weeks per year and receiving a minimum of an a priori defined frequency of infusions for at least 45 weeks (85%) of the year under consideration.

Large joints = ankles, knees, hips, elbows and shoulders.

Administration and Dosing Schedules

Prophylactic Therapy Dose

Factor VIII 25 to 40 u/kg thrice a week
Factor IX 25 to 40 u/kg twice a week

These dosage schedules are expensive. Therefore, different countries follow different protocols for prophylaxis, and the optimal regimen remains to be defined.

Thus we now have the concept of low dose prophylaxis, using 10 u/kg twice a week, which is gaining popularity in developing countries.

Muscle Bleeds and Hematomas (Figs 5 and 6)

- Seventy-five percent of patients present with soft tissue bleeds
- The most common site is the ileopsoas muscle
- This leads to reflex muscle spasm and joint flexion deformity
- It may lead to complications such as compression of adjacent nerves, vessels; muscle atrophy; contractures
- Large bleeds may produce fever, hyperbilirubinemia and neutrophilia.

Treatment

- Factor correction—soft tissue bleeds generally require more factor correction, about 50 to 60 percent. One can try with lesser doses if availability or finances are a problem.

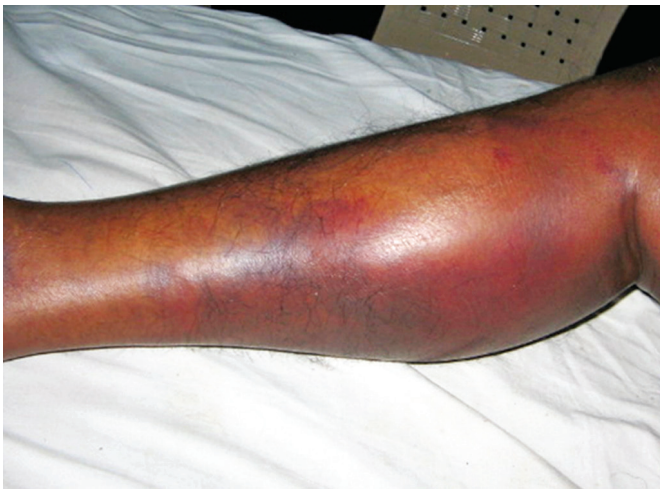


Fig. 5 Muscle hematoma in a severe hemophilia patient. Responded to conservative management

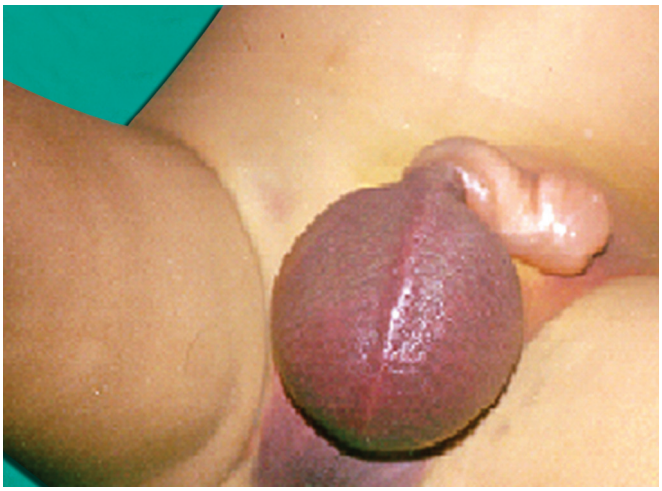


Fig. 6 Scrotal hematoma in a severe hemophilia patient. Responded to conservative management

- Patients may require the factor to be given for at least 3 to 4 days or more, depending on the response. However, once the patient responds, further factor need not be given.
- Once the pain subsides the patient must be given supervised physical therapy to prevent complications.

CNS Bleeds

CNS bleeds are a frequent occurrence in hemophiliacs and one of the major causes of mortality.

- They develop in 10 to 20 percent of patients
- However less than 50 percent give history of trauma
- They may be subdural, intracranial or subarachnoid
- They require full factor replacement for a longer duration.

Treatment

- It should be noninvasive as far as possible; surgical intervention is rarely required except in those patients with neurological deficit or an altered sensorium.
- Aggressive 100 percent factor correction should be given and continued for at least 8 to 10 days.
- Antifibrinolytic drugs such as trenexamic acid, may be given for 6 to 8 weeks.

Gastrointestinal Bleeds

- They are seen in 15 to 20 percent of patients
- Bleeding may be exacerbated by an underlying peptic ulcer; in fact peptic ulcer disease is more common in hemophiliacs than in the general population
- The patient must also be investigated for an underlying disease such as ulcer, chronic liver disease, GI malignancy, etc. especially in patients who are HbSAg or HCV positive.

Treatment

- Factor concentrates of about 40 to 60 percent correction may be required
- They are given till the bleeding stops
- H_2 receptor blocking agents may be given simultaneously
- Antifibrinolytic drugs can also be given.

Urinary Tract Bleeding

- Sixty-six to ninety percent of patients will experience at least one episode of hematuria
- Most patients present with spontaneous painless hematuria
- All patients must also be investigated to rule out an underlying organic cause, e.g. calculus, malignancy.

Treatment

- Recommended first line treatment for hematuria is to increase fluid intake to 2 to 3 liters per day, either oral or parental
- Most patients will respond to conservative therapy
- If hematuria persists, factor correction 50 to 80 percent should be given, till bleeding stops
- Antifibrinolytic drugs are contraindicated in hematuria.

Mucous Membrane Bleeds

- These commonly present as epistaxis, gum bleeds, tongue bleeds or from the frenulum in infants, and in children following loss of decidous teeth, etc.
- Whenever a clot forms, the body attempts to break it down within the vessel so that blood flow can be restored. This process of clot breakdown called fibrinolysis is very active on mucous membrane surfaces. In people with hemophilia, this process can prevent a bleed from stopping.
- Thus these bleeds especially those following injury are often difficult to treat and may end up consuming a lot of factor.
- The problem is worse in children as they tend to continuously disturb the clot with their tongue.

Treatment

Fibrinolysis can be inhibited by drugs, and tranexamic acid is the most widely used drug for this.

- It is advisable to treat with local measures such as EACA application to the site of bleeding or fibrin glue where feasible since the drug is absorbed from the buckle mucous membrane and then secreted into the saliva
- Oral EACA tablets or a mouthwash prepared by crushing or dissolving the tablets can be used
- If this does not arrest the bleed, then factor replacement 30 to 40 percent correction is given till the bleed stops.

Chronic Hemophilic Arthropathy

This is a common problem we face in our country, due to inadequate treatment of an acute hemarthrosis. Patients often come with deformed joints and marked restriction of movement. Treatment of this can be conservative or surgical, depending on the individual patient.

Conservative Treatment

- Factor replacement with gradual supervised physical therapy
- Synovectomy to control bleeding and prevent progressive destruction of the articular cartilage.

Types of Synovectomy

- *Conventional arthrotomy:* Articular debridement and synovectomy
  Disadvantage: Loss of range of motion.
- *Arthroscopic synovectomy:*
  - Low morbidity
  - Early rehabilitation
  - Better range of motion

Both the above procedures are not recommended today as nonsurgical synovectomy is a much better treatment option.

- *Nonsurgical synovectomy:* This is done by the intra-articular injections of drugs or radioactive material
  Chemical: Rifampicin
  Radioactive: Au 198 colloidal gold, Y colloidal yttrium, Re colloidal rhenium, P 32 colloid

Of these using intra-articular rifampicin is a safe and effective means of causing a chemical synovectomy.

Pseudotumors (Figs 7A to D)

Hemophilic pseudotumors are large encapsulated hematomas that represent progressive cystic swelling from persistent bleeding and incomplete resorption. This serious complication is evident in approximately 1 to 2 percent of severely affected patients. The pseudotumor is composed of clot and necrotic tissue.

Three types of pseudotumors predominate in hemophilia.

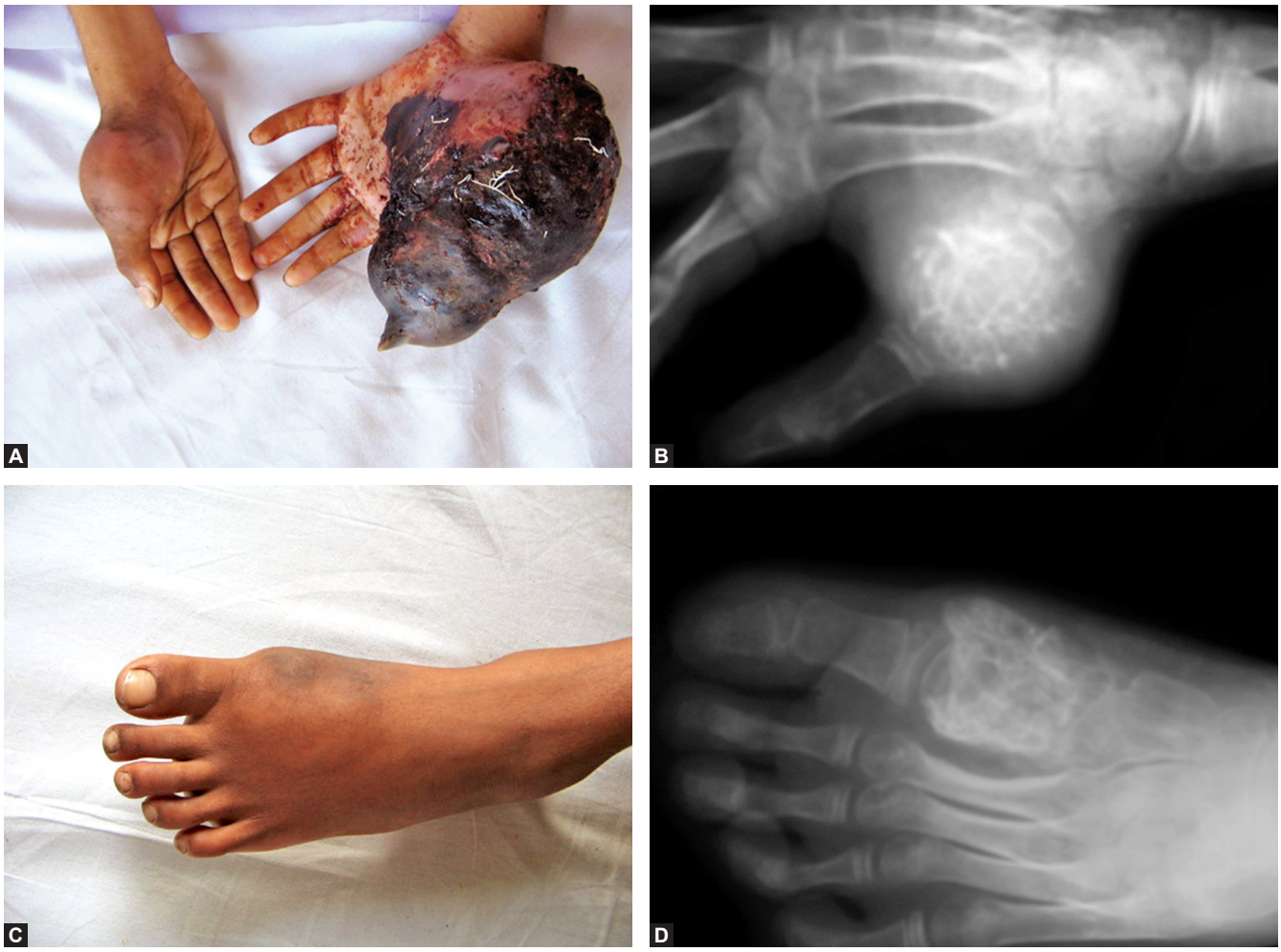
1. The most common type arises from repeated hemorrhage and inadequate clot resorption. They are usually confined within facial and muscle planes. Radiologically, they appear as simple cysts.
2. The second type involves large muscle groups, such as the gluteus maximus and iliopsoas. These lesions are especially problematic because they may gradually enlarge, develop a fibrous capsule, and eventually destroy adjacent underlying structures by pressure necrosis. Skeletal fractures and bony deformities produced by cortical erosion may result.
3. The third and rarest type of pseudotumor arises from within bone itself, often secondary to subperiosteal bleeding. This lesion typically is observed in the long bones of the lower extremities and pelvis but has been reported to occur within the calcaneus, cranium, and mandible.

Most such pseudotumors arise in adults and occur in proximal skeletal structures; distal lesions occur more frequently in children before skeletal maturity and are associated with a better prognosis.

Treatment

Distal pseudotumors respond well to conservative treatment consisting of aggressive clotting factor replacement and cast immobilization. Because conservative treatment has not been nearly as successful for lesions of the proximal musculoskeleton, and because complete regression is very rare, this approach has been reserved primarily for patients with high-titer inhibitors.

High- and low-intensity radiotherapy regimens have been successful in eradicating pseudotumors in the long bones and may offer an alternative conservative approach.



Figs 7A to D Pseudotumors of hand and foot in hemophilia A

Surgical extirpation is the most effective therapy for pseudotumors and is the treatment of choice when it can be carried out in major hemophilia centers. Nevertheless, the operative mortality rate approaches 20 percent.

Depending on the size and location of the pseudotumor, percutaneous evacuation of the cavity and subsequent introduction of fibrin sealant or cancellous bone can be considered.

Surgery in Hemophilia

- Surgeries in hemophilia patients are a major challenge in our country due to the poor availability of factor and the expense, as majority of our patients may not have access to it or are unable to afford the factor.
- Optimal care requires cooperation between the hematologist, surgeon, blood bank and physical therapist
- Thus it is best to carry out surgeries in hemophilia patients only at specialized centers where all facilities are at hand.

Before operating on a patient certain pre-requisites must be ensured:

- Accurate diagnosis
- A baseline factor level
- Rule out presence of an inhibitor
- Adequate stocks of factor

It is best to do the surgery in the morning as that gives adequate time to take care of any unforeseen postoperative complications.

Recommended Dose of Factor

Using 100 percent factor correction as recommended is not always financially feasible for our patients. Thus,

we need to modify our strategies of treatment to suit the patients' finances and also give him optimum treatment.

- Depending on the type and site of the operation one would preoperatively raise the factor levels to 70 to 100 percent and try to continue the dose for about 48 hours in the perioperative period.
- Subsequently, levels may be maintained around 50 percent or even less, for 7 to 15 days, depending on the type of surgery.
- There is generally no need to routinely measure factor levels in the postoperative period. Factor levels should be measured only, if required.
- Adjuvant use of antifibrinolytic drugs where possible, considerably reduces the requirement for factor.
- Also intraoperative use of local agents such as antifibrinolytic drugs/fibrin glue can minimize bleeding.

Use of DDAVP in Hemophilia A

- Infusion of desmopressin—a synthetic analog of vasopressin causes a 3 to 5 fold rise in factor VIII levels by release of von Willebrand factor (vWF) from endogenous stores in the endothelial cells.
- It is effective in mild/moderate hemophilia and therefore it can only be used in patients those patients.

Disadvantages

- Variability in response, and needs to be individually assessed in each patient
- Transient flushing/headaches/palpitations
- Fluid retention, hyponatremia
- Tachyphylaxis
- Seizure activity in infants
- Thrombocytopenia in Type 2B and platelet type vWD
- It is contraindicated in patients with hypertension/CAD/elderly persons.

Route of Administration

- *Spray:* The intranasal dose for patients <50 kg is 150 μg and for patients over 50 kg—300 μg.
- IV—The usual dose is 0.2 to 0.3 μg/kg IV in a volume of 50 to 100 mL infused over 30 minutes.
- Increase in factor levels is seen within 15 to 60 min
- Effect intranasal lasts for 6 to 8 hours
- It may be given daily for 2 to 3 days.

Pharmacologic Options for Controlling Bleeding

- *Tranexamic acid:* Tranexamic acid is an antifibrinolytic agent that inhibits the activation of plasminogen to plasmin. It promotes clot stability and is useful as the adjunctive therapy in hemophilia. It is especially valuable in controlling bleeding from mucosal surfaces (e.g. oral bleeding, epistaxis, menorrhagia).
  Dose: Oral tablets are freely available.
  - For an adult 1 g is administered every 6 hours
  - For a child is 20 mg/kg.
- *Fibrin sealant:* Fibrin sealant has hemostatic, sealing, and healing properties. It is made by mixing fibrinogen and thrombin, which mimics the last step in the blood coagulation cascade. A semirigid to rigid fibrin clot consolidates and adheres to the application site and acts as a fluid-tight sealing agent able to stop bleeding. Fibrin sealant can be used for:
  - Dental extraction (Fig. 8)
  - Circumcision
  - To stop bleeding from mucous membranes
  - Postoperatively over suture lines, etc.
  - Commercially available fibrin sealants are prohibitively expensive.
- *Cryoprecipitate and plasma:* Cryoprecipitate, fresh frozen plasma (FFP), and cryo-poor plasma are sometimes the only affordable treatment options in many developing countries. However, they are usually not treated to eliminate blood-borne viruses. Because of the risk of transmitting disease, the use of plasma and cryoprecipitate which has not been viral inactivated should be considered a temporary measure until adequate amounts of factor can be made available.

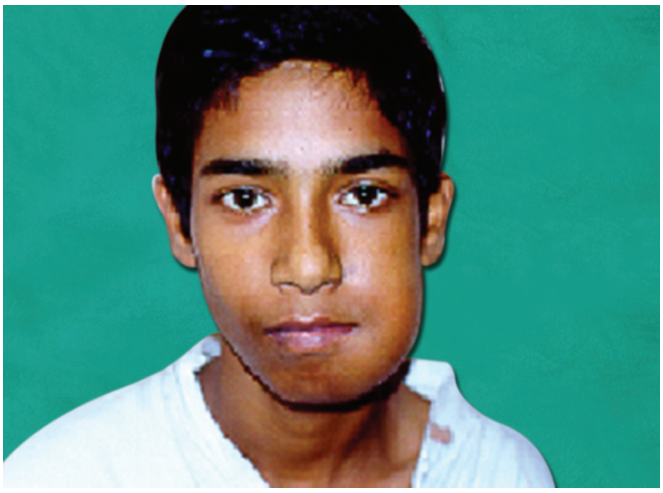


Fig. 8 Hematoma following tooth extraction without factor correction

PREVENTION

Carrier Detection and Prenatal Diagnosis

In developed countries, where hemophilia care has progressed to such an extent that a child can live a near

normal life with safe and effective therapy, the need for carrier detection and prenatal diagnosis may not be important.

However, these services are necessary in developing countries so that individuals and families can be evaluated, informed of their carrier status, and be allowed to make an informed choice on whether they will risk having a baby with hemophilia or not.

If there is an affected family member then the antenatal diagnosis for hemophilia A and B is now available for those parents who wish to opt for it and has more than 99% accuracy. It can be done at 10–11 weeks of gestation on chorionic villous samples or at 18–19 weeks on cord blood samples.

Take home message

The key to success in hemophilia management lies in early diagnosis and treatment. This will reduce the morbidity and mortality from a disease which is treatable. Though factor accessibility remains a major issue due to financial constraints, even with a limited amount of factor concentrates, it is possible to improve the lives of people with hemophilia in developing countries through education, prevention, and ancillary care. Hemophilia services must emphasize on education, physiotherapy, laboratory diagnosis, and simple measures to manage bleeds, along with a supply of safe concentrates.

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CHAPTER 29

von Willebrand Disease and Other Rare Coagulation Disorders

Kana Ram Jat, Ram Kumar Marwaha

Among the hereditary coagulation disorders, hemophilia is the most common in which one of the clotting factors has either quantitative deficiency or absent or has qualitative (functional) abnormality, as a result of other medical conditions, it can also be acquired.

COMMON HEREDITARY COAGULATION DISORDERS

The three most common hereditary bleeding (coagulation) disorders are:

1. Hemophilia A (factorVIII deficiency)
2. Hemophilia B (factor IX deficiency)
3. von Willebrand disease.[1]

Factors other than factor FVIII, FIX and von Willebrand factors are classified as rare coagulation factor disorders[2] apart from inherited deficiencies of coagulation.

Rare coagulation factor (deficiencies) disorders include:

- Fibrinogen
- FII
- FV (parahemophilia)
- FV+FVIII
- FVII
- FX
- FXI (Hemophilia C)
- FXIII

Kashyap et al.[3] reported a comprehensive study of 24 cases with rare coagulation disorders from India of which factor X deficiency was found in 8 patients, 7 had factor XIII deficiency, fibrinogen and factor VII deficiency was found in 4 cases each and factor V deficiency was found in 1 case.

von WILLEBRAND DISEASE (VWD)

In 1926, Erik von Willebrand first described a unique bleeding disorder, in a 5-year-old girl from Finland, inherited as an autosomal dominant or recessive pattern in patients with normal platelet counts which is due to an abnormality, either of quantitative (Type 1 and Type 3) and/or qualitative (Type 2)of the von Willebrand factor.[4]

Pathophysiology

Bleeding occurs due to abnormalities in platelet adhesion and aggregation, and decreased factor VIII levels, because of decrease in quantity or a dysfunction of von Willebrand factor (vWF) in this disease.

Extremely large multimeric glycoprotein complex; low, intermediate, and high molecular weights of multimers are the constituents of von Willebrand factor. It is synthesized in endothelial cells and megakaryocytes and after cleavage of a large propeptide, is released as a series of multimers, including ultralarge forms that are rapidly cleaved to a slightly small size.

Type of Hemostasis

Primary hemostasis: vWF in the subendothelium and plasma bind to the platelet receptor glycoprotein Ib (GPIb) and to subendothelial structures, such as collagen, during normal hemostasis consequent to an injury, and serve as a bridge between platelets and subendothelium in damaged vessels.

Higher the molecular weight of the multimer, higher would be the number of platelet-binding sites and adhesive properties. Each multimeric subunit has binding sites for the receptor glycoprotein Ib on nonactivated platelets and the receptor glycoprotein IIb/IIIa on activated platelets, this facilitates both platelet adhesion and platelet aggregation, making high molecular weight multimers most important for normal platelet function.

Secondary hemostasis: von Willebrand factor protects factor VIII from degradation in secondary hemostatis and delivers it to the site of injury. Binding enhances the localization of platelets as fibrin is formed at the site of the injury promoting aggregation.

The small multimers function mainly as carriers for FVIII: The size of the multimers determines the binding of vWF. Patient might have a bleeding diathesis in spite of a normal concentration of vWF when there is a decrease in the more functional large vWF multimers. Thrombus formation as in thrombocytopenic purpura can happen when the ultra large vWF forms that initially released are sticky and are capable of binding the platelets in the circulation spontaneously.

Due to decrease in quantity or a dysfunction of von Willebrand factor (vWF) there will be abnormalities in platelet adhesion and aggregation and decreased factor VIII levels, thus causing bleeding in this disease. The small multimers function mainly as carriers of FVIII.

Prevalence

The prevalence of clinically significant cases of vWF in humans are 1 per 10,000. It is detected more in women and adolescent girls whose bleeding tendency shows during menstruation. Most forms of vWF are mild. The level of vWF varies depending upon the blood group, for instance people with type "O" are approximately 25% less affected than those of the other blood groups.[5] vWF is reported to affect animals including dogs (especially Doberman Pinschers), rarely it affects wine, cattle, horses and cats.

Inheritance

In types I and II of von Willebrand disease is inherited in an autosomal dominant pattern. In type III and sometimes in type II it is inherited in autosomal recessive pattern. However penetrance may widely vary in a single family. Also on different occasions even the clinical and laboratory findings may vary in the same patient.[2]

CLINICAL PRESENTATION

Clinical symptoms have a wide spectrum. One end of the spectrum many children with von Willebrand disease (vWD) are asymptomatic and are diagnosed as a result of a positive family history or during routine preoperative screening. vWD is of various types and each type is presented with varying degree of bleeding tendency. Due to its wide range and severity of symptoms, vWD patients may present at any age. In majority of the cases the bleeding is of mild to moderate severity reflecting the predominance of type 1 vWD.[6] Individuals with decreased or qualitatively abnormal vWF function, usually present earlier in life. Type 3 vWD is more serious in nature.

Other end of the spectrum when associated with angiodysplasia vWD, serious gastrointestinal bleeding can occur.[7] Abnormally heavy bleeding during menstruation (menorrhagia), blood loss during childbirth, hematuria, hemarthroses, intramuscular, intracerebral and retroperitoneal hemorrhages, cephalhematomas in newborns (more common in Type 3 vWD).[8] Severe internal or joint bleeding is uncommon (except for in vWD type III).

In between are the children who present with *symptoms usually involving skin and mucosal bleeding like:* Easy bruising, excessive nose bleeds (epistaxis) bleeding from the gums, bleeding during procedures like tooth extractions, tonsillectomy, menorrhagia, etc.

Classification

International Society on Thrombosis and Haemostasis's (ISTH) classification.

There are four types of hereditary vWD, described are type 1, type 2, type 3 and pseudo or platelet-type.

- vWD Type I
- vWD Type II-4 subtypes-A, B, M, and N
- vWD III
- Pseudo or platelet-type
- Within the three inherited types of vWD there are various subtypes
- Platelet type vWD is also an inherited condition
- Most cases are hereditary, but acquired forms of vWD have been described.

Type 1 vWD

- This is the most common type which accounts for approximately 75–80% of patients and is characterized by quantitatively decreased and qualitatively and structurally vWF
- Many patients are typically asymptomatic or may have mild to moderate bleeding
- Often incidentally identified when other medical procedures requiring, a blood work-up is done.

Laboratory Assays

- Decrease in vWF activity and antigen levels
- Decrease in the ristocetin-inducecl platelet aggregation and vWF multimers show normal distribution, due to lower vWF concentration their intensity may be diminished
- Decreased binding of vWF to platelets factor VIII is decreased or in the low normal range
- A normal multimer size distribution, though vWF bands with abnormal migration may be present. Abnormal multimers may be seen (Vicenza variant)
- Caused by a defect in the von Willebrand factor gene that produces decreased or absent binding to

platelet glycoprotein 1b. The vWF gene is located on chromosome twelve (12p13.2). It has 52 exons spanning 178 kb
- By performing a binding assay for factor VIII that uses the patient's vWF as the binding partner, the diagnosis is established.

von Willebrand Disease Type 2

Type 2 vWBD is associated with primarily qualitative defects of von Willebrand protein and is most common. Its incidence is 20–30% and vWBF levels are normal. Bleeding tendency can vary between individuals as it is a qualitative defect, however subgroups of large or small multimers may be absent or structurally abnormal multimers are present.

Type 2A von Willebrand Disease Includes Four Subtypes (A, B, M and N)

- **Type 2A.** In type 2A vWD the qualitatively defective vWF's ability to bind to glycoprotein 1 receptor on the platelet membrane is enhanced abnormally, leading to spontaneous binding to platelets and rapid and subsequent clearance of the bound platelets and of the large vWF multimers. There are chances for thrombocytopenia. vWF antigen assay is normal but qualitatively defective and decreased capability at multimerization. Large vWF multimers are reduced or absent from the circulation
- Large vWF multimers are reduced or absent and ristocetin cofactor activity is low. Substitution within a normal cleavage site in the A2 domain of vWF is the effect of the majority of mutations in 2A. Some make it more susceptible to proteolysis by the vWF cleaving protease (ADAMTS13)
- The mutations in type 2A vWD may either cause a defect in intracellular transport (2A, type 1) or render the molecule more susceptible to proteolysis (2A, type 2).

Laboratory Findings

- Its ability to bind to the glycoprotein 1 (GP1) receptor on the platelet membrane is reduced. This results in abnormally low ristocetin cofactor activity and decreased platelet adhesiveness and aggression. The defective vWFs ability to coalesce and form large vWF multimers is also impaired which would lead to decreased quantity of large vWF multimers and detected in the circulation are only small multimer units
- Because of the loss of high molecular-weight multimers which are more functional there is a decrease in vWF activity assays compared with antigen. There is an absence of high-molecular weight multimers on agarose gels
- Decreased RIPA
- The factor VIII may be normal or decreased.

TYPE 2B von WILLEBRAND DISEASE

- Type 2B accounts for approximately 5% of vWD
- Inherited either autosomal dominant or autosomal recessive pattern
- The removal of platelets aggregates with bound vWF may result in hemostatic defect caused by qualitatively abnormal vWF and intermittent thrombocytopenia. The ability of the qualitatively defective von Willebrand factor to bind to glycoprotein 1 (GP1) receptor on the platelet membrane is abnormally enhanced, leading to its spontaneous binding to platelets and subsequent rapid clearance of the bound platelets and of the large vWF multimers. From the circulation, large vWF multimers are reduced or absent.

Laboratory Assays

- Low concentrations of ristocetin in ristocetin-induced platelet (RIPA) show an increased reactivity, similar to type 2A, vWF ristocetin cofactor activity shows marked decrease than in antigen level and high molecular weight multimers of vWF are decreased
- Thrombocytopenia may be present in some patients. Low or normal factor VIII
- Bleeding symptoms may be moderate to severe
- Platelet-related vWF function is normal

Type 2M von Willebrand Disease

Type 2M is very rare type of vWF and is a qualitative defect. In this type of vWD there is normal capability at multimerization and a decreased ability to bind to glycoprotein (GP1) receptor on the platelet membrane. This is characterized by a decreased platelet-directed function which is not because of the decrease of high-molecular weight multimers. Though vWF bands with abnormal migration may be present and abnormally large multimers may be seen, there would be a normal multimer size distribution (Vicenza variant).

Caused by a defect in the von Willebrand factor gene that produces decreased or absent binding to platelet glycoprotein 1b characterized by its decreased ability to bind to glycoprotein 1 (GP1) receptor on the platelet membrane and normal capability at multimerization. This results from mutations affecting the A1 domain in a different area from those mutations in type 2B.

Decreased ristocetin cofactor activity and decreased vWF antigen and activity, and high molecular weight large vWF multimers are present in the circulation.

Laboratory studies show that there is normal vWF function and antigen, and RIPA and multimer distribution, however shows decreased factor VIII which is between 2% and 10%.

Type 2N von Willebrand Disease

- The cause of type 2N vWD is by mutations in the amino terminus of the mature vWF monomer and this is an uncommon variant. This may be initially confused with hemophilia A as patients may have mild to moderate bleeding which is related to the decreased factor VIII. The presence of affected females in the family is a vital indication that this diagnosis should be considered
- Decrease binding for factor VIII result in rapid clearance of factor VIII. This is a deficiency of the binding of vWF to coagulation factor VIII. Soft tissue and joint bleeding are apparent symptoms, as expected with decreased factor VIII
- Normal platelet-related vWF function
- Laboratory studies show decreased factor VIII (2–10%) and normal vWF function and antigen, RIPA and multimer distribution
- Performing a binding assay for factor VIII that uses the patient's vWF as the binding partner is the diagnosis to establish type 2N vWD.

Type 3 von Willebrand Disease

- Type 3 vWD is characterized by complete absence of production of vWF and it is the most severe and rare form of von Willebrand disease. Manner of inheritance of type 3 vWD is homozygous or doubly heterozygous. Consanguinity is common. A variety of mutations, including larger deletions are the causes of type 3 vWD
- From proteolytic degradation, the vWF protects coagulation factor VIII, extremely low factor VIII level is caused due to total absence of vWF, severe clinical bleeding which is similar to severe hemophilia, e.g. hemarthrosis, intramuscular bleeding, etc., would be the clinical presentation
- It is identified by marked deficiencies of both FVIIIc in the plasma and vWF, the absence of vWF from both endothelial cells and platelets and lack of response to DDAVP, therefore platelets cannot clot. It is difficult to stop bleeding in patients with type 3 vWD as bleeding is severe. Patients may present with cephalohematomas in newborns, hemarthroses, hematuria and intramuscular, intracerebral and retroperitoneal hemorrhages.

Platelet-type vWD (pseudo-vWD)[6]

- Pseudo-vWD is another name of platelet type vWD
- It is a genetic defect of the platelets which is autosomal dominant
- The vWF is qualitatively normal
- No mutational alteration is revealed while genetically testing the von Willebrand gene
- Thrombocytopenia, and diminishing or absence of large vWF multimers result as large platelet aggregates and high molecular weight vWF multimers are removed from the circulation. The ristocetin cofactor activity and loss of large vWF multimers are similar to vWD type 2B and can be distinguished from type 2B vWD by mixing studies with patient platelets and normal plasma using RIPA
- Because of the genetic mutation in factor VIII binding region of vWF it is characterized by a marked decrease affinity of vWF for FVIII. Bleeding is related to the decreased factor VIII. Symptoms include as soft tissue and joint bleeding and with decreased factor VIII may initially be confused with hemophilia A. This is suspected when patients with mild FVIII deficiency and a bleeding disorder which is not clearly transmitted as X-linked disorder or when patients do not completely respond to hemophilia.

A significant indication is the presence of affected females in the family. It is usually presented before adulthood with symptoms such as moderate to severe bleeding in affected patients.

Laboratory Findings

Due to the loss of the more functional high molecular weight multimers, laboratory testing shows more marked decrease in vWF activity assays as compared with antigen.

- Decreased RIPA
- High molecular weight multimers on agarose gels are absent
- Normal-to-reduced plasma levels of factor VIIIc and vWF (in moderate/severe disease it is abnormal).

DIAGNOSIS OF von WILLEBRAND DISEASE

Accurate Personal and Family Bleeding History

- Increased or easy bruising and bleeding from wound
- Gingival bleeding and recurrent epistaxis
- Menorrhagia, postpartum bleeding
- Postoperative bleeding (particularly after tonsillectomy or dental extractions):

Clinical Evaluation

Laboratory Assays Tests for vWD Include

- Routine screening test for coagulation disorders like
- CBC, platelet count
- *Bleeding time:* Bleeding time tests are not sensitive and are not done as often as they once were
- *Prothrombin time, APTT:* Characteristically there is marked prolongation of the PTT, PT and the thrombin time (TT)
- *vWF antigen level:* vWF antigen (vWF Ag) is a quantitative test that is usually carried out in an ELISA format using antibodies specific for vWF
- *vWF multimer distribution by gel assays and RIPA:* This occurs in type 2B vWD
- The patient's plasma which is the source of vWF and platelets are used in RIPA. To assess whether the platelet aggregation is present or absent, different concentrations of ristocetin are added to aliquots of the platelet-rich plasma of the patient. Aggregation will cause in patients with type 2B vWD if, concentrations of ristocetin is approximately below 0.6 mg/mL, but will not cause aggregation in normal subjects. The "gain of function" can be assessed primarily through RIPA
- *Ristocetin-induced platelet aggregation (RIPA), collagen binding:* Ristocetin is the antibiotic that promotes the binding of vWF to platelets. In the presence of ristocetin the vWF have the functional ability to bind to platelets. If concentration of ristocetin is approximately below 0.6 mg/mL, it will cause aggregation in patients with type 2 vWD. "Gain of function" mutation in patient's vWF is primarily assessed through RIPA.

Factor VIII Activity

In moderate and severe disease factor VIII would be abnormal, otherwise it would be normal or decreased.
Factor VIII activity is usually performed in the traditional coagulation factor assay.

- vWF multimer study and ristocetin—Additional tests useful in classifying the type of vWD include–ristocetin induced platelet aggregation (RIPA) performed in a platelet function analyzer. In this test, the patient's own platelets and vWF are employed, so the test is not specific for vWF abnormalities.[9]
- To assess whether high molecular weight multimers are decreased or absent, vWF multimer gels are used as they provide visualization of the size distribution of vWF multimers in plasma
- vWF is visualized using antibodies to vWF and immunofluorescence end point by performing electrophoresis on diluted plasma in agarose gels and the proteins are transferred to a membrane.[10]

Genetic Testing–Mutation in the Patient's vWF

The gene defect in the majority of type 1 patients is unknown. Specific gene defect in type 2 and type 3 vWD patients is available in specialized laboratories and research centers.

Specialized laboratories and research centers are available where genetic testing for diagnosis of specific gene defect in type 2 and type 3 vWD patients are available. Direct sequencing of the suspect area of the patient's gene is done to identify the specific defect. Usually genetic testing is not usually performed in type 1 patients as it is unknown at that time.

Medical Care

Treatment for von Willebrand disease depends on type and severity of the disorder. vWD, the patient has dual defect of hemostasis, i.e.

- Defect in platelet adhesiveness and aggregation which can be corrected by raising the level of von Willebrand factor
- Low factor VIII activity.

Treatment

The three major treatment modalities used for patients with vWD is detailed in Table 1[6]

- Desmopressin acetate (DDAVP)
- Replacement therapy with plasma-derived factor VIII vWF concentrates
- Adjunctive therapies such as antifibrinolytic agents and topical therapies.

Specific treatment is not required if there is minor bleeding problems, such as bruising or a brief nose bleed, etc., in patients with vWD. The main aim in case of serious bleeding is to limit the patient's bleeding, by medications that can raise the vWF level.

Desmopressin Acetate (DDAVP)

Desmopressin is a synthetic analog of antidiuretic hormone. It is considered that for patients with mild vWD, the primary treatment and mainstay of therapy is desmopressin [1-deamin-8-d-arginine vasopressin (DDAVP)]. It stimulates the release of vWF from the Weibel Palade bodies of endothelial cells (storage site). In type 1 vWD patients who have normal vWF in storage sites DDAVP (desmopressin acetate) is most effective.

It may not be effective in vWD type 2M and is hardly effective in vWD type 2N and in severe forms of vWD 1 and 2. It is totally ineffective in vWD type 3.

Table 1 Classification and treatment of von Willebrand disease

Type	*Description*	*vWF activity*	*Ag*	*RIPA*	*Multimer pattern*	*Treatment*
Type 1	Partial quantitative deficiency of vWF	↓	↓	↓	Uniform ↓	DDAVP 0.3 µg/kg IV in 50 mL saline over 20 minutes, or nasal spray 300 µg for weight >50 kg or 150 µg for <50 kg. Replacement vWF concentrate at 20–30 IU/kg q12h
Type 2	Qualitative vWF defect					
2A	Decreased vWF-dependent platelet adhesion with selective deficiency of high molecular weight multimers	↓↓	↓	↓	↓ Large and intermediate	DDAVP as in type 1. Replacement vWF concentrate
2B	Increased affinity for platelet GPIb	↓↓	↓	↑	↓ Large	Possibly DDAVP (may worsen thrombocytopenia) as in type 1. Replacement vWF concentrate
2M	Decreased vWF-dependent platelet adhesion without selective deficiency of high molecular weight multimers	↓	↓	↓	Normal	DDAVP as in type 1. Replacement vWF concentrate
2N	Markedly decreased binding affinity for FVIII	N	N	N	Normal	DDAVP as in type 1. Replacement vWF concentrate
Type 3	Virtually complete deficiency of vWF	↓↓↓	↓↓↓	↓↓↓	Undetectable	Replacement vWF concentrates platelet transfusions if inadequate response to vWF replacement

Abbreviations: DDAVP: Desmopressin acetate; N: Normal; IV: Intravenous; RIPA: Ristocetin-induced platelet aggregation; vWD: von Willebrand disease; vWF: von Willebrand factor.

DDAVP indirectly causes release of vWF and factor VIII from storage sites, increasing the levels of both factors 2- to 5-fold within 45 minutes following intravenous administration; the effect usually lasts about 6 hours.

Route and Mode of Administration and Dose

Administration of DDAVP can be either intravenously or intranasaly or subcutaneously. If administered slowly through intravenous infusion or subcutaneous mode, the recommended dosage is 0.3 µg/kg.

Intranasal Preparation

If DDAVP is administered through intranasal mode in the form of nasal spray the levels peak approximately 2 hours after intranasal delivery.[12] A high concentration preparation (i.e. stimate 1.5 mg/mL) available and this allows home treatment for bleeding symptoms. At the start of the bleeding episode, through intranasal dosing the patient can have rapid access to medication at home. It is recommended that DDAVP be administered at 8–12 hours interval for 2–3 doses and then at intervals of 48 hours in case of tachyphylaxis and serious hyponatremia, where bleeding can occur even after repeated doses.

Dosage: Fixed doses of 300 µg in adults and 150 µg in children by intranasal spray.[11]

Common Side Effects Include

Frequent side effects of intranasal spray include transient headache, facial flushing and mild tachycardia, but are usually well-tolerated by patients. More often in infants and in young children severe symptoms such as cerebral edema and seizures are reported as a result of water intoxication. Water retention and dilutional hyponatremia with consequent convulsion can occur due to over use of DDAVP.

Type 2B patients are at risk for worsening thrombocytopenia after DDAVP and in type 2B patients, platelet count should be evaluated along with vWF levels.

vWF Concentrates

When there is more severe bleeding and could not controlled by DDAVP then vWF concentrates should be used. They are also given prophylactically and following surgery or trauma for 2–14 days, as dictated by the clinical situation.

Intermediate-purity plasma-derived factor VIII concentrates, administered intravenous mode in an interval time of approximately 12 hours, contains vWF (not recombinant or monoclonally purified factor VIII concentrates).

Cryoprecipitate

Due to lack of viral inactivation, this product is generally not recommended.

Nonreplacement Therapy

Aminocaproic acid and tranexamic are both drugs that steady the clots formed by platelet by preventing fibrinolysis. The antifibrinolytic agents epsilon amino caproic acid and tranexamic acid are useful adjuncts in the management of vWD complicated by gum bleeding, bleeding from mucous membrane, menorrhagia, etc. Common side effects include nausea, vomiting, and clot complications.

Estrogen-containing oral contraceptive medications are effective in reducing the frequency and duration of the menstrual periods for women with heavy menstrual bleeding. Estrogen compounds available for use in the correction of menorrhagia are:

Ethinyl Estradiol and Levonorgestel (Levona, Nordette, Lutera, Trivora)

Stabilization of the endometrial surface of the uterus is done by administration of ethinyl estradiol which in turn diminishes the secretion of luteinizing hormone and follicle stimulating hormone from the pituitary.

Use of Topical Thrombin

Are effective adjuncts for correction of hemorrhage from wounds.

Replacement Therapy Plasma Products

- Plasma derived-derived factor VIII (FVIII) concentrates
- Human derived medium purity factor VIII concentrates, contain von Willebrand factors. This can be used as prophylaxis to treat patients with vWD who do not respond to DDAVP. It can also be used for patients with vWD scheduled for surgery, patients with rare types 2B or 3. vWD and cases of vWD complicated by clinically significant hemorrhage
- However, most available FVIII concentrates do not contain sufficient von Willebrand factor to be used in von Willebrand disease e.g. Humate-P, Alphanate, Wilate Alphanate and Koate also contain vWF in high molecular weight form. These concentrates are especially useful in types 2B and 3 vWD and are available commercially for prophylaxis and treatment of vWD. Insignificant quantity of vWF is present in monoclonally purified factor VIII concentrates and recombinant factor VIII, hence are not clinically useful.

In 10–15% of patients receiving human derived medium purity factor VIII concentrates development of alloantibodies occur. Other side effects include allergic reactions including anaphylaxis and increased risk of venous thromboembolic complications.

Cryoprecipitate Contains Multimeric von Willebrand Factor

In general, the dosage of cryoprecipitate or FVIII to be used is calculated on the basis of FVIII units. Other blood products are rarely required for patients with von Willebrand disease.

Type 3 vWD patients or platelet-type vWD who do not respond to vWF containing concentrates or cryoprecipitate may be benefitted by platelet transfusion.

Other supportive line of treatments includes blood transfusions for hypotension secondary to hypovolemia to correct anemia. For correction of hemorrhage associated with platelet type vWD, infusion of platelet concentrates is recommended.

Adjunctive Therapies

Antifibrinolytic agents such as epsilon amino caproic acid and topical agents such as topical thrombin, gelfoam, and fibrin sealant are used as adjunctive therapies.

For dental procedures, epsilon amino caproic acid may be helpful.

Acquired von Willebrand Disease

Acquired von Willebrand syndrome (AvWS) is a rare bleeding disorder and is distinguished from the congenital form by several factors such as age at presentation, absence of personal and family history of bleeding disorders.[13]

Acquired vWD can occur in patients with autoantibodies. Antibodies, binding mechanism responsible for decreased vWF, proteolysis, decreased

production of vWF are the mechanisms responsible for decreased vWF.[14] There is a rapid clearance of vWF antibody complex from the circulation.

Often Associated with Underlying Diseases Like

- Lymphoproliferative (48%)
- Myeloproliferative disorders (15%)
- Neoplasia (5%)
- Immunological (2%)
- Cardiovascular (21%), aortic valve stenosis, left ventricular assist device (LVAD)
- Miscellaneous disorders including hypothyroidism (9%), Wilms' tumor and mesenchymal dysplasias.

Laboratory Findings

Laboratory features are the same as in congenital von Willebrand disease. Only the measurement of vWF propeptide (also known as vWF Ag II) has been suggested as helpful to discriminate between congenital and acquired vWD. In AvWS, the propeptide levels remain normal because vWF synthesis is normal or higher and it is not targeted by antibody and not consumed or bound.

In less than one-third of the cases, antibodies are found and are difficult to demonstrate in the laboratory.

Treatment

Treatment of vWS has two main objectives:

1. To control the bleeding episode.
2. To treat the underlying associated disease.

Initial treatment is usually administration of DDAVP, however, if the response is not adequate, either replacement therapy (FVIII/vWF concentrates) or intravenous immunoglobulin (IVIg) is used.[14] Despite the possible presence of antibodies to vWF, the response to replacement therapy is usually satisfactory.

Drugs to Avoid

As aspirin and nonsteroidal anti-inflamattory drugs can increase bleeding complications, these drugs are to be avoided. These children must inform about their health problems to health providers, including their dentists of their condition as well as teachers in the school, family members and close friends.

Rare Coagulation Disorders

Rare coagulation disorders due to deficiencies of coagulation factors other than factor FVIII and FIX and von Willebrand disease, include deficiency of coagulation factors like fibrinogen, FII, FV, FV+FVIII, FVII, FX, FXI, FXIII.

FIBRINOGEN DEFICIENCIES (F1-5)

Fibrinogen deficiencies are inherited disorders of fibrinogen defect.[2] They may be classified as:

Quantitative Defect

- Hypofibrinogenemia, with fibrinogen levels lower than 1.5 g/L
- Afibrinogenemia, characterized by the complete deficiency of fibrinogen.

Qualitative Defect of the Circulating Fibrinogen

- Dysfibrinogenemia
  or
- Both hypo/dysfibrinogenemia

Congenital fibrinogen disorders are relatively rare.

Clinical Features

- Symptoms in afibrinogenemia are not as severe as those seen in classic hemophilic disorders
- Congenital afibrinogenemia is an autosomal recessive disorder. Hereditary dysfibrinogenemia are usual autosomal dominant
- It may manifest in the neonatal period with gastrointestinal hemorrhage or hematoma (cephalhematoma) after normal vaginal delivery
- Thrombosis is another cause of concern. Patients with congenital fibrinogen disorders may paradoxically suffer from severe thrombotic episodes. The etiopathogenesis is poorly understood except for a few cases having a severe thrombophilic disorder concomitantly.[15]
- The clinical complication which is common in case of fibrinogen deficiency is pregnancy loss.

DIAGNOSIS[16,17]

- There is marked prolongation of the PT, PTT and the thrombin time (TT)
- Fibrinogen is the ligand for the glycoprotein IIb-IIIa receptor which enables platelet aggregation. In fibrinogen deficiency, the bleeding time and the platelet aggregation tests are abnormal
- The best screening tests are the TT and the reptilase time, which measures the time required for the conversion of fibrinogen in plasma to a fibrin clot.

Unlike the TT, the reptilase time is unaffected by heparin treatment
- The bleeding phenotype is difficult to predict even by the characterization of the molecular defects responsible for afibrinogenemia or hypofibrinogenemia
- Congenital fibrinogen disorder patients may paradoxically suffer from severe thrombotic episodes, at times independent of any fibrinogen substitution. Few cases having a severe thrombophilic disorder concomitantly.

Management

During bleeding due to congenital fibrinogen deficiency, fibrinogen levels should be increased and maintained above 1.0 g/L until hemostasis is secured and it should be maintained above 0.5 g/L until wound healing is complete.[18,19] A dose of 50 mg/kg is required to increase the fibrinogen concentration to 1 g/L.

Fresh Frozen Plasma or Cryoprecipitate

- The plasma half-life of fibrinogen is between 2 days and 4 days and the availability of fibrinogen concentrates is rare. Treatment with either fresh frozen plasma (FFP) or cryoprecipitate is effective. Each bag of cryoprecipitate contains 100–150 mg of fibrinogen. Efficiency of viral inactivation process is not that effective as it is for fibrinogen concentrates. Transfusion-related acute lung injury or TRALI complication can be caused due to cytotoxic antibodies contained in the infused plasma
- To treat mucosal bleeding and to prevent bleeding following procedures, e.g. dental extraction, antifibrinolytic agents may be given
- Agents such as tranexamic acid should also be considered
- For treating superficial wounds or wounds following dental extraction fibrin glue will be useful
- For selected patients gene therapy could be the future.[20]

FACTOR II—PROTHROMBIN DEFICIENCY

Factor II (FII) deficiency also called hypoprothrombinemia or prothrombin deficiency and is a rarest coagulation disorder first, identified in 1947 by Dr Armand Quick. Prevalence of 1:2,000,000 in the general population.[21]

The mode of inheritance is autosomal recessive. At least 32 different mutations have been identified.

FXa activates prothrombin on the surface of platelets in the presence of FV and calcium.

Clinical Phenotypes

Two clinical phenotypes are recognized.
1. *Hypoprothrombinemia (type I deficiency)*, in which prothrombin antigen and activity levels are reduced concomitantly.
2. *Dysprothrombinemia (type II deficiency),* in which prothrombin activity is reduced but antigen levels are normal.

Clinical Features

- Hemarthrosis and muscle hematomas are most frequent bleeding manifestations in this group of patients
- Intracranial bleeds and umbilical bleeding have been reported in neonates
- Postoperative bleeding and mucosal bleeding are other manifestations.

Diagnosis

- High index of suspicion and family history helps in early diagnosis. Prolonged PT and a normal TT is diagnostic criteria although both PT and APTT may be prolonged in FII deficiency. It should also be noted that the degree of abnormality may be minimal and results can be within the normal range
- A specific FII assay confirms the diagnosis
- In premature and young neonates where vitamin K deficiency may complicate assessment, the diagnosis of mild prothrombin deficiency is difficult. Reassessment after vitamin K replacement may be necessary.

Management[22,23]

There are no specific prothrombin concentrates available.

Prothrombin Complex Concentrates are therefore Treatment of Choice

- As a basis for dosage usually approximately 1 unit of prothrombin per unit of FIX and can be used. Doses of 20–30 IU/kg seem to be effective as relatively low levels are required for normal hemostasis. The plasma prothrombin level is estimated to rise by 1 IU/dL with one unit of prothrombin
- An alternative source of prothrombin is fresh frozen plasma (FFP). Around 72 hours is the half-life period of prothrombin. This eases comparatively occasional dosing, usually every 2–3 days. Depending on the frequency and type of bleeding, prohylaxis should be

used in older children. To prevent the development of chronic arthropathy, prophylaxis should be used in cases where recurrent joint bleeding is a feature.

FACTOR V DEFICIENCY (PARAHEMOPHILIA)

In 1943, a Norwegian patient was found to have factor V deficiency and was reported by Dr Paul Owren in 1947. Factor 5 deficiency is also called as parahemophilia, Owren's disease, labile factor deficiency and proaccelerin deficiency.

Clotting factor: FV is a large glycoprotein of molecular weight 249 kDa, is synthesized by hepatocytes and megakaryocytes. Encoded by a gene on chromosome 1, hereditary FV deficiency is a very rare autosomal recessive condition. The prevalence of the homozygous state is approximately 1 per million.

FV is activated by thrombin and the resulting heterodimer FVa acts as a cofactor for FXa in the conversion of prothrombin to thrombin.

Clinical Features

Homozygous deficiency is associated with a moderately severe bleeding disorder with easy bruising and mucous membrane bleeding, epistaxis and oral cavity bleeding[24], postoperative, postdental extraction and postpartum bleeding, etc. Hemarthroses and muscle hematomas are often related to trauma rather than being spontaneous. Gastrointestinal bleeding and hematuria may occur rarely. Intracranial bleeding especially in the antenatal and neonatal periods have been reported.[25]

Diagnosis

Factor V deficiency is characterized by prolongation of both the PT and APTT but a normal TT. By mixing with normal plasma, both PT and APTT are corrected. By FV assay or by immunological assessment of FV levels, deficiency of FV is confirmed. FV assay has to be performed on individuals with reduced FV levels to exclude combined FV and FVIII deficiency.

Management

- There is no FV concentrate available
- FV replacement is done in patients presenting with a bleeding episode by administering a dose of 15–20 mL/kg of FFP
- Use of agents such as tranexamic acid should also be considered
- In patients who are not responding to FPP, use of recombinant activated factor VII (rFVIIa) should also be considered[26]
- A potential complication of hereditary FV deficiency is the development of alloantibodies to FV in FFP
- It is suggested that low-level inhibitors can be used to neutralize large amounts of FFP in case of bleeding problem[27]
- Immunoglobulin administered intravenous may be effective in eliminating FV inhibitor.[28]

COMBINED DEFICIENCY OF FACTORS V AND VIII

In 1954, Oeri et al. first described combined FV and FVIII deficiency, which is a rare autosomal recessive disorder. History of consanguinity present. It is likely to be due to a single gene defect (located on the long arm of chromosome 18), leading to deficiency of a transport protein, rather than due to coinheritance of separate defects of the FV and FVIII genes.

Affected individuals have reduced plasma levels of both FV and FVIII.[29]

Shetty et al. from India reported nine patients from five unrelated families of combined FV and FVIII deficiency, youngest being an 8-year-old girl.[20]

Clinical Features

Mild bleeding symptoms, such as easy bruising and epistaxis are present. In 9 patients from India, the most common manifestations observed were prolonged bleeding from cuts, easy bruisability, bleeding gums and postdental extraction bleeding. Following a dental extraction or a surgery, bleeding is common phenomena. In affected woman menorrhagia and postpartum hemorrhage is seen.

Diagnosis

The combined deficiency disorder is associated with a prolongation of both the PT and APTT, with the APTT prolongation disproportionate to that of the PT. By using normal plasma, both the test times are corrected.

APTT-based activity assays and antigen assays reveal levels of between 5 IU/dL and 20 IU/dL for both FV and FVIII.

Management

- Both FVIII concentrates and FFP are to be used for treating patients with combined FV and FVIII deficiency who have spontaneous bleeding episodes. (as a source of FV)
- FVIII levels should be raised to at least 30 IU/dL for minor bleeding episodes and for more severe bleeding atleast 50 IU/dL with rFVIII concentrate

FFP should be administered for patients with FV deficiency, in order to increase the FV level to at least 25 U/dL.

Rather than intramuscular vitamin K, affected babies should receive oral vitamin K.[18]

There is no indication for routine prophylaxis with plasma and FVIII. Neonatal intracranial hemorrhage has not been described in this condition.

FACTOR VII DEFICIENCY

Also known as proconvertin, deficiency, Alexander's disease.

Among the rare inherited coagulation disorders the most common is factor VII deficiency. Factor VII is a vitamin K-dependent glycoprotein with a MW of approximately 50 kDa.

FVII deficiency is inherited in an autosomal recessive manner.

Estimated prevalence of FVII deficiency 1:300000–1:500000. First recognized in 1951, circulates in plasma in two forms—the majority in a single chain inactive form with a concentration of 10 nmoles/L (0.5 μg/mL) and a much smaller amount (approximately 10–110 pmoles/L as the active two-chain form.

Clinical Features

- Common manifestations include; epistaxis, gum bleeding, menorrhagia and other mucous membrane-type bleeding.[30] Increased risk for developing intracranial hemorrhage is reported in neonates who are diagnosed with factor VII deficiency
- Although it is not a consistent finding joint bleeds are reported in some patients with severe FVII deficiency
- Bleeding into the central nervous system is common in patients with severe FVII deficiency (FVII:C <2 IU/dL), and is reported to be between 15% and 60%
- Although the mechanism is unclear, thrombosis in association with FVII deficiency is also reported.[31]

Diagnosis

Characteristic features of factor VII deficiency is finding of a prolonged PT, which corrects, unless an inhibitor is present, in a 50:50 mix with normal plasma.

FI concentration, APTT and TT are found to be normal. Before making the diagnosis of FVII deficiency, it is critical to exclude vitamin K deficiency or any other clotting disorder which is acquired.

A therapeutic trial of vitamin K may be of value.

Using a one-stage PT-based assay, the functional FVII activity (FVII:C) is measured. Cold activation of FVII and substantial overestimation of the true FVII level can happen if the blood samples collected for the determination of FVII:C are stored at 4°C. An enzyme-linked immunosorbent (ELISA) assay or immunoradiometric assay (IRMA) assay and monoclonal or polyclonal antibodies are used frequently to measure FVII antigen (FVII:Ag). Such assays can detect as little as 0.01 IU/dL of FVII. Functional FVII assay should be preferred over immunological assay. Due to the low physiological levels of the neonate, diagnosis of factor VII deficiency may be difficult in neonates. Age- and gestation-related reference ranges must be used in such cases for this reason.

Management

Current therapeutic options to manage patients with FVII deficiency include fibrinolytic inhibitors (tranexamic acid), plasma, intermediate purity FIX concentrates (prothrombin complex concentrates), FVII concentrates and recombinant factor VIIa (rFVIIa).

Plasma FVII has a short *in vivo* half-life of approximately 5 hours; plasma infusions may not achieve adequate levels for normal hemostasis. With levels of FVII:C in the range of 10–15 IU/dL, efficient hemostasis can be achieved. For patients requiring replacement therapy due to FVII deficiency, rFVIIa is recommended.[22]

FACTOR X DEFICIENCY

It is an autosomal recessive disorder which is also called severe (homozygous). In general population its incidence is 1:1,000,000. Factor X or Stuart-Prower factor, deficiency was first identified in the 1950s in the US and England in two patients: Rufus Stuart and Audrey Prower. Kumar et al. from India reported three pediatric cases with FX deficiency and two out of them were product of consanguineous marriage.[32]

In the coagulation cascade, factor X occupies a unique position, as the first enzyme in the common pathway of thrombus formation. Following secretion into plasma, FX synthesis occurs in the liver, at a concentration of 10 μg/mL.

Either a quantitative deficiency or a dysfunctional molecule can be the cause of this deficiency. Systemic amyloidosis, although rare in children, may be associated with factor X deficiency owing to the adsorption of factor X on the amyloid protein.

Clinical Features

FX deficiency may present at any age in individuals. With umbilical stump bleeding, factor X deficiency may be present in the neonatal period too. Easy bruising may be experienced in patients who are mildly affected by factor X deficiency. Epistaxis is the most frequent symptom

in patients with FX deficiency and other mucosal-type bleeding is less frequent. Women of reproductive age may present with menorrhagia. Rarely reported presentations include central nervous system hemorrhage, hemarthroses and severe postoperative hemorrhage. Severe arthropathy may be due to recurrent hemarthroses.

Bleeding only after hemostatic challenge is seen in moderately affected patients (FX:C 1–5 IU/dL), for example, trauma or surgery.

During routine screening or family studies, mild FX deficiency (FX:C 6–10 IU/dL) may be identified incidentally.

Diagnosis

Following the finding of a prolonged PT and APTT, which corrects in a 50:50 mix with normal plasma, the diagnosis of FX deficiency is suspected. By measuring the plasma FX levels, the FX deficiency diagnosis is confirmed. the one-stage PT- and APTT-based assays, a chromogenic assay, an assay employing Russell viper venom (RVV) and an immunological assay are the five different assays available for measuring plasma FX levels. However, for the diagnosis of FX deficiency, one-stage PT- or APTT-based assay are sufficient. Before the diagnosis of FX deficiency is made, it is crucial to exclude other deficiencies such as vitamin K and other acquired causes of a clotting disorder. However, a therapeutic trial of vitamin K may be of value.

Management

Management of patients with FX deficiency includes fibrinolytic inhibitors, plasma and intermediate purity FIX concentrates (prothrombin complex concentrates). To treat acquired FX deficiency secondary to amyloidosis rVIIa is used.[33] Even in the immediate postoperative period, for hemostasis, factor levels of 10–20 IU/dL are generally sufficient. For management of the acute bleed and the treatment of choice is prothrombin complex concentrates. The biological half-life of FX is 20–40 hour, so infusion of approximately 20 mL of FFP per kg of body weight followed by 6 mL/kg every 12 hours increases the level FX sufficiently to achieve hemostasis for minor bleeding episodes.[34]

FACTOR XI DEFICIENCY (HEMOPHILIA C)

Factor XI (FXI) deficiency or hemophilia C was described for the first time in 1953 in a Jewish family in the United States by Dr Rosenthal. It is particularly common in Ashkenazi Jews in whom the heterozygote frequency is 8 percent. Factor XI deficiency is generally transmitted as an autosomal recessive trait, and both sexes are affected; however, cases of dominant transmission have also been reported. FXI can be activated to FXIa by FXIIa, thrombin, or by autoactivation. FXIa promotes coagulation by activating factor IX (FIX).

This disorder is divided into two categories:

1. Type I deficiency, which corresponds with low activity and levels of FXI antigen.
2. Type II deficiency, with low activity and normal FXI antigen levels.[35]

FXI deficiency has been described in all racial groups.[36]

Gene defect is seen in the F11 gene, located on the long arm of chromosome 4 [4q35]. This gene encodes an 18-amino acid signal peptide and a 607-amino acid mature protein. More than 100 mutations causing FXI deficiency have been reported which are distributed all over the gene.[37]

Clinical Features

It can affect both men and women and is associated with mild to moderate bleeding, especially after trauma or surgery.

There are no reports of presentation of spontaneous bleeding in the neonatal period. No instances of neonatal intracranial hemorrhage resulting from FXI deficiency have been reported. Epistaxis, soft tissue hemorrhage, and bleeding after dental extraction may occur, but hemarthroses and concomitant arthropathy are not seen.

Diagnosis

The APPT is prolonged in factor XI deficiency, whereas the PT is normal.

Management

Minor surgeries can be controlled with local pressure; dental extraction can be monitored closely and the patient treated only if hemorrhage occurs. Fibrinolytic inhibitors (Tranexamic acid-15 mg/kg, 8 hourly) are useful. The IV preparation is given orally in this situation although this is not a licensed use of the product.[18]

Plasma infusion of 1 mL/kg body weight can increase the circulating factor by about 1.5 U/dL.

A loading dose of 15–20 mL of plasma/kg will result in plasma level of 20–30 U/dL, a level that is usually sufficient to control moderate hemorrhage. The half-life of FXI is 48 hours or greater.

FACTOR XIII DEFICIENCY (FIBRIN STABILIZING FACTOR DEFICIENCY)

Inherited factor XIII (FXIII) deficiency is an autosomal recessive bleeding disorder, mostly because of defects in the FXIII-A gene resulting in FXIII-A deficiency.[38]

Jayandharan et al. reported nine mutations in coagulation factor XIII A gene in eight unrelated Indians and five out of them were novel.[39]

Clinical Manifestations

Factor XIII deficiency is charecterized by delayed hemorrhage. Patients develop a bruise or hematoma after delay of some time interval of injury. Bleeding from the umbilical stump in the first few days of life with delayed separation is common. It is characteristic that prolonged bleeding following trauma, after an intracranial hemorrhage, ecchymoses, hematomas.[40] Hemarthroses and bleeding into the muscles are less common than in hemophiliacs. Delayed wound healing also occurs. In affected females, habitual abortions are commonly observed. It may either be due to intrauterine bleeding or impaired formation of cytotrophoblastic shell leading to detachment of the placenta and miscarriage.

Diagnosis

The normal factor XIII deficiencies are PT and APTT. Due to the failure of cross linking there is an increased solubility of clot and screening tests for factor XIII deficiency are based on this observation. FXIII deficiency is demonstrated by increased clot solubility in 5 M urea, dilute monochloroacetic acid or acetic acid. To determine FXIII activity quantitatively, measuring the incorporation of fluorescent or radioactive amines into proteins is adopted. Specific ELISA tests are required to assess FXIII-A and FXIII-B antigen lvels.

Management

Replacement therapy for FXIII deficiency is highly satisfactory because of the small quantities of FXIII needed for effective hemostasis (5%) and the long half-life of FXIII (10–14 days). Prophylactic therapy with plasma-derived, virus-inactivated FXIII concentrate at a dose of 10–20 U/kg every 5–6 weeks has been successful in achieving normal hemostasis.[41] A new recombinant FXIII-A2 (rFXIII-A2) concentrate appears to be safe and appropriate for monthly prophylactic administration in patients with FXIII-A deficiency[42]. Characteristic features of rare coagulation disorders are shown in Table 2.

CONCLUSION

The most frequent bleeding disorders are the von Willebrand and disease, hemophilia A and B. Inherited deficiencies of coagulation factors other than factor (F) VIII and FIX, the so-called rare coagulatuion disorders (fibrinogen, FII, FV, FV+FVIII, FVII, FX, FXI, FXIII deficiencies), generally leads

Table 2 Characteristic features of rare coagulation disorders

Disorder	*Inheritance*	*Clinical features*	*APTT*	*PT*	*TT*
Factor I deficiency	Autosomal recessive 4q23-34	Predisposition to thrombosis; may suffer from little, moderate or severe bleeding	Prolonged	Prolonged	Prolonged
Factor II deficiency	Autosomal recessive 11p11-q12	Umbilical cord bleeding and intracranial bleeds in neonates; bleeding after trauma or surgery; easy bruising	Prolonged	Prolonged	Normal
Factor V deficiency	Autosomal recessive 1q21-25	Bleeding into the skin; nose bleeds; bleeding of the gums; prolonged/excessive bleeding with minor injuries, surgery or trauma	Prolonged	Prolonged	Normal
Factor VII deficiency	Autosomal recessive 13q34	Spectrum variable. Bleeding of mucous membranes; excessive bruising; bleeding into muscles and/or joints; CNS bleeding	Normal	Prolonged	Normal
Factor X deficiency	Autosomal recessive 13q32	Umbilical stump bleeding. Mucous membrane bleeding; bleeding into joints; muscle bleeding; CNS bleeding	Prolonged	Prolonged	
Factor XI deficiency	Autosomal recessive 4q35	Mild-to-moderate bleeding. Prolonged/ excessive bleeding with surgery or trauma; bruising; hematuria; delayed bleeding	Prolonged	Normal	
Factor XIII deficiency	Autosomal recessive A-subunit- 6p24-25 B-subunit- 1q31-32	Delayed bleeding; bleeding from umbilical stump after birth with delayed separation; prolonged bleeding from trauma; delayed wound healing	Normal	Normal	Normal

to lifelong bleeding disorders. These disorders are largely inherited by autosomal recessive genetics. As these are not well-characterized clinically in comparison to common bleeding disorders, they do not have well-established treatment strategies. High index of suspicion is required to diagnose rare coagulation disorders and a sophisticated laboratory support is essential to confirm the clinical diagnosis.

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CHAPTER 30

Acquired Inhibitors of Coagulation

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Acquired inhibitors of coagulation are antibodies that develop against coagulation proteins when there is either a congenital deficiency of coagulation proteins or when there is an underlying disease process that precipitates the formation of these antibodies. The resultant functional deficiency of coagulation factors causes altered coagulation profiles leading to, at times, severe bleeding disorders.

Coagulation inhibitors are basically antibodies against naturally occurring clotting factors and may occur as alloantibodies in patients with congenital factor deficiencies or as autoantibodies in patients with previously normal coagulation who have associated underlying diseases.[1] Bleeding occurs from multiple sites and is often compounded by the simultaneous deficiency of natural clotting factors. As acquired inhibitors of coagulation are quite rare, a high index of suspicion is required to recognize and treat the disorder effectively. Multiple mechanisms are involved in the pathogenesis and hence treatment modalities need to attend to all the mechanisms in order to be effective.

Among all the clotting factor inhibitors, the most commonly occurring inhibitor is against factor VIII and the resultant clinical syndrome is referred to as "acquired hemophilia".[2] Autoantibody inhibitors against factor II, factor V, factor VII, factor IX, factor X, factor XI, factor XIII and the von Willebrand factor proteins have also been reported.[3]

ACQUIRED HEMOPHILIA

Inhibitors to FVIII are the most common in clinical practice, but the diagnosis of acquired hemophilia is difficult owing to its rarity and because the patient does not have the usual precedent personal or family history of bleeding as seen in congenital hemophilia.[2] Moreover, the clinical signs and symptoms as well as the severity of acquired hemophilia differs quite significantly from that of hereditary hemophilia.

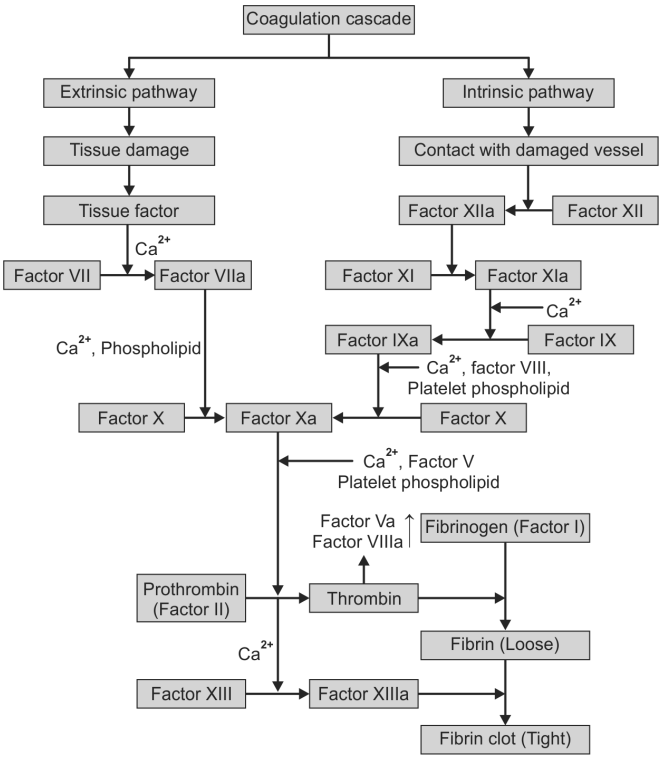
Pathophysiology

Acquired hemophilia is a spontaneous autoimmune disorder in which patients with previously normal hemostasis develop antibodies against clotting factors, most frequently FVIII.[4] The development of antibodies against FVIII leads to functional FVIII deficiency, which results in insufficient generation of thrombin through the intrinsic pathway of the coagulation cascade (Flow chart 1). Patients with this disorder are thus at an increased risk of both spontaneous as well as post-traumatic bleeding.

At the chromosomal level, the most common sites coding for the development of these offending antibodies appear to occur in the **A2 and A3 domain on the heavy chain of FVIII and in the C2 domain on the light chain.**[3,5,6] These sites when activated produce anti A2, A3 and C2 antibodies which are alloantibodies in nature in primary coagulation deficiencies and autoantibodies when associated with underlying disease.

Anti-A2 and Anti-A3 antibodies impede the binding of FVIII to activated factors X and IX of the 'Factor X activation Complex' in the intrinsic pathway while Anti-C2 antibodies inhibit the binding of FVIII to phospholipids and may also interfere with the binding of FVIII to Willebrand factor protein.[7]

Flow chart 1 Coagulation cascade



Among the antibodies, the mechanism of factor VIII inactivation differs.[8] For example, alloantibodies inactivate FVIII activity in totality according to type 1 kinetics and this total inactivation is independent of the titer or concentration of circulating antibody. In contrast, autoantibodies typically exhibit more complex type II kinetics causing an initial rapid inactivation of factor VIII followed by a slower inactivation reaction and results in some residual FVIII activity which can be detected in the blood but is not useful clinically to prevent bleeding.[8,9] The end result is that severe or partial deficiency of Factor VIII occurs leading to its associated clinical syndromes.

EPIDEMIOLOGY

Acquired hemophilia has a worldwide distribution. In the United Kingdom, the incidence has been reported to be **1.48 per million persons per year[10] while in the United States, it is 0.2 to 1.0 case per million persons per year.** These figures may, however, underestimate the true incidence of the disorder given the difficulty in making the diagnosis.[2] Further, some patients with acquired hemophilia and low titers of inhibitors may not be diagnosed, unless they bleed after surgery or trauma.[2]

The incidence of acquired inhibitors to clotting factors other than FVIII is unknown, although it is significantly lower than reported with acquired hemophilia A. There is no known association between the tendency to develop these acquired antibodies and ethnicity and these inhibitors have been seen with no specific genetic inheritance pattern in all racial groups.

ETIOLOGY

Acquired hemophilia results from the development of antibodies (mostly of the IgG_1 and IgG_4 subclasses) directed against various clotting factors.[3,8,11]

Numerous conditions that have been associated with acquired inhibitors to FVIII (Table 1) include:

- Frequent blood transfusions (as in hemolytic anemias)
- Pregnancy
- Autoimmune disorders
- Inflammatory bowel disease
- Dermatologic disorders
- Respiratory diseases
- Diabetes mellitus
- Infections
- Malignancies
- Rarely, FVIII antibodies arise as an idiosyncratic reaction to medications.

However, in approximately 50 percent of cases, no underlying or precipitating factor can be found.[2,12]

CLINICAL FEATURES

History

Unlike patients with hereditary hemophilia, patients do not have a personal or family history of bleeding episodes.[2] About half of the cases are associated with other conditions, such as autoimmune disease, and cancer[2,12] and history may often reflect the underlying disease.

Physical Findings

In these patients, instead of the intra-articular bleeding episodes, which are typical in congenital FVIII deficiency, hemorrhages occur into the skin, muscles, soft tissues and mucous membranes.[2] Bleeding episodes are often more frequent and severe than in congenital hemophilia.

On Examination

Typical signs include epistaxis, gastrointestinal and urological bleeding and rarely cerebral hemorrhage.[2,7,11] Spontaneous bruising and muscle hematomas are also quite frequent.[3] Other manifestations include prolonged bleeding following trauma or surgery and iatrogenic bleeding, particularly following attempts to insert intravenous lines.[2]

Table 1 Conditions associated with acquired inhibitors to factor VIII

S. No.	*System involved*	*Disease state*
1.	Frequent blood transfusions	Hemolytic anemias (Thalassemia)
2.	Autoimmune disorders	Rheumatoid arthritis Systemic lupus erythematosus Autoimmune hemolytic anemia Goodpasture syndrome Myasthenia gravis Grave's disease Autoimmune hypothyroidism
3.	Inflammatory bowel disease	Ulcerative colitis
4.	Dermatologic disorders	Psoriasis Pemphigus
5.	Respiratory diseases	Asthma
6.	Drugs	Penicillin and its derivatives Sulfonamides Phenytoin Chloramphenicol Methyldopa Interferon-Alfa
7.	Malignancies and premalignant conditions	Solid tumors Chronic lymphocytic leukemia Non-Hodgkin lymphoma Waldenström macroglobulinemia Myelodysplastic syndrome Myelofibrosis Erythroleukemia
8.	Infections and vaccinations	Acute hepatitis B infection Acute hepatitis C infection BCG vaccination
9.	Idiopathic	

INVESTIGATIONS

- An isolated prolongation of the activated partial thromboplastin time (aPTT) that is not corrected when the patient's plasma is incubated with equal volumes of normal plasma in a mixing study is pathognomonic of acquired inhibitors to factor VIII.[2,11] Because the action of the inhibitor is often delayed, incubation for 2 hours at 37°C is required before the correction study is initiated.[13]
- **Bleeding time, prothrombin time, and platelet counts are normal.**
- **Reduced factor VIII levels and evidence of a factor VIII inhibitor are diagnostic.** Although acquired hemophilia A is a rare condition, FVIII inhibitors in very low concentrations and not typically detected by screening coagulation assays have been detected by specific assays in 17 percent of healthy individuals with normal FVIII levels and no bleeding symptoms or history.[14,15]
- Acquired hemophilia can occasionally be confused with disseminated intravascular coagulation because of its clinical presentation and a prolonged aPTT; however, the absence of a prolonged prothrombin time (PT), low fibrinogen, elevated fibrin degradation products and D-dimers and thrombocytopenia[7] should help distinguish between the conditions.
- Among the common causes of isolated prolonged aPTT is **lupus anticoagulant**.[16] Presence of lupus anticoagulant is suggested when aPTT values during the mixing study are similar at time zero and after incubation at 37°C[8] and can be confirmed by specific tests, such as the dilute Russell viper venom time and the kaolin clotting time.[17,8]
- As heparin administration can also prolong aPTT, a thorough treatment history and relevant tests

for heparin effects are indicated. The presence of heparin is suggested by a prolonged thrombin time in association with a normal reptilase time.[8]

- The levels of other intrinsic pathway factors (factors IX, XI, and XII) may also be reduced by antibodies against these factors in patients with acquired hemophilia A.[14,12] Therefore, it is important to repeat factor assays[8] using increasing dilutions of patient plasma to establish the specificity of the inhibitor. Once detected, the acquired inhibitor should be quantified to assess the severity of the disorder and the risk of hemorrhage. Methods used for quantifying factor VIII inhibitors are the Bethesda assay and the Nijmegen modification of the Bethesda assay.[18] **One Bethesda unit (BU) is the quantity of inhibitor that inactivates 50 percent of factor VIII in normal plasma after incubation at 37°C for 2 hours.** However, both the Bethesda assay and the Nijmegen modification may underestimate the potency of the inhibitor due to its nonlinear complex reaction kinetics.[18] As a result of its kinetic profile, the recovery and half-life of exogenous FVIII may be considerably reduced, even in patients with low inhibitor titers. This has significant implications for therapy.
- *Imaging studies:* MRI, CT scan, and ultrasound may be needed to localize, quantify, and serially monitor the location of bleeding and response to therapy. Other imaging tests can be used as needed to diagnose associated diseases.
- *Other tests:* Testing patients with pregnancy-associated acquired hemophilia, for autoimmune disorders such as lupus and rheumatoid arthritis is recommended because the presence of an autoimmune disorder may require a change in therapeutic approach.[19]

DIFFERENTIAL DIAGNOSES

- Lupus anticoagulant
- von Willebrand disease
- Disseminated intravascular coagulation
- Dysfibrinogenemia
- Heparin administration
- Congenital hemophilia

MANAGEMENT

Management of acquired inhibitors involves three strategies:

1. Management of acute bleeding
2. Eradication of the inhibitor
3. Management of the etiological cause

The approach to these objectives usually depends on the natural history of the disease, the clinical presentation, and the titer of the inhibitor. Frequently, treatment of the underlying disorder or the discontinuation of an offending drug may be all that is required.[7]

MANAGEMENT OF BLEEDING

Management of Mild Bleeding

Patients with mild or minimal bleeding rarely require specific treatment to control bleeding and require only immunosuppressive therapy for the inhibitors. Studies have shown that there is no correlation between the titer of the inhibitor and the severity of bleeding hence treatment should be based on symptomatology rather than on the inhibitor titers.[10]

Management of Moderate-to-Severe Bleeding

In patients with moderate-to-severe bleeding, the management depends upon the inhibitor titer.[10] In patients with very low titer inhibitors (<3 BU) treatment with Desmopressin has been found to be useful in augmenting residual factor VIII activity.[7] IV infusion of desmopressin (0.3 mcg/kg) may result in a 2- to 3- fold temporary increase in plasma levels of FVIII and von Willebrand factor.[19] However, in many patients, Desmopressin treatment alone will not ensure hemostasis.[7]

In cases where the inhibitor **titer is between 3 and 5 BU**, increasing the levels of factor VIII in the plasma by factor VIII infusions may suffice to control the bleeding.[7,12] These patients may need a higher than usual dose of factor VIII, as much as double or triple the dose compared to patients of congenital hemophilia of the same body weight.[19,20] An arbitrary dose of FVIII 200 IU/kg IV bolus every 8–12 hours has been recommended.[21] There are no published studies on the use of human FVIII in acquired hemophilia to guide its dosing.[14]

Patients in whom the levels of Factor VIII inhibitor is higher (> 5 BU) require other modalities of treatment to control bleeding:

- **Recombinant activated factor VII (FVIIa):** Here the requirement of factor VIII in the coagulation cascade is bypassed by FVIIa binding to activated platelets and promoting thrombin synthesis, thereby controlling bleeding. Studies have shown that there is dramatic control of bleeding in more than 90 percent of cases within a few hours of infusion.[22] FVIIa has also been found to have very few side effects, is free of anamnestic reactions and does not transmit blood borne diseases.
- **Activated prothrombin complex concentrates:** Activated prothrombin complex concentrates (APCCs) are also used to manage bleeding episodes in acquired hemophilia. The mechanism of action is by bypassing the requirement of factor VIII and promoting thrombin

synthesis in the coagulation pathway. Studies in adults have shown a response rate of 86 percent.[23] No studies are yet available on children. There is a potential risk of anamnestic reaction and transmission of blood-borne diseases with APCCs which has restricted their universal use.

- **Immunoadsorption/plasmapheresis:** Selective removal of the inhibitor using immunoadsorption has been found to be useful especially in cases where there is severe hemorrhage, no response to the above modalities of therapy or when the concentration inhibitors is very high. After the inhibitors are removed, factor VIII infusions are given to control the bleeding.

ERADICATION OF THE INHIBITOR

Eradication of the inhibitor is achieved by stopping the production of the inhibitor by immunosuppressive therapy.

The various modalities available are:

- **Steroids:** These have been used as first line therapy in the eradication of the inhibitors. Methyl prednisolone (oral or IV) or oral prednisolone are the drugs of choice and have shown response in 60 to 70 percent of adult cases.
- **Cytotoxic drug therapy** with **cyclophosphamide and azathioprine** has been used to control inhibitor production. However, the side effects of these drugs and their low safety profiles restrict their universal use.
- Other drugs which have also been used are **mycophenolate mofetil, vincristine and 2-chlorodeoxyadenosine.**
- **Cyclosporine** has also been found to have good immunosuppressive effect on the inhibitors, however due to its toxicity and side effects, its use in children is restricted. It has been tried both as monotherapy and as an adjuvant drug to steroids, showing best results in cases of systemic lupus erythematosus.[7]
- **Immunoglobulins** have been used as a second line treatment option in patients not responsive to other modalities.[7] However, studies have shown an equivocal response of immunoglobulins in eradicating inhibitors, and have found best response in those with low titers of the inhibitor.[15]
- **Biological therapy-Rituximab, an anti-CD20 monoclonal antibody,** has shown promising results in eradicating inhibitors in acquired hemophilia.[7,24-26] Given in the dose of **375 mg/m^2 on D1 and D15**, it has shown excellent results in refractory cases.
- *Surgical management:* May be required in cases where there are life-threatening bleeding episodes. Treatment options vary according to the site of bleeding and can be mechanical (ligature placement, selective embolization), thermal (electrocautery, cryotherapy) or chemical (fibrin glues, micronized collagen).[27]

ACQUIRED INHIBITORS TO VON WILLEBRAND FACTOR

Acquired inhibitors to the von Willebrand factor (vWF) are infrequently encountered and have been seen in association with:

- Autoimmune disorders, monoclonal gammopathies, lymphoproliferative diseases
- Epidermoid malignancies, Wilm's tumor
- Hypothyroidism
- Myeloproliferative disorders
- Certain medications.

The incidence of these acquired antibodies is especially high in children with Wilm's tumor, which makes identifying such patients important due to the inevitable associated hemorrhagic complications that occur during surgery. The exact mechanism of synthesis of this antibody is unclear, however, an interaction between a plasma factor secreted by the tumor and the naturally occurring vWF in the blood is thought to result in premature clearance of the vWF.[28] Treatment options include Desmopressin, infusion of FVIII that contains vWF (cryoprecipitate), platelet transfusions, intravenous immunoglobulin and plasma exchange. Acquired anti-vWF antibody usually disappears after treatment of the tumor.

ACQUIRED INHIBITORS TO FACTOR V

Acquired inhibitors to factor V are rare and are seen in lymphoproliferative disorders, adenocarcinoma, tuberculosis, prolonged aminoglycosides (particularly streptomycin) usage and topical exposure to bovine thrombin.[29] The clinical presentation of children with this inhibitor is varied, some children bleed while others do not which is probably because patients with antibodies that bind to the factor V present on the platelets bleed more profusely than those where the antibody binds to factor V present in the plasma.[30] Treatment is by transfusing fresh frozen plasma and recombinant factor VIIa.

ACQUIRED INHIBITORS TO PROTHROMBIN

Acquired inhibitors to prothrombin occur in patients with systemic lupus erythematosus, in children treated with bovine fibrin glue after surgery for congenital heart disease or exposure to procainamide. A small number are idiopathic in origin. In children in whom the concentration of lupus anticoagulant is high, acquired inhibitors to prothrombin are also present. These inhibitors are difficult

Table 2 Conditions associated with acquired inhibitors to other clotting factors[11, 31]

Coagulation factor	*Associated disorders*
VII	Bronchogenic carcinoma, idiopathic
IX	Systemic lupus erythematosus, acute rheumatic fever, hepatitis, collagen vascular diseases, multiple sclerosis, and postpartum
X	Amyloidosis, carcinoma, acute nonlymphocytic leukemia, acute respiratory infections, fungicide exposure, idiopathic
XI	Autoimmune diseases, prostate carcinoma, chronic lymphocytic leukemia, chlorpromazine
XIII	Idiopathic, isoniazid, penicillin

to measure as they do not neutralize coagulant activity in activity dependent inhibitor assays and hence do not cause clinically significant spontaneous bleeding. However, they may cause bleeding during surgery or after trauma. Treatment is by using fresh frozen plasma or activated prothrombin complex concentrates. These inhibitors usually disappear spontaneously within 7 to 21 days or else can be treated using plasmapheresis, corticosteroids and immunosuppression.

ACQUIRED INHIBITORS TO OTHER FACTORS VII, IX, X, XI, XIII

Inhibitors to these factors occur in various conditions (Table 2) and are not common in children. Treatment options include fresh frozen plasma, activated prothrombin complex concentrates, recombinant factor VIIa along with inhibitor eradication using plasmapheresis, corticosteroids and immunosuppression.

CONCLUSION

Acquired inhibitors to naturally occurring clotting factors are commonly encountered in clinical practice and must be considered in the differential diagnoses of any child with an altered bleeding profile not responding to the standard therapy. Acquired inhibitors are associated with numerous common underlying conditions and require to be managed aggressively in order to prevent mortality and morbidity. Early recognition of the presence of inhibitors helps to institute appropriate management to control the bleeding as well as prevent further episodes.

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CHAPTER 31

Immune Thrombocytopenic Purpura—Diagnosis and Management

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Immune thrombocytopenic purpura (ITP) in children is an acquired hemorrhagic disorder occurring in an apparently healthy child, usually due to transient postviral autoimmune phenomenon, characterized by acute onset of petechiae, bruising and mucosal bleeding. It is associated with isolated thrombocytopenia (platelet count <1,00,000/cumm) with normal or increased megakaryocytes in an otherwise normal marrow without evidence of concurrent abnormality or disease process that might account for the thrombocytopenia. Despite major advances in our understanding of the molecular basis of many blood disorders, despite major advances in our understanding of basic underlying pathophysiology for more than 50 years, the diagnosis of ITP still remains one of exclusion and currently no confirmatory clinical or laboratory diagnostic parameters exist.

There is no single simple test for the diagnosis of ITP. Hence, there are many unresolved issues pertaining to its diagnosis and management of this disease.

Immune thrombocytopenic purpura (ITP) was first described as Morbus hemorrhagicus maculosus by German physician Paul Gottlieb Werlof in 1735.[1–3] Immune thrombocytopenic purpura (ITP) is most common acquired autoimmune bleeding disorder and is characterized by a low platelet count and mucocutaneous bleeding.[1-9,3a]

CLASSIFICATION

ITP may be classified as:[4-9]

- Acute
- Chronic
- Recurrent.

These differ especially in respect to patient's age at onset, sex, medical events preceding the onset, duration of thrombocytopenia and response to treatment. It is not possible to distinguish them at the onset of symptomatology. However, in the age group above 13 years, the incidence of chronic ITP is higher. In this age group 80 to 90 percent cases continue to have active disease for 6 months to one year.[4-9]

Acute ITP is common after the age of 3 months through childhood with a peak incidence between 2 and 5 years of age which also is the age at which children are most susceptible to viral infections, a major etiological factor.[4-9]

Acute ITP is generally a benign and self-limiting condition, with 90 percent of them making an uneventful recovery within 3 weeks to 6 months with or without specific treatment. Only 10 percent of ITP cases progress to chronic ITP. 65-95 percent of prepubertal children who develop ITP have the acute form of the disease. Of these 55 to 75 percent in < 1 month, 80 to 90 percent in < 4 to 6 months resolve, only 10 percent of ITP cases progress to chronic ITP.[2-6] ITP occurring for the first time before the age of 10 years is predominantly acute whereas after 20 years of age chronic ITP is almost the rule, though the disease is not limited to any age.[7,9] Although there is no sex predilection for ITP in childhood, in the adult form of the disease, there is 3:1 predominance in women.

In recurrent ITP there is recurrence of thrombocytopenia after a sustained normal platelet count. It is precipitated each time usually following a viral infection. Boys and girls are equally affected.

DIAGNOSIS OF ITP[1-9,12-19]

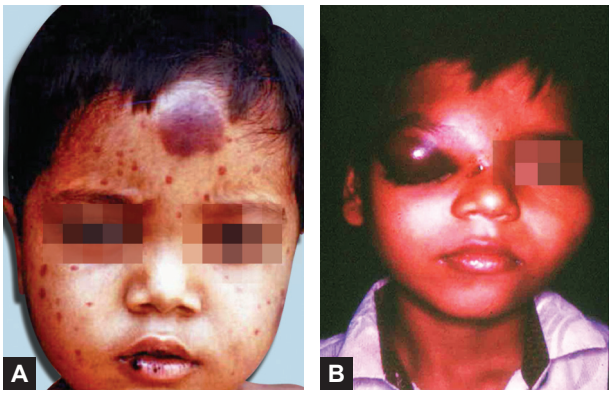
There is no single simple test for the diagnosis of ITP. The diagnosis of ITP is still a clinical one, based on the patient's history, physical examination and complete blood cell count as well as examination of peripheral blood smear and bone marrow examination (If done.).[12-16] There is no "gold standard" test that can reliably establish the diagnosis. The usual presentation is sudden onset of mucocutaneous bleeds (Figs 1A and B) in an otherwise healthy child (the child may be considered healthy in the absence of fever and any systemic abnormality). The diagnosis is more by exclusion. A typical case is a child with ITP, characterized by isolated thrombocytopenia, with normal counts in otherwise healthy child without any hepatosplenomegaly, no lymphadenopathy and bony tenderness and may be preceded by viral infection 2 to 3 weeks earlier. However, patients with risk factors for human immunodeficiency virus, should be tested for HIV antibodies.

It is usually a benign disorder.

ITP may be (Table 1):[4-12]

- *Asymptomatic:* Incidentally detected low platelet count. Systemic examination is usually normal. No bleeding manifestations may be present.
- *Mild symptoms:* Bruising/petechiae, minor epistaxis, little/no interference with daily living.
- *Moderate symptoms:* Skin bleed, epistaxis and menorrhagia, mucosal bleeds more troublesome.
- *Severe symptoms:* Severe bleeding episodes include ICH requiring hospitalization and/or transfusions. GI bleeding, severe epistaxis, hematuria, prolonged menorrhagia.

Usually there is no hepatosplenomegaly; spleen may be just palpable in 10 to 12 percent of the even normal children.



Figs 1A and B Mucocutaneous bleeds (hematoma, petechiae and purpura)

Table 1 Incidence of symptoms in ITP[10]

	Common bleeds UK survey		*Our series Total 132*
Bruising and skin bleeds	386	(90%)	122
Nose bleeds	95	(20%)	31
ICH		1%	1
Mouth, gum, tongue bleed	68	(16%)	35
GI bleed	10	(2%)	5
Conjuctival hemorrhage	7	(2%)	5
Hematuria	6	(1%)	7
Heavy periods	3	(0.7%)	2
Bleeding ear/eye	2	(0.5%)	3
No bleeding symptoms	8	(2%)	1
Preceding viral infection/immunization	245	(57%)	30

- If child is clinically ill, with moderate or massive splenomegaly, sternal tenderness/bony tenderness or joint pains then it suggests an alternative cause, sinister causes like malignancy, leukemia.
- Constitutional symptoms, such as fever or weight loss, hepatomegaly or lymphadenopathy might indicate underlying disorder such as HIV, viral infection, systemic lupus erythematosus (SLE), or a lympho-proliferative disease.
- Anemia disproportionate to severity of bleeding, is unlikely to be due to ITP and should consider aplastic anemia, leukemia.
- Atypical rash should lead to suspicion of an alternate diagnosis like viral infections.
- If child is clinically ill, then it is unlikely to be ITP, and should consider other etiology like infection—meningococcal infection, septicemia and other sinister diseases like aplastic anemia, leukemia, etc.
- Presence of significant lymphadenopathy, hepatosplenomegaly should lead to the suspicion of infectious diseases like EBV or CMV or any other alternative diagnosis like leukemia.
- Response to specific therapy, for example, intravenous immunoglobulin (IVIg) and intravenous anti-D is supportive of the diagnosis, but a response does not exclude secondary ITP.

On examination, these children may have petechiae, purpura and/or bruises. Serious bleeding is rare. Only 4 percent have serious symptoms such as severe epistaxis, GI bleeding or hematuria. Less than 1 percent children with ITP develop intracranial bleeds.

Presence of fever, weight loss, bony pains, hepatosplenomegaly or lymphadenopathy and anemia disproportionate to amount of bleeding, would suggest diagnosis other than ITP (e.g. leukemia, lymphoma, viral infections, malaria, aplastic anemia, etc.). However, a just palpable spleen may be normally present in 10 percent of pediatric population.

When platelets are reduced in number (Thrombocytopenia) or defective in function (thrombasthenia), bleeding may occur. Bleeding typically involves skin and mucous membranes including petechiae, purpura, ecchymosis and epistaxis, hematuria and gastrointestinal hemorrhage. Intracranial hemorrhage can occur rarely.

Most thrombocytopenia in children are the result of increased platelet destruction. The bone marrow in such cases responds with compensatory increase in the rate of production with increased number of immature megakaryocytes. The increased mean platelet volume provides supportive evidence of the larger size young platelets, which are functionally very active, are more prominent in the peripheral blood smear. The increased mean platelet volume provides evidence of the larger size. Normal MPV is 6.0–10 fL.

In disorders with decreased platelet production, the decreased platelet number is associated with small sized platelets, a decreased mean platelet volume and a longer bleeding time relative to platelet number. The megakaryocytes are decreased in number or absent in bone marrow aspirate.

In the diagnostic evaluation of thrombocytopenia, it is important first to determine whether other blood components are involved. Co-existing abnormalities of the white blood cells or red cells may indicate other causes of diseases involving bone marrow like aplastic anemia (Fig. 7), leukemia (Fig. 8).

Abnormalities in coagulation, in association with thrombocytopenia, suggest disorders of consumption including DIC, liver disorder.

Platelets are one of the important components in the first phase of hemostasis and platelet plug formation.

The characteristics of platelets are (Figs 2 to 5):

Number: 150,000 to 400,000/mm^3, out of which 2/3rd circulate in blood stream and 1/3rd located in spleen. Life span is 7 to 10 days.

Mean platelet volume (MPV)—7.1 fL.

Most thrombocytopenia in children is the result of increased platelet destruction. The bone marrow in such cases responds with compensatory increase in the

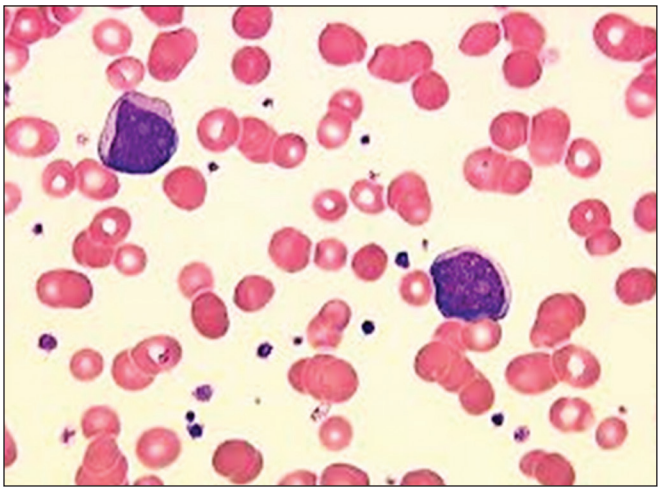


Fig. 2 Normal blood smear with normal platelet count

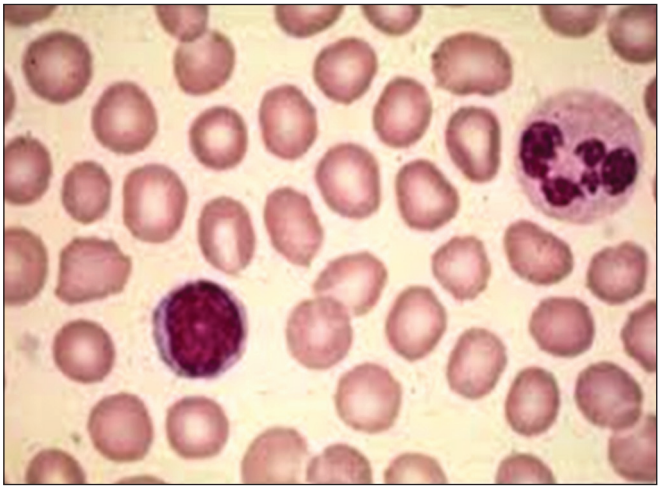


Fig. 3 Blood smear with decreased platelet count

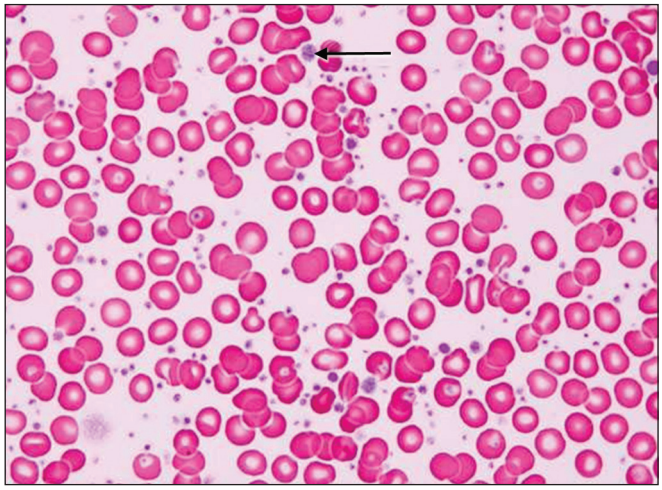


Fig. 4 Blood smear with increased platelet count

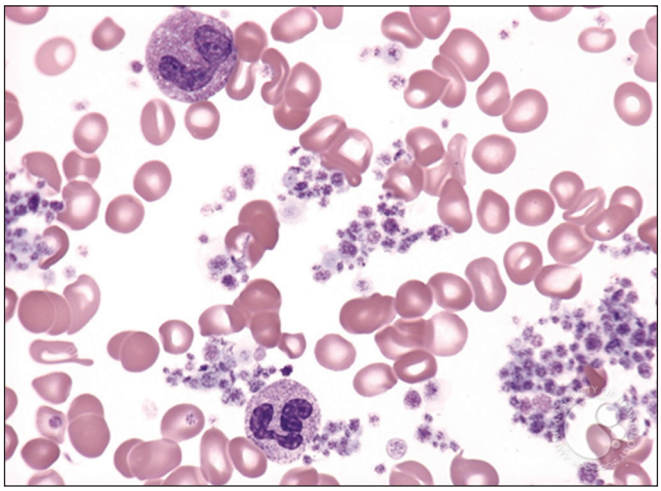


Fig. 5 Blood smear with increased platelet count and platelet in clumps

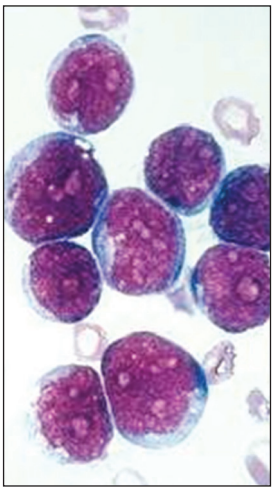


Fig. 8 Bone marrow aspiration smear in leukemia

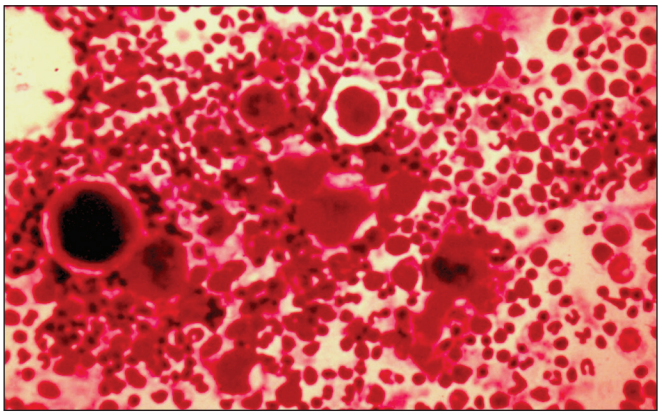


Fig. 6 Bone marrow aspiration smear—increased megakaryocytes

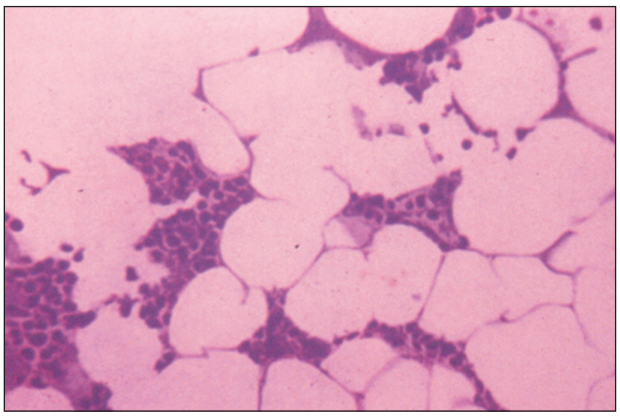


Fig. 7 Bone marrow aspiration smear in aplastic anemia

rate of production with increased number of immature megakaryocytes (Fig. 6). The large young platelets, which are functionally very active, are more prominent in the peripheral blood smear. The increased mean platelet volume provides supportive evidence of the larger size.

In disorders with decreased platelet production, the decreased platelet number is associated with small sized platelets, a decreased mean platelet volume and a longer bleeding time relative to platelet number. The megakaryocytes are decreased in number or absent in bone marrow aspirate.

Platelet count less than 100,000/cumm is called thrombocytopenia

- 80 percent of children with acute ITP have platelet count less than 40,000/cumm.
- In chronic ITP, platelet count at the time of presentation is usually higher (20,000–75,000/cumm).

Thrombocytopenia is evident on peripheral smear and is accompanied by bizarre shaped or giant forms of platelets (Fig. 3).

In chronic ITP both mean platelet volume (MPV) and number of large platelets are significantly increased compared to control values. However, these values are unchanged in acute ITP.

The presence of low or normal MPV in a case of thrombocytopenia suggests aregenerative thrombocytopenia, i.e. bone marrow suppression or marrow infiltration.

Platelet distribution width (PDW) may be more discriminating than MPV in detection of compensated thrombocytopenic states.

Leukocyte Count

The total leukocyte count is usually normal though mild to moderate lymphocytosis with increased number of atypical lymphocytes may be seen especially when preceded by viral infection. Mild peripheral eosinophilia may be seen in 20 percent of children but is of no diagnostic or prognostic value.[2]

Anemia

Anemia because of blood loss is seen in about 20 percent of children with ITP. However, if the degree of anemia is disproportionate to amount of bleeding seen then, other sinister conditions like leukemia, aplastic anemia or occult blood loss, Evan's syndrome should be kept in mind. Bone marrow aspiration, trephine biopsy, Coomb's test, etc. are of immense value in confirming the diagnosis and ruling out above conditions.

Antiplatelet Antibody[13-25]

Understanding of pathophysiology and incite in the clinical and laboratory aspect started with published series of observation by Harington in 1951[13] which revealed transferable plasma factor mediated the disease in many patients. This was accomplished by infusion of plasma from ITP patients into healthy volunteers which lead to acute thrombocytopenia in recipient. Shulman et al. and others subsequently confirmed that this factor as IgG antibodies. Platelet associated with IgG antibody (PAIgG) is present in 80 percent of thrombocytopenic children with ITP. However, PAIgG is also found in other immune thrombocytopenic states. Although these tests are highly sensitive they have very low specificity as the patients with both immune and non-immune thrombocytopenia have elevated PAIgG. In Evan's syndrome, as it is associated with autoimmune hemolytic anemia, Coomb's test is helpful in the diagnosis.

Bone Marrow Examination[27-29]

In a typical case of ITP bone marrow evaluation is unnecessary. Thus, acute onset of bruising following a viral infection in a previously healthy child without any significant hepatosplenomegaly, anemia not disproportionate to amount of bleeding, bony tenderness or lymphadenopathy and appearance of mega thrombocytes without any abnormal premature cells on peripheral smear does not need evaluation of bone marrow aspirate.

Indication for Bone Marrow Aspiration

- *In patients with atypical features:* The clinical presentation suggests leukemia or aplastic anemia as a differential diagnosis, associated with hepatosplenomegaly/Lymphadenopathy, (Sternal) bone tenderness or painful joint, atypical rash
- *Abnormal leukocyte count* (leukopenia and leukocytosis) and/or abnormal (premature) cells on peripheral smear
- *Anemia (Low Hb)* disproportionate to amount of bleeding.
- Prior to the initiation of the corticosteroid therapy or blood transfusion for presumed ITP as this may lead to a temporary remission and may mask the presence of blast cells
- Lack of response to specific therapy like IVIg /Anti-D globin.
- Prolonged thrombocytopenia (> 6 months)

  A possibility of missing the diagnosis of rare conditions like a amegakaryocytic thrombocytopenic purpura should be kept in mind if bone marrow examination is not done.
- It is not necessary to perform bone marrow aspiration if IVIg therapy is contemplated.

Bone marrow examination in ITP will show normal or increased megakaryocytes with normal erythroid and myeloid maturation. Cytoplasm of megakaryocytes is decreased, often vacuolated, less granular and stains more basophilic. They may have increased nuclear lobe count. Platelet production and turnover is increased up to 8 times the normal.

Other Investigations[23,30,31]

Plasma glycocalicin (a fragment of platelet membrane glycoprotein Ib levels) are significantly below the normal range (5–27%) in a regenerative thrombocytopenic conditions like aplastic anemia and amegakaryocytic thrombocytopenic purpura. The levels are above the normal range (48–261%) in thrombocytopenia associated with normal or increased megakaryocytes in bone marrow. Over the last few years platelet survival studies using the radioisotope chromium-51, Indium-111 (In-111) have become available. There are characteristic patterns of platelet recovery and survival. Immune thrombocytopenic disorders like ITP have nearly normal platelet recovery but a very short platelet survival, whereas markedly reduced platelet recovery with normal platelet survival is seen in hypersplenism.

Glycoprotein specific acute antibody assay: Early studies showed encouraging results with sensitivity of 75-85 percent and specificity of almost 100 percent. Unfortunately recent large studies showed low (40–60%) sensitivity but high specificity (78–92%) However, patients with myelodysplastic and lymphoma with thrombocytopenia were also tested positive. Further studies perhaps using

new technology may give better sensitivity and specificity and needs further study.[24]

Management of ITP[2-9,32-53,57,58, 66,69,70-80]

The main strategy of treatment of ITP is to administer least amount of therapy "In ITP treat the child and not the platelet count." Why certain patients bleed but most do not, remains unclear. Some patients may have marked thrombocytopenia yet normal or near normal hemostasis, because of increased young platelets having better functional capacity.

On the other hand some patients (5% of all children with ITP) may have impaired platelet function as a result of antibodies and these children have prolonged bleeding time and increased bleeding tendency in spite of having near normal platelet count. Therefore, platelet count as well as bleeding time estimation is recommended prior to the decision regarding management of ITP.

Therapy depends on whether it is acute or chronic ITP.

Acute ITP[2-9]

In general 70 to 80 percent of children with acute ITP will have complete remission and permanent recovery without sequelae with or without treatment. 55 to 75 percent of those who recover do so within the first month and 80 to 90 percent within 4 to 6 months of diagnosis and rest beyond 6 months to 1 year sometimes even beyond 10 years.

Chronic ITP

However, in chronic ITP only 1/3rd go into remission spontaneously, that too usually late in the course of the disease, i.e. between 1 and 10 years after the diagnosis.

ITP patients may not need any treatment but reassurance. Children with chronic ITP whose platelet count remains within a relatively safe range (more than 10–30,000/cumm) and whose bleeding time is fairly normal need no therapy except defensive management. Remission is known to occur in about one-third of these children, sometimes as late as 10 to 20 years postdiagnosis.

The treatment of a benign disease like acute ITP should be decided after balancing the risk of treatment vs no treatment. The mainstay of treatment in majority of cases of childhood acute ITP hence is a **"Defensive management and nonfrantic watchful waiting."** During the initial period following the onset of ITP, restriction of physical activity and complete avoidance of all contact sports and playground activities. Use of helmets to prevent trauma especially head injury and using knee-cap during the phase of thrombocytopenia are indicated.

Certain antiplatelet drugs like aspirin, phenacetin, antihistaminics, phenothiazines, nonsteroidal anti-inflammatory drugs, etc. should be avoided.

Deep intramuscular injections should be avoided and if has to be given, then pressure over the injection site should be maintained minimum for 10 minutes, continuously without trying to see in between whether bleeding is present or not.

Immunization with live viral vaccines (polio, measles, MMR) preferably should be avoided during the period of severe thrombocytopenia.

Previous studies of ITP have not addressed the risk of hemorrhage during various sport activities. Restrictions of contact sports (football, soccer, kabaddi, etc.) advocated until platelet count is above 100,000. Most noncontact sports can be safely enjoyed with platelet count greater than 30,000/cumm. Serious athletes may need frequent platelet count measurement and treatment during their participation.

SPECIFIC THERAPY IN ACUTE ITP

Corticosteroid Therapy (Oral)[32-36]

The use of corticosteroids in the management of ITP is a matter of considerable controversy. In a heterogeneous disease that usually sooner or later gets better on its own and gives rise to little morbidity and low mortality, it is difficult to evaluate the modality of the treatment. It has been estimated that to have statistical significance, a randomized trial of corticosteroid versus placebo, would require some 14,000 patients. A double blind randomized prospective study is more likely to give the truth and eliminate both physician and patient bias. Normalization of platelet counts as well as reduction in prolonged bleeding time occurs earlier in the steroid treated group as compared to the untreated group. However, it takes 8 to 10 days before significant changes are noticed. In a randomized double blind and placebo controlled trial, platelet counts reached a level of 30,000/cumm or more (safe range) significantly earlier, with corticosteroids. Platelet survival increased in ITP after steroids.[17-21]

Steroids in ICH

No proof exists that use of steroids reduces the incidence of intracranial hemorrhage (ICH) or death.

Walker and Walker[3] while reviewing the data of ITP in children from England and Wales noted that 11/12 children died of ICH, 8 of whom had received corticosteroids.

Lusher and Zuelzar[4] reported ICH in only one child who died despite immediate treatment with steroids.

Table 2 Controversies in ITP in children

	No. of patients	*No. of ICH ITP*	*%*	*Steroid*	*<1 M*	*1–6 M*	*>6 M*	*Not known*	*Mortality*	*Platelet count*	*History of trauma*
Lokeshwar et al.	122	1	0.81	0/1	1	-	-	-	1/1	<20000	1/1
Walker and Walker[3]	181	1	0.5	1/1	1	-	-	-	-	<20000	-
Lusher[4] et al.	465	0	0	-	-	-	-	-	-	-	None
Choi and McClure[5]	413	6	1.4	0/6	2	4	1	-	2/6	<20000	1/6
Benham and Taft[62,63]	132	2	1.5	½	-	1	-	1	0/2	-	0/2
Simon et al.[7]	95	1	1.1	0/1	1	-	-	-	1/1	<20000	0/1
Lammi and Lovric[6]	152	1	0.7	0/1	-	1	1	-	1/1	-	0/1
Zerella et al.[6a]	183	6	3.3	4/6	2	4	1	-	2/6	<20000	2/6
Imbach et al.[64]	108	1	0.9	0/1	1	-	-	-	1/1	-	None
Total	**1851**	**19**	**1.02**	**6**	**8**	**10**	**3**	**1**	**8/19 42%**	**-**	**4**

Benham and Taft[62] reported 132 children with ITP, with 2 cases having ICH, one each in steroid treated and untreated group.

Review literature done in 1851 (Table 2) cases showed 19 cases with ICH (1.026%) and 6 cases had ICH when they were on steroid therapy. Eight children had within 1 month of diagnosis and 10 children had intracranial hemorrhage after 1 month of onset of ITP. Few of precipitating factors included hypertension, aspirin ingestion, platelet count less than 20,000/cumm and trauma.

Table 2 shows the ICH in children with ITP.

Indications for Steroids in ITP

Though there is a lot of controversy whether steroids should be given to children with acute ITP or not, there is uniformity in the opinion that large doses of steroids for a prolonged period should not be given, since steroids may in fact, suppress platelet production.

Adverse effects of steroid include: Hyperglycemia, hypertension, fluid electrolyte imbalance, psychosis, and osteoporesis, etc. Hence, the clinician should balance the benefits of the treatment against the risk.

"The child and not the platelet count should be treated." In children who present with mild illness no therapy other than purely defensive management is required.

A child with severe thrombocytopenia with a platelet count of less than 10,000 to 30,000/cumm with generalized petechiae and purpura, wet purpura with mucosal bleeding, gastrointestinal hemorrhages and fundal hemorrhages and ICH should be treated with steroids. Active young children less than 3 to 4 years with low platelet count also may be treated with steroids, because of fear of trauma induced severe bleeds.

Dose of Steroids

Prednisolone is used in the dose of 2 mg/kg/day for two to three weeks followed by tapering of dose over the next week irrespective of platelet count. However, as steroids are being tapered some patients may develop a drop in their platelet count. This is usually transitory and not an indication to step up the dose to previous levels since clinically purpura often improves. In severe cases, for initial 4 to 5 days, prednisolone may be given in a dose of 4 mg/kg followed by reduction in the dose thereafter to conventional levels.[2] A small number of patients with chronic ITP with recurrent mucosal bleeds or severe thrombocytopenia can be managed successfully with small maintenance dose (0.5–1.0 mg/kg/alternate day or even less) of corticosteroids.

Intravenous Pulse Methylprednisolone Pulse Therapy[37,38,42]

Pulses of few days' duration—single dose short course of methylprednisolone 25 mg/kg/day on 3 consecutive days resulted in early response lasting for 3 months or more. Lusher et al. (1984)[42] have used this therapy to raise

platelet counts prior to splenectomy. One of our patients, 16-year-old with chronic ITP for 8 years responded well to 3 doses of methylprednisolone and platelet count increased from 5,000/cumm to 1,50,000/cumm following which tooth extraction could be done. Similar results have been reported by other authors. A short intravenous course of high-dose methylprednisolone is effective as initial treatment of ITP. Toxicity of long-term treatment with prednisone can be avoided in a number of patients with ITP. In patients refractory to treatment with methylprednisolone, the response rate to second-line treatment with prednisone was not negatively influenced, since two-thirds of these relapsing patients subsequently responded to prednisone. Both IVIgG and methylprednisolone produce a significant early rise in platelet count that is somewhat greater with IVIgG. However, the higher platelet counts produced by IVIgG may not justify the additional cost and potential risks of this agent.

Intravenous Immunoglobulin[43-48]

The major goal in the treatment of acute ITP is to restore the platelet count to relatively safe levels as soon as possible so as to prevent ICH and life-threatening hemorrhages.

Fifty-five to seventy-five percent children with ITP recover within first month of illness, irrespective of treatment. An increase in the platelet count to a safer level of more than 30,000 to 1,80,000/cumm was noted within 1 to 2 days following IVIgG therapy. Bussel et al.[44] used 1 g/kg/day for 2 to 3 consecutive days followed by maintenance infusion. Imbach et al.[43] described randomized multicentric trial in which IVIgG was compared with steroids. Eighty percent of children in each group responded to the therapy with mean time for the peak platelet count being 12 days in the group receiving corticosteroids versus 9 days in IVIgG. Thus, the effect of corticosteroids and IVIgG were identical for children who responded rapidly to the treatment and IVIgG does not offer a major advantage over corticosteroids. However, in steroid nonresponders, IVIgG can produce better remissions. Reactions seen in 20 percent of children trivial such as headache, fever, vomiting, fatigue, etc. But there is a potential problem of transmission of plasma-borne infections like hepatitis, AIDS and other viral infections. In addition, the cost is prohibitive. (The total cost for a 10 kg child for one course will be about ₹ 30,000/- onwards). As the chance of spontaneous recovery is high and chances of ICH are very low (0–3.3 %) routine administration of IVIgG is not recommended. IVIgG should be considered for any patient with ITP in whom rapid rise in platelet count is deemed essential such as before surgery, after significant trauma, especially a child with head injury, menorrhagia, delivery, life-threatening bleeds like gastrointestinal bleeding and ICH and during pregnancy as steroids are contraindicated. In addition, young children below the age of 5 years with severe, recurrent hemorrhage may be given IVIgG to postpone/avoid splenectomy. Bussel[44] et al. were able to avoid splenectomy in about 75 percent of patients with ICH. IVIgG acts by causing temporary reticuloendothelial blockade. This might be due to two separate effects, a decrease in Fc receptor affinity for platelet associated IgG and competition for Fc receptors by the increased serum IgG.

IVIgG in Chronic ITP

IVIgG is effective for temporarily raising the platelet count in 70 to 80 percent of children with chronic ITP. Platelet count rises within 1 to 3 days of infusion. Permanent remission occurs only in minority (0–20%) of these children. A safe count however (above 20,000–30,000/cumm) may be achieved with periodic booster doses.

However, for the child with bleeding symptoms and not responding to steroid therapy as whose platelet count remain precariously low (less than 10,000/cumm) IVIgG now has become the initial therapy of choice prior to splenectomy in about 70 percent of patients with chronic ITP.

Anti-D in ITP[49-53]

Rh anti-D globulin has been recommended as an alternative to IVIgG in treatment of chronic ITP. Rh anti-D globulin have been tried in varying doses intravenously. Responses are usually slower in onset when compared to IVIgG and are transient. However, in some patients sustained responses have been seen, lasting for 6 months to 3 years.

- Splenectomized and Rh-ve patients respond less well
- Though occasionally a complete remission has been observed after a single course of anti-D globulin, repeated booster doses at intervals of more than 3 weeks may be required to maintain platelet count at a safe level.
- A number of children are able to discontinue the therapy during the first year of treatment.
- The drug is administered slowly in 20 to 50 cc of saline over 2 hours or can be administered fast over 3 minutes. Though the peak platelet count occur at a mean of 8 days following initial infusion, platelet counts increase significantly in 72 hours.
- Hypersensitive reactions like for any other plasma product are known and may cause shaking and chills. Transmission of HIV and hepatitis after infusion of anti-D is uncommon.
- Hemolysis has been observed and patient may need blood transfusions due to anemia caused by IV anti-D globulin.

- IV anti-D appears to be useful in treatment of ITP as it is cheaper and effective in steroid-refractory patients prior to splenectomy.

Splenectomy in ITP[54-65]

Spleen is the most important site for the destruction of antibody coated platelets (Graveyard of platelets.). It is one of the major sites of antiplatelet antibody production. Reported efficacy rate in regard to achieving a stable increased platelet count have varied for 40 to 86 percent. With most reporting approximately 60 percent platelet count increased to normal range of 150,000 to 400,000 in 5 to 60 days. No response was observed in 21 percent (6–40%) of patients, morbidity of splenectomy 10 percent and mortality less than 2 percent.

Indication of Splenectomy ITP

- As an emergency measure for life-threatening ICH, and in adolescent girls with chronic disabling menorrhagia. If patient do not afford or failure to IVIg therapy
- Some clinicians prefer to perform an emergency splenectomy during life-threatening hemorrhages because they believe surgical procedure produces more rapid rise in platelet count than IVIgG and occasionally patient may fail to respond to IVIgG within first 24 hours
- Less convincingly perhaps, in those with persistent thrombocytopenia to avoid prolonged disruption of lifestyle caused by limitation of activity and avoidance of contact sports
- Non responding chronic ITP.

For children with bleeding symptoms whose count remains precariously low (less than 10,000/cumm) or with recurrent mucosal bleeds and who do not respond to steroids and IVIgG (medical line of treatment), splenectomy is the alternative. Response rate to splenectomy in chronic ITP is about 65 to 88 percent.[2,5,6,9,10] There is no definite test by which one can predict response to splenectomy. But some authors have found that the initial response to steroids and thrombokinetic studies could demonstrate predictive relationship.[2,26] At the time of splenectomy the surgeon should look for accessory spleens which if missed may result in relapse of ITP. Administration of platelet transfusions prior to surgery usually is not required as platelet count starts rising immediately following surgery (Clamping splenic radicles) reaching as high as 3,00,000/cumm in the immediate postoperative period and reaching a peak within 1 to 2 weeks and then gradually dropping to normal values by 4 to 8 months. Though platelet count can rise as high as 1 to 2 millions, there are no reported cases of thrombosis and hence routine administration of antiplatelet drugs like aspirin or dipyridamole is not recommended.

Problems after Splenectomy

Risk of postsplenectomy infection is related to age; it is very high in early infancy and in those with underlying disorders. As compared to ITP, the risk of infection is more when splenectomy is done for diseases like thalassemia major, sickle cell disease and Hodgkin's disease. A detailed review in 1973 estimated the risk of fatal sepsis following splenectomy for ITP to be 1 to 4 percent. *Pneumococcus* was the most common infecting organism with adrenal hemorrhages occurring in over 25 percent of fatal cases. Most deaths occur within first 2 to 3 years following splenectomy, though deaths are reported as late as 30 years after splenectomy. Children rapidly develop progressive, overwhelming sepsis presenting initially with acute onset of fever, nausea, vomiting and then rapidly progressing with altered sensorium, confusion and leading to coma and death within few hours. It may be associated with DIC, electrolyte imbalance, shock, etc.

Parents of splenectomized children should be educated and instructed to seek immediate medical attention whenever child develops febrile illness. Broad spectrum antibiotics by intravenous route are recommended after collecting blood for cultures. Combination of ampicillin/amoxycillin or 3rd generation cephalosporins should be administered. Prophylactic antibiotics, particularly during infancy, are recommended for several years after splenectomy. Pneumococcal vaccine should be administered at least 2 to 8 weeks prior to the surgery. Revaccination after 2 to 5 years is recommended. Other vaccinations such as *H. influenzae* and meningococcal vaccine also may be given.

Thus, splenectomy should not be first treatment initiated in the management of patients with ITP particularly in children. It should be performed only after all other therapeutic modalities have been exhausted and patients have platelet count less than 10 to 25,000/cumm and has mucosal bleed. It has fairly favorable response rate of 60 to 80 percent. Acute and late morbidity of splenectomy, low rate of mortality of ITP and the chance of late spontaneous remission of thrombocytopenia have lead to differ splenectomy indefinitely particularly in childhood ITP. Splenectomy is thus differed for as long as possible in pediatric population.

Role of Nonsteroidal Immunosuppressant Drugs in Chronic ITP

A small percentage of children (less than 2%) with severe chronic ITP do not respond either to steroids, IVIgG, anti-D

globulin or splenectomy and continue to have bleeding tendencies with a platelet count of less than 10 to 30,000/cumm. In these patients immunosuppressive therapy is the alternative. Nonsteroidal immunosuppressive agents used are vincristine, vinblastine, azathioprine, 6-mercaptopurine, cyclophosphamide[67,68,68a] and cyclosporine,[69] etc.

Vinca alkaloids act more quickly but cyclophosphamides have a more lasting effect. Vincristine (0.025 mg/kg not over 2 mg) or vinblastine (0.125 mg/kg) IV is given at weekly intervals for 3 to 4 doses. Recently a constant infusion of vincristine has been tried, however, overall advantage of this method has not been established.

Target delivery of the drug by infusing platelets incubated with vinblastine has not been found to be effective as binding of vinblastine is easily reversible. Our observations are discouraging with none out of 5 patients in the age group of 3 to 8 years showing any significant response to Vincristine therapy. Only one child showed marginal rise in platelet count and did not show a lasting improvement in the course of the disease.

Studies using azathioprine in chronic ITP refractory to splenectomy show marked variation in the rate of remission. The dose used ranged from 1 to 4 mg/kg/day which may cause mild neutropenia. Three to six months of treatment may be necessary before maximum response is observed. The major side effects which are encountered relate to granulocytopenia and other features of bone marrow suppression.

Cyclophosphamide[67,68] has been used in ITP with variable success rates. It may be given either orally 1 to 2 mg/kg/day or intermittently intravenously in a dose of 750 to 1000 mg/m^2 every three weeks. Response occurs 2 to 10 weeks after the initiation of therapy.

Cyclosporin[69] has been recently tried in refractory ITP with transient increase in platelet counts after the treatment. It is known to modulate cell-mediated immunity or alter T-helper cell aspect of humoral immunity. It is given in the dose of 8 to 10 mg/kg/day in 2 divided doses continued over 2 to 3 months. The drug is usually tolerated well without major side effects but a significant rise in glutamine transpeptidase may be present. Nonsteroidal immunosuppressive drugs should be used with greater caution in children as alkylating agents are known to be mutagenic and increase the risk of subsequent malignancy. Secondary lymphomas have been reported. Close monitoring of these patients, including WBC count is necessary.

Alpha Interferon[70,71]

Alpha interferon has recently been used to treat refractory ITP. Interferon alpha-2b therapy is indicated to treat patient with life-threatening hemorrhage and those patient not responding to steroids, IVIgG or splenectomy. Alpha-2b interferon is a nontoxic alternative that may modify B-cell activity involved in antibody production. Responses were similar in splenectomized and non-splenectomized patients.

Dose used is 2.5 mu/m^2 3 times a week given subcutaneously for 12 weeks. The course may have to be repeated in some cases. The response is usually transient with a mean duration of fewer than 14 days. Alpha interferon therapy is well-tolerated and side-effects are mild. It may cause increase in tendency of clinical bleeding. There may be a significant fall in granulocyte count. Fortunately this has not been associated with an increase in the incidence of bacterial infections. Flu-like symptoms may develop particularly in older patients for which acetaminophen may be used.

Monoclonal anti-FcR III antibody has been found to be effective in 6 to 10 patients with long-term response.[54]

Rituximab[72-76]

Rituximab binds to the transmembrane antigen CD20 located on Pre B and mature B lymphocytes. CD20 antigen found on both normal and malignant cells but not on hematopoietic stem cells, Pro B Cells, normal plasma cells or any other normal tissue.

Rituximab in ITP available as Mabthera (Roche) 1 vial contain 10 mg in 10 mL or 500 mg in 50 mL.

Rituximab dose 375 mg per meter square was administered as IV infusion at weekly interval for 4 doses.

Methods of administration: IV infusion should be administered through a dedicated line. Administration IV push or bolus should not be done.Infusion should be administered where full resuscitation facilitates are immediately available.

Premedication consisting of an analgesic/antipyretic (paracetamol) and an antihistaminic drug (e.g. diphenhydramine) should always be administered before giving each infusion of Rituximab.

Initially start in micro drip 10 drops/min for 15 minutes then 15 drops/min for 15 minutes then 20 drops/min be continued.

Adverse events: Common symptoms are fever, chills rigor, flushing, nausea, vomiting, urticaria, rash, pruritus, angioedema headache, throat pain, abdominal pain, myalgia, rhinitis, hypotension, bronchospasm, arthalgia.

Prepared solution is stable for 24 hours at 2 to 8° C and subsequently 12 hours at room temperature. Store the vials in refrigerator in the carton do not freeze.

Brox et al.[75] in 1988 reported beneficial response to ascorbic acid in 3 out of 11 patients with chronic ITP.[56] But, this has not been confirmed by others.

Since children with chronic ITP can have a spontaneous remission even many years after their initial diagnosis, it is important to reduce or withdraw the drugs periodically. It has been reported that approximately 0.5 to 3 percent of children with chronic ITP will eventually develop autoimmune diseases and hence it is necessary to evaluate children with chronic ITP particularly adolescent females, for evidence of concomitant autoimmune disease.

Thrombopoietin in ITP

AMG 531 (Romiplostim, Nplate) and **Eltrombopag** (Promacta).[77,78]

Stimulating the thrombopoietin (TPO) receptor increases the platelet production has been successful by drugs or various agents.[79]

First-generation agents—recombinant human thrombopoietin (rHuTPO) and pegylated recombinant human megakaryocyte growth and development factor (PEG rHuMGDF).

- **First-generation agents**
  Recombinant human thrombopoietin (rHuTPO) and pegylated recombinant human megakaryocyte growth and development factor (PEG rHuMGDF)--showed promise, however antibody formation to PEG rHuMGDF led to the discontinuation of both agents.
- **Second-generation agents**
  TPO agonist antibodies--have been developed to reduce or eliminate the problem of antigenicity.
  - TPO peptide mimetics
  - TPO non-peptide mimetics.
  - AMG 531 (romiplostim, Nplate) and
  - Eltrombopag (Promacta).

AMG 531 and eltrombopag are able to stimulate platelet production in patients with ITP.

Clinical studies for some of these agents, such as AMG 531 (romiplostim, Nplate) and eltrombopag (Promacta), are demonstrating their relative safety and efficacy in increasing platelet counts in patients with ITP. There are currently seven second-generation TPO receptor agonists that have been reported in the literature, representing the potential advantages.

Dose

AMG 531 (romiplostim, Nplate) and eltrombopag (Promacta) (0.2 to 10 microg - 1 or 3 ug per kilogram of body weight six weekly subcutaneous injections).
No major adverse reactions.

CONCLUSION

'ITP in children' constitutes 90 percent cases which are acute and most of them go into remission in 1 to 6 months period. Since practically none of them in one's experience go into ICH (less than 1% cases) and morbidity is minimum, the dictum of the treatment of ITP should be the too frequently forgotten maxim 'first do not harm'.

In mild ITP with cutaneous bleeding and a platelet count of more than 50,000/cumm—no drug therapy—defensive management—nonfrantic watchful waiting is the rule.

In severe ITP with mucosal bleeding and platelet count of less than 20,000/cumm, prompt vigorous treatment with steroids should be started.

In nonresponders, and during emergency, IVIgG should be given. If IVIgG is not available or if the patient cannot afford, in life-threatening conditions, splenectomy may be done, particularly in children above 6 to 7 years. Intravenous anti-D globulin and intravenous methylprednisolone bolus dose may be tried before splenectomy.

In Chronic ITP

"Treat the child and not the platelet count." No treatment is required for cutaneous purpura and ecchymosis with a platelet count above 20 to 30,000/cumm. For platelet counts less than 20,000/cumm and mucosal bleeding, 1 to 2 courses of steroids may be given for 3 to 4 weeks each time. Low minimum required maintenance doses may be continued in partial responders. Avoid long-term high dose steroids. Nonresponders may be treated with IVIgG and booster dose if required or anti-D globulin may be given intravenously along with maintenance booster dose if required. Pulse methylprednisolone therapy is other alternative for nonaffording patients. Avoid splenectomy in children below 5 to 6 years and before 1 year of onset of the disease. In 2 percent of cases who do not respond to above therapy, immunosuppressive drugs may be used with caution.

ITP in children are generally benign conditions leading to only in few patients, serious complications and long term squeal. 10 to 20 percent of children ultimately develop chronic ITP whose platelet count majority of times remains in relatively safe range more than 30,000/cumm and whose bleeding time is fairly normal hence needs only defensive management and nonfrantic watchful waiting. IVIg or high dose steroid may benefit some patients who have evidence of clinical bleeding and severe thrombocytopenia and splenectomy may be of value in patients with chronic ITP with recurrent manifestations of bleeding or very low count. In acute ITP with severe bleeding like intracranial hemorrhage or severe menorrhagia, splenectomy may be indicated above the age of 5 years amongst those children who cannot afford IVIg or are nonresponsive to steroids. In some children with recurrent thrombocytopenia periodic booster dose of IVIgG or methylprednisolone may be

required so as to keep the platelet count in the safe range. However, there is no way to predict which patients are at high-risk for the development of ICH/severe hemorrhage and which patient will respond to which type of therapy and hence the decision to treat the child with ITP is more often based on entire clinical picture and severity of the bleeding and experience of the clinician rather than only the platelet count.

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CHAPTER 32

Platelet Function Disorders

Shanaz Khodaiji

Platelet function disorders are difficult to diagnose because the laboratory assays are time-consuming, and require skilled technical staff to standardize and perform. Mild platelet function disorders such as primary secretory defects are rare and are a diagnostic challenge. They may be missed due to their heterogeneity and failure to perform the appropriate tests for their diagnosis.

The latest guidelines on platelet function testing have been published in 2011, a long time after the previous guidelines, which were published by the British Committee for Standards in Hematology (BCSH) in 1988.[1] Lack of standardization of platelet function tests necessitated the publication of the new guidelines. The "gold standard" for platelet function testing is platelet aggregometry, which was first described in the 1960s.[2]

In Germany, Austria, and Switzerland, it is estimated that 2 children per million are affected by this disorder. Ethnicity and consanguinity have a role to play in this condition. A study carried out by Israels et al. has shown that abnormalities of platelet function are as common as von Willebrand disease (vWD) in patients with muco-cutaneous bleeding.[3] Platelet function disorders are more common than previously diagnosed as has been reported by a Canadian registry having 577 cases since 2004 (http://www.fhs.mcmaster.ca/chr/data.html). Many of these patients have incompletely characterized platelet defects, thus suggesting that these disorders are not as rare as previously believed.

PLATELET STRUCTURE

Platelets are discoid smooth surfaced cells, 3–4 μm in diameter that are present in whole blood in a concentration of 150,000–400,000/μL.

The major structural features of platelets include:

- *Cell membrane* which is a bilipid membrane and is the site of some complex coagulation activities (Fig. 1). It contains several glycoproteins that function as surface receptors. The two membrane systems are the endoplasmic reticulum or dense tubular system and the plasma membrane-derived, surface connected canalicular system (Fig. 1).
  The glycoprotein Gp Ib is a binding site for von Willebrand (vWF). It is an intrinsic transmembrane protein with a molecular weight of 140 kilodaltons. The vWF is necessary for platelet adhesion, an important first step in platelet function.
  The other glycoprotein GpIIb/IIIa is a prominent calcium dependent membrane protein complex that functions as a fibrinogen receptor. Fibrinogen binding is necessary for platelet aggregation to occur.
- *Circumferential microtubular system and microfilaments:* Microtubules lying just beneath the platelet membrane form a circumferential band round the platelet (Fig. 1). The microtubules are composed of tubulin which plays a role in cytoskeletal support and in contraction of the stimulated platelet. Closely associated microfilaments contain actin and participate in platelet pseudopod formation
- *Dense tubular system* is so named because of the presence of an amorphous electron-dense material. This system selectively binds divalent cations and serves as the platelet calcium reservoir (calcium flux is critical to platelet function). The dense tubular system is also the site of platelet cyclo-oxygenase and prostaglandin synthesis (Fig. 1).
- *Platelet granules:* Granules store various substances that are secreted during platelet aggregation or are

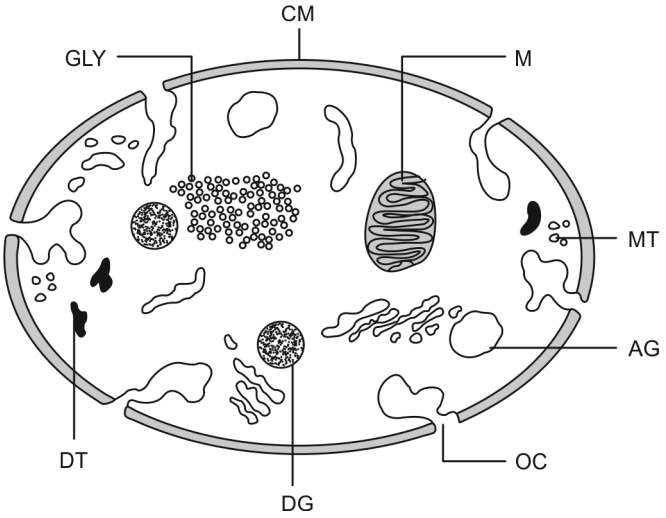


Fig. 1 Schematic diagram of morphology of a platelet. *Abbreviations:* AG: α granules; CM: Cell membrane; DG: Dense granule; DT: Dense tubule; GLY: Glycogen; M: Mitochondria; MT: Microtubule; OC: Open canaliculus.

instrumental to aggregation. The four types of granules are:

1. α *granules* store a variety of proteins that are secreted by stimulated platelets. These include platelet factor IV, factor V, vWF, fibrinogen, β-thromboglobulin, and platelet derived growth factor (PDGF). Various glycoproteins important to adhesion are also contained in these granules e.g. osteonectin, fibronectin and thrombospondin (Figs 1 and 2).
2. *Dense granules* are electron dense particles that contain high concentration of ADP and Ca^{2+} as well as serotonin and other nucleotides. It also contains ATP. These substances are released upon platelet stimulation and enhance platelet aggregation (Figs 1 and 3).
3. *Lysosomes* contain hydrolytic enzymes.
4. *Peroxisomes* contain catalase.

- *Externally communicating open canalicular system* (Fig. 1).

Platelets perform many functions and are therefore associated with many pathological processes.

Platelet function in bleeding and clotting: When platelets come in contact with a damaged vessel wall, they undergo shape change, adhesion, release of secretory granules and aggregation leading to exposure of the procoagulant surface. As a result, a hemostatic plug is formed that prevents further blood loss by occluding blood vessels at the site of injury. If this sequence of events is disturbed, clot formation is impaired and patient is at increased risk of bleeding.

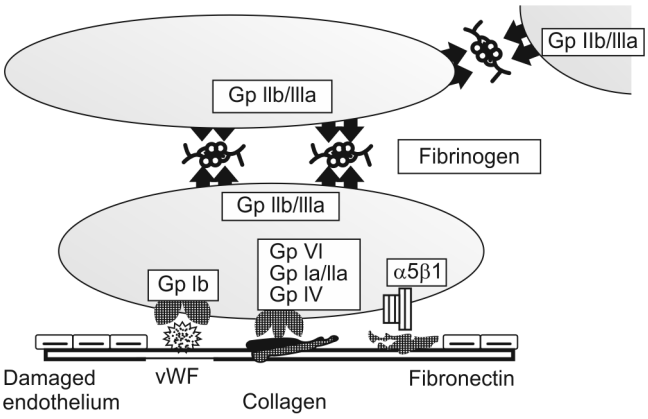


Fig. 2 Interaction between vessel wall and platelets following injury (adhesion and aggregation)[4]

Sequence of events occurring when platelets come in contact with injured vessel wall

1. Collagen and endothelial proteins are exposed on the internal surface of the affected vessel (Fig. 2).
2. Platelet adhesion is initiated when vWF binds to collagen (from exposed endothelium) and Gp Ib/V/IX complex on the platelet surface (Fig. 2). Simultaneously platelets bind directly to collagen via Gp VI membrane receptor complex and the Gp Ia/IIa integrin which is a collagen receptor (Fig. 2). This in turn binds to the G chain of fibrinogen and links the platelets to one another (Fig. 2). The platelets form a layer on the breached surface of the vessel wall causing platelet activation, which leads to the next step of primary hemostasis (Fig. 2).

   Since Bernard–Soulier syndrome (BSS) is characterized by the absence of the platelet membrane GP Ib complex, normal adhesion to the vWF-A1 domain cannot take place.
3. The platelets change shape rapidly from discoid to round and spread with filopodia and lamellipodia (stellate form). Platelet adhesion initiates intracellular signaling and platelet activation, by which substances such as ADP are released from platelet granules and thromboxane, (TXA_2) are generated (Fig. 3).
4. Platelets stagnate and transiently stick to (adhere) or roll along the vessel wall.
5. Platelet aggregation occurs when vWF and fibrinogen bind to activated platelets through the Gp IIb/IIIa complex (Fig. 2). Calcium ions are necessary for this process. This is the final step in the formation of the platelet plug. Absent or severely reduced platelet aggregation is seen in glanzmann thrombasthenia (GT), which is due to decreased levels/function of the Gp IIb/IIIa complex.

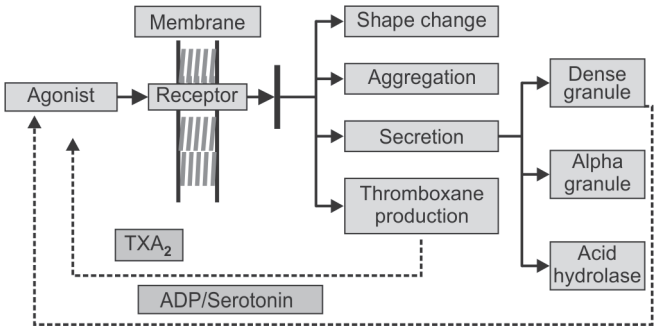


Fig. 3 Platelet response to activation[4]

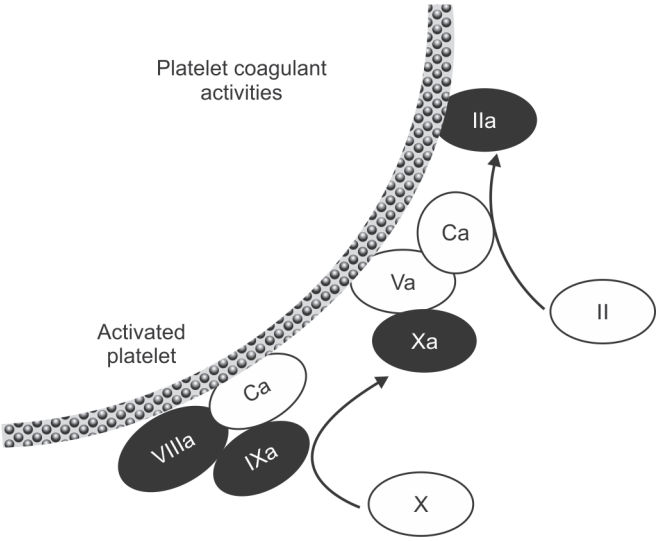


Fig. 4 Formation of a procoagulant surface[4]

6. The hemostatic plug is stabilized (Fig. 4) by reactions such as activation of Gp llb/IIIa complex, exposure of anionic phospholipids on the platelet surface and production of procoagulant microvesicles.
7. As the activation process proceeds, the actin-myosin complexes within each platelet shorten and draw the platelet mass together, which results in clot retraction.
8. TXA_2 generation induces further platelet aggregation (Fig. 2) and vasoconstriction, slowing the flow of blood and increasing the shear forces.
9. Platelet function is regulated *in vivo* by the presence of nitric oxide.

While coagulation screening tests such as the PT and the APTT are standardized in all labs, and not costly, there are no easy diagnostic screening tests for platelet function defects. Owing to the heterogeneity of these disorders it is difficult to find a platelet function test which is 100% sensitive. Moreover, these tests are laborious, expensive and require trained and experienced persons to perform and interpret. Newer instruments are now available such as the lumiaggregometer, which can assess platelet function from whole blood and flow cytometry, which can detect the presence or absence of antigens on the surface of the platelet as well as some secretory pathway defects. These are also used for monitoring antiplatelet therapy in patients of coronary artery disease.

Initial Approach to Diagnosing Platelet Dysfunction

Though there are not many studies comparing platelet function in pediatric age group and adults, all studies conclude that with the exception of neonates, there is no difference in aggregation patterns between the two groups.[3]

The first step in investigating a suspected bleeding disorder is to take a detailed personal and family history followed by examination and ordering of appropriate lab tests.

Clinical Features of Platelet Defects

Primary hemostasis involves formation of the platelet plug and secondary hemostasis is represented by the initiation of the coagulation process up to formation of the fibrin clot. Defects of both these functions have characteristic features, which can distinguish these 2 conditions (Table 1).

In addition, mucosal membrane bleeding can occur from the gastrointestinal and genitourinary tracts and pulmonary sites in patients with platelet functional defects.

The petechiae and purpura are usually symmetrical and found on the extremities as well as the torso, unlike the vascular disorders where petechiae and purpura are seen in dependent areas.

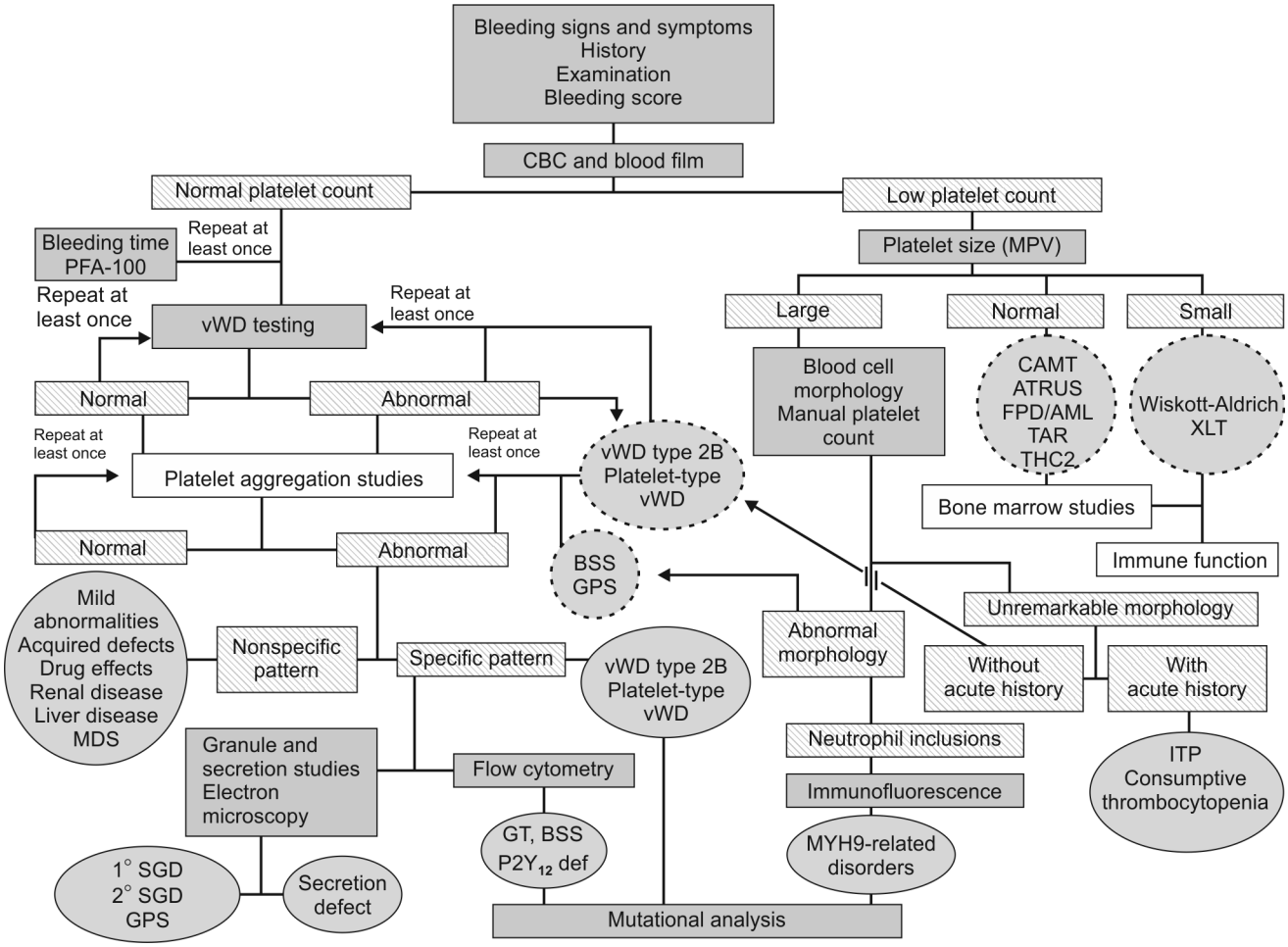
Platelet Function Defects may be Hereditary, Acquired or Drug-Induced

Acquired platelet function defects are associated with many diseases and are more commonly seen than the hereditary disorders. They often cause clinically significant bleeding.

Table 1 Characteristic bleeding patterns seen in primary and secondary hemostatic defects

Primary/platelet defect	*Secondary/coagulation defect*
Immediate excessive bleeding after trauma	Delayed bleeding
Petechiae, epistaxis, menorrhagia, gingival bleeding (mucocutaneous bleeds) accompanied by a history of easy, spontaneous bleeding	Bleeding in the deeper tissues, joints (hemarthrosis), and intramuscular hematomas are seen commonly

Flow chart 1 Approach to diagnosis of platelet function disorders in children[3]



Key: Gray boxes – Investigations; hatched boxes – results; circles – diagnosis; dotted circles - suspected diagnoses.
Abbreviations: BSS: Bernard–Soulier syndrome; CAMT: Congenital amegakaryocytic thrombocytopenia; ATRUS: Amegakaryocytic thrombocytopenia with radio-ulnar synostosis; FPD/AML: Familial platelet disorder and predisposition to acute myelogenous leukemia; GT: Glanzmann thrombasthenia; GPS: Gray platelet syndrome; SGD: Storage granule disorder; TAR: Thrombocytopenia with absent radii; THC2: Autosomal dominant thrombocytopenia; XLT: X-linked thrombocytopenia.

An algorithm for evaluation of children with suspected platelet disorders is described in flow chart 1.

CLASSIFICATION OF HEREDITARY PLATELET FUNCTION DEFECTS[3]

- Receptor abnormalities affecting platelet adhesion
  - Gp Ib–IX–V abnormality causing Bernard–Soulier syndrome or platelet-type vWD.
  - Gp IIb-IIIa (αIIbβ3) defect causing Glanzmann thrombasthenia
  - Gp Ia-IIa (α2β1) defect
  - Gp VI defect
  - Gp IV defect
- Abnormalities of receptors for
  - Thromboxane A_2
  - P2Y12
  - α_2-adrenergic receptor
- Platelet granule defects
  - Dense granule defects leading to δ-storage pool deficiency, Hermansky–Pudlak syndrome, Chediak–Higashi syndrome, thrombocytopenia with absent radii (TAR) syndrome
  - Alpha granules defects such as Gray platelet syndrome ARC syndrome, Quebec platelet disorder, Paris–Trousseau–Jacobsen syndrome
  - α and δ granules storage pool deficiency

- Abnormalities of signal-transduction
  - Primary secretion defects
  - Abnormalities of the AA or TXA_2 pathway
  - Gαq deficiency
  - Partial selective PLC-β2 deficiency
  - Defects in pleckstrin phosphorylation
  - Defects in Ca^{2+} mobilization
- Cytoskeleton related abnormalities
  - Disorders of MYH9 e.g. May–Hegglin anomaly, Fechtner syndrome, Epstein syndrome, Sebastian syndrome
  - Wiskott–Aldrich syndrome
  - X-linked thrombocytopenia
- Membrane phospholipid abnormalities
  Scott syndrome

These disorders also have thrombocytopenia in addition to functional defects.[3]

Secondary aggregation defects occur more frequently than primary or hereditary platelet disorders. Storage pool defects are the most common in the hereditary as well as acquired groups.

Other rare disorders are:

- Quebec platelet syndrome characterized by delayed-onset bleeding, impaired epinephrine induced platelet aggregation. The diagnosis is confirmed by presence of platelet urokinase by immunoblotting or ELISA
- Scott syndrome presenting with mucocutaneous bleeding but normal aggregation with all agonists. The confirmatory test is by demonstrating absence of annexin A5 binding to activated platelets by flow cytometry
- Thromboxane A2 receptor defect presents with mucocutaneous bleeding, absent or decreased aggregation with AA and U46619. The molecular assay used for confirmation of this disorder is mutational analysis of TBXA2R gene.

Secondary or acquired defects of platelet function are encountered more frequently than hereditary disorders, the most common being storage pool defects (SPD).

These divisions are somewhat arbitrary but are convenient for categorizing the defects and interpreting laboratory tests.

The hereditary disorders are discussed below:

- *von Willebrand disease (vWD):* von Willebrand disease can present in children with mucocutaneous bleeding in the absence of a family history, in which case it is difficult to differentiate it from platelet function disorders. Testing for both vWD, and platelet function defects is helpful. Both conditions are common specially in children, and combined disorders may be present. Quiroga et al. demonstrated that in 11.5% of 113 individuals (ages 4–50 years) with mucocutaneous bleeding and laboratory evidence of primary hemostatic defects both vWD and platelet dysfunction are found. VWD can also present with thrombocytopenia. Macrothrombocytopenia can be seen in patients of type 2B vWD.[3]
- *Bernard Soulier syndrome (BSS):* BSS is a manifestation of a defect in platelet adhesion and is inherited as an autosomal recessive trait. Heterozygotes are often asymptomatic. The platelets in this syndrome lack membrane glycoproteins Ib, V and IX. It mimics vWD in that the clinical manifestations are similar.
  - ***Clinical manifestations:*** Patient can present with, epistaxis, menorrhagia, petechiae and purpura. Easy bruising, which is sometimes spontaneous can be the presenting feature.
  - ***Laboratory manifestations:***
    - Thrombocytopenia (mild/moderate) with macrothrombocytes
    - Abnormal template BT but clot retraction is normal
    - Abnormal petechiometer test (in children)
    - Reduced aggregation response to ristocetin. The response may be abnormally increased in type 2B vWD.

The template BT is not standardized in children; hence a petechiometer is more useful in children for assessing platelet function defects or vascular type bleeding.

Peripheral blood smear examination shows macroplatelets along with mild thrombocytopenia in most patients of BSS but not in vWD. Tests for diagnosing vWD are the factor VIII coagulant activity (factor VIII:C), factor VIII related antigen (factor VIII:RAg) and ristocetin cofactor activity.

Treatment: Platelet concentrates are given only if bleeding is life threatening.

GLANZMANN'S THROMBASTHENIA

Glanzmann's thrombasthenia (GT) and essential athrombia are very rare primary aggregation disorders. Patients of GT lack the membrane glycoprotein Gp IIb/IIIa complex.

Clinical Findings

Patients present with history of easy and sometimes spontaneous bruising and petechiae, menorrhagia and very occasionally hemarthroses. The symptoms of bleeding usually disappear as the patient grows older. This feature is common to most of the hereditary hemostasis defects.

Laboratory Findings

The BT is prolonged. Platelet function defects such as reduced or absent primary aggregation with ADP,

epinephrine, thrombin and collagen and reduced platelet factor 3 availability are seen.

Clot retraction is impaired in GT but is normal in essential athrombia.

Therapy

Treatment for both the disorders is platelet transfusion only if the bleeding is severe and life threatening. Some workers prefer to infuse platelets till the bleeding stops rather than relying on empirical monitoring of aggregation patterns or the template bleeding time. The vast majority of patients with hereditary platelet function defect of any type will stop bleeding immediately with the appropriate use of platelets.

HEREDITARY STORAGE POOL DEFECT (SPD)

Clinical Features

These patients have a variable pattern of inheritance and commonly present with mucocutaneous hemorrhage, hematuria and epistaxis. Petechiae are not so common in these disorders as in other platelet function disorders. These patients usually present with spontaneous bleeds.

Laboratory Findings

Prolonged BT and abnormal collagen-induced aggregation is noted (absent or markedly reduced). Absent secondary aggregation wave to ADP and epinephrine is seen although the primary waves are present. Normal ristocetin aggregation and usually normal response to AA is observed.

Therapy

In case of heavy bleeding, patients can be given platelet transfusion.

ASPIRIN-LIKE DEFECTS

This is an inherited condition which mimics the aspirin-induced acquired platelet function defect. It is very rare and is inherited as an autosomal dominant trait.

Clinical Features

Like other platelet function defects these patients present with easy, spontaneous bruising and bleeding from mucocutaneous areas, epistaxis, menorrhagia, petechiae and purpura. This defect may be due to a hereditary deficiency of the enzyme cydo-oxygenase or the enzyme thromboxane synthetase.

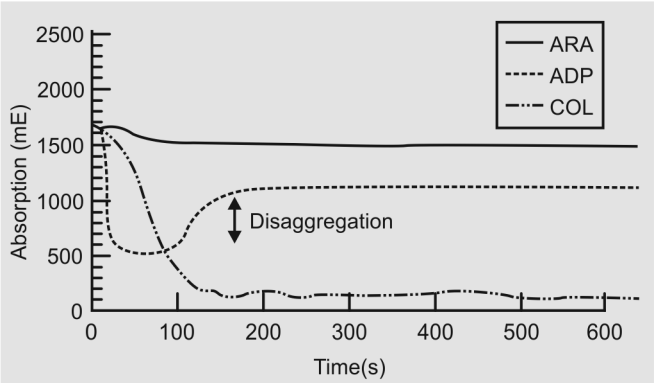


Fig. 5 Aggregation pattern in patients on acetylsalicylic acid (ASA)[5]

Abbreviations: ADP: shows a disaggregation in the curve; Collagen (COL) normal aggregation; Arachidonic acid (ARA), aggregation shows reduced response.

Laboratory Findings

These patients have a prolonged BT and show abnormal collagen adhesion. Secondary aggregation response to ADP (Fig. 5) and epinephrine is absent. Aggregation to collagen is normal or absent. Aggregation response to AA is absent (Fig. 5) along with absence of cyclo-oxygenase and/or thromboxane synthetase enzymes. The same findings are seen in patients taking aspirin or COX-2 inhibitors.

Therapy

When clinically significant bleeding occurs, treatment consists of infusing platelet concentrates. Steroids have been used in some patients with beneficial effects.

ACQUIRED PLATELET FUNCTION DEFECTS

Patients with acquired platelet function defects present with profuse bleeding during surgery or trauma. This is a common cause of bleeding in adults. Acquired platelet function defects can be seen in a variety of conditions but the platelet aggregation abnormalities are not specific to any condition, unlike the hereditary disorders.

Causes of acquired platelet function defects:

- Myeloproliferative syndromes[7]
  - Essential thrombocythemia (ET)
  - Agnogenic myeloid metaplasia
  - Paroxysmal nocturnal hemoglobinuria (PNH)
  - Polycythemia vera (PV)
  - Chronic myeloid leukemia (CML)
  - MDS, RAEB
  - Sideroblastic anemia
- Uremia

- Malignant paraproteinemias
- Waldenström's macroglobulinemia
- Multiple myeloma
- Leukemic reticuloendotheliosis
- Autoimmune disorders
  - Collagen vascular disease
  - Antiplatelet antibodies
- Presence of FDPs
  - Disseminated intravascular coagulation
  - Primary fibrinolysis
- Anemia
  - Severe iron deficiency
  - Severe folate or B12 deficiency
- Drug induced

MYELOPROLIFERATIVE SYNDROMES

Acquired platelet function defects are often seen in these disorders. However, the platelet aggregation pattern is not characteristic of any one disorder.

UREMIA[7]

Almost all patients of uremia have a platelet function defect. It is thought that the circulating guanidinosuccinic acid and/or hydroxyphenolic acid in these patients cause a platelet function defect by reducing platelet factor 3 activity. The aggregation pattern is not diagnostic of the condition.

Dialysis can reverse this effect and normalize platelet function.

Other mechanisms such as altered prostaglandin metabolism have also been proposed in uremic patients.

Treatment

Platelet concentrates are recommended for life threatening bleeding. Cryoprecipitate, desmopressin and estrogen compounds can also be used to correct bleeding time and control the hemorrhage.

PARAPROTEIN DISORDERS

A majority of patients with malignant paraproteinemias have platelet function defects. Paraproteins coat the platelet membrane in malignant and benign states, thereby impairing their function, which manifests as significant bleeding and abnormal platelet aggregation curves.

MISCELLANEOUS CAUSES

Autoimmune disorders: Systemic lupus erythematosus, rheumatoid arthritis and scleroderma.

Fibrinogen degradation products: Fragments D and E appear to bind to the platelet membrane causing a very severe bleeding and abnormalities of platelet aggregation.
Nutritional anemia: Iron deficiency or vitamin B12 and folic acid deficiency also demonstrate a platelet function defect, with or without clinical bleeding.

DRUG-INDUCED PLATELET FUNCTION DEFECTS[7]

Numerous drugs cause defective platelet aggregation; though not all of them cause significant clinical bleeding.

Drugs act through the following pathways:

- *Interaction with platelet membrane or receptors.* The drugs acting through this mechanism are propranolol, ampicillin, penicillin, diphenhydramine, phenylephrine plus promethazine and alcohol. Other drugs included in this class are reserpine, nitrofurantoin, dextran and hydroxyethyl starch
- *Inhibition of prostaglandin pathways.* The most common drugs in this category are aspirin, indomethacin, phenylbutazone, ibuprofen, sulfinpyrazone and furosemide. Other, not commonly used drugs in this category are quinacrine, mefenamic acid, tocopherol, hydrocortisone, methylprednisolone and cyclosporine
- *Inhibition of phosphodiesterase activity.* The most common drugs are caffeine, dipyridamole, aminophylline, theophylline and papaverine
- *Drugs with unknown mechanism.* Acetazolamine, chlortetracycline, hydroxychloroquine and nitroprusside.

COMMONLY USED DRUGS WHICH INHIBIT PLATELET FUNCTION[7]

- Anti-inflammatory drugs
- Psychiatric drugs
- Cardiovascular drugs
- Antibiotics
- General and local anesthetics
- Antihistamines

HYPERACTIVE PLATELETS[7]

Macrothrombocytes are commonly seen in hypercoagulable states and in patients with thromboses. Large platelets are immature platelets with more RNA content and this makes them prothrombotic.

Microvesicles and Platelet Procoagulant Activity

A major advance in the last several years has been the discovery of small, membrane-bound microparticles

which are shed from plasma membrane of activated platelets. These microparticles are rich in binding sites for factor Va and Xa, thus conferring on them a procoagulant property. This shedding is accompanied by local collapse of the normal asymmetrical distribution of plasma membrane phospholipids and the exposure of phosphatidylserine and phosphatidylethanolamine on the outer surface.

PLATELET FUNCTION DEFECTS IN INFANTS AND SMALL CHILDREN[1]

Hereditary platelet disorders such as GT and BSS are usually seen in infants and young children. They pose a diagnostic dilemma in very young children mainly due to preanalytical errors occurring at this age. Blood collection for platelet function tests should be done on a free-flowing venous sample and not form heel or finger prick. At least 20 mL of blood is required for these assays; this may be 8-10% of the total blood volume of a neonate and could result in hypovolemic shock. Generally, 19-21G needles are used in adults but these are too big for use in infants as they are likely to cause significant trauma to subcutaneous tissues if a severe platelet disorder is present. For blood collection in infants and smaller children, a 23G needle is used. The control sample should also be collected with a 23G needle.[1]

There is a paucity of data regarding aggregation patterns in normal neonates due to difficulty in collecting large volume of blood form healthy children. Few studies conducted in this group of patients suggest that the platelets of infants are hyporeactive to all agonists except ristocetin and sometimes collagen. Later, platelet aggregation patterns and nucleotide release reactions reach adult levels. Therefore, platelet function should be assessed in children above 1 year of age. Family studies are recommended to assess inheritance pattern. Severe platelet function defects such as in GT or BSS show characteristic patterns, which are easy to diagnose. Methods using less blood volume like PFA-100/200 and flow cytometry can be used in children prior to performing platelet aggregation assay for confirmation of the disorder. In BSS, PFA reveals a severe defect of primary hemostasis and this along with macrothrombocytopenia and demonstration of absence or very low levels of the defective receptor, is sufficient to start appropriate treatment for bleeding. Both GT and BSS show prolonged closure times on the PFA. In severe unexplained bleeding (intracranial) when an inflicted injury has to be differentiated from spontaneous bleeding in a patient with a severe bleeding diathesis, this approach is very helpful.[1]

LABORATORY TESTS FOR PLATELET DISORDERS

Diagnosing these disorders in the laboratory is a challenging task, more so in children because large volumes of blood are required for testing. Newer testing modalities are not universally available and need proper validation especially in the pediatric age group. Standardization of platelet function assays has recently been undertaken and this effort is bound to improve the quality of testing and diagnosis of these disorders. Specialized tests should be made available to those requiring a diagnosis of rare platelet function defects. [3]

- *Basic clotting tests* such as PT and APTT are mandatory to exclude coagulation defects.
- *Measuring platelet number and size:* A peripheral blood smear examination is necessary to assess platelet size, and granularity. Platelet morphology is helpful in diagnosis of gray platelet syndrome (GPS) in which platelets are large and appear gray in color due to lack of granules, or a borderline thrombocytopenia with large platelets suggests BSS. The diagnosis of platelet function defects can be excluded in acute leukemias which present with petechiae or purpura by examination of the peripheral smear. Accurate platelet counts are now possible with introduction of fluorescence flow cytometry in hematology analyzers, by improving the ability to distinguish large platelets from RBCs. The international reference method (IRM) for platelet counting is by flow cytometry. If the platelet count is normal, but signs of platelet dysfunction are present, the differential diagnosis lies between a platelet function defect and a vascular defect, both of which may cause a prolonged bleeding time by the standardized template method
- *Global screening tests of platelet function:* They are normally performed as first line tests for assessing platelet function. Screening tests should be done to exclude the diagnosis of platelet function disorder so that further specialized testing can be avoided. Thus, global platelet function tests can be performed along with routine coagulation assays such as PT, APTT, and some specialized coagulation tests such as vWF assay. The vWF panel of tests include vWF:Ag, vWF:RCo and F:VIII:C assays and an accurate platelet count. The template bleeding time is the simplest screening test for platelet function and is easy to perform in all laboratories. A prolonged BT can be followed up by testing on the PFA-100.[1]

  Apart from the PFA analyzers, other instruments are available to test platelet function. These include

those that can assess the effect of antiplatelet drugs. Thromboelastography (TEG) and Rotational Thromboelastometry (ROTEM) are tests of coagulation and platelet function which have found favor with surgeons. Though used extensively in a surgical setting, there is no proper validation of these systems, and their routine use for diagnosing platelet function defects is therefore not recommended currently.[1]

Template Bleeding Time

The oldest test of platelet function is the BT, which was described by Duke in 1910. It was previously recommended for diagnosis of platelet function by the BCSH hemostasis and thrombosis Task Force and is widely used in the UK. However, there is lack of standardization of this test between laboratories.[1] It is technologist dependent and highly subjective. It varies according to certain patient characteristics such as age, gender, hematocrit, vascular pattern, skin thickness and skin temperature. Therefore, it is not reproducible and has a low sensitivity and specificity. Also, it is invasive and for these reasons it is not routinely recommended and performed.

The template BT is standardized; therefore it possible to compare this parameter from different labs. It is performed by tying a sphygmomanometer cuff on the upper arm of the patient, which is inflated to 40 mm Hg. Using a spring-loaded template, an incision 5 mm long and 1 mm deep is made on the extensor surface of the forearm. Avoids going into scar tissue and blood vessels, and make the incision within 60 seconds of inflating the sphygmomanometer. The edges of the wound are blotted by filter paper at 30 second intervals until the bleeding stops. Normal template BT is 2–9 minutes.[1]

Platelet Function Analyzer PFA-100[1]

Assay Principle

The PFA-100 analyzer is made up of disposable cartridges containing apertures coated with either collagen-epinephrine (CEPI), or collagen-ADP (CADP). Blood collected in citrate (0.8 mL per cartridge) is aspirated at high shear rates (5000-6000s-1) into the PFA analyzer. These agonists on the aperture initiate platelet adhesion, activation and aggregation leading to rapid closure of the aperture. The end-point for each agonist is time taken to obstruct the blood flow. Nonclosure of the aperture is seen if the closure time (CT) exceeds 300 seconds. Since small quantities of blood are required, it is useful for pediatric patients too.

It has been observed that a low platelet count (<100 × 10^9/L) and anemia (<20% hematocrit) often cause a prolonged CT. The CT also correlates inversely with plasma vWF activity and may therefore be longer in patients with blood group O. The CEPI-CT is usually prolonged in patients taking COX-1 inhibitors such as aspirin. This is not the case with the CADP-CT,

PFA-100 in vWD [1]

Abnormal CT on both CEPI and CADP cartridges is seen in of vWD types 2A, 2B, 2M and 3 with a sensitivity of >98%. The overall sensitivity of CT to vWD is lower (85-90%) if type 1 vWD is also considered. There seems to be a significant correlation between vWF level and CT. Type 2N vWD shows normal results. The PFA-100 can also be used for monitoring desmopressin therapy in vWD.

PFA-100 in Diagnosis of Hereditary Platelet Function Defects

Nonclosure is typically seen in both cartridges in patients with severe platelet function defects such as GT, BSS and platelet type or pseudo-vWD. The PFA-100 CT is not very sensitive for mild platelet dysfunction. A recent study showed that the overall sensitivity was 83% and specificity 89% of the CEPI cartridge for primary hemostatic disorders. CADP sensitivity was lower. The PFA-100 has shown a sensitivity of >90% in screening patients with menorrhagia for vWD and platelet function disorders.

However, if the platelet defect is strongly suspected, a normal PFA result should be overlooked and specific platelet function assays should be performed.

PLATELET FUNCTION ASSAYS

Platelet Light Transmission Aggregometry (LTA)[1]

Platelet aggregometry is considered the **gold standard for platelet function testing**. It was invented in the early 1960s. However, this test varies widely in laboratory practice due to lack of standardization of the assays for many years. Recently, new guidelines for platelet aggregometry have been published.

Principle

Platelet aggregometry works on the principle of optical density in which upon addition of agonists the platelets undergo a change in shape from discs to round structures with extended filopodia. This results in a transient decrease in light transmission, followed by an increase as the platelets aggregate. The increase in light transmission (% aggregation) is measured at 37^0C by a photometer.

A secondary aggregation response curve is seen with a higher concentration of ADP and epinephrine. This is due

to TXA_2 formation and release of the contents of platelet granules. Platelet agglutination by ristocetin, which changes the conformation of plasma vWF thereby making it suitable to bind to the Gp Ib–IX–V complex, can also be assessed by platelet aggregometry.

Platelet aggregometry is carried out with a complete panel of agonist and require at least 15 mL of blood, which is a major disadvantage in children. A recently introduced method for testing platelet function is whole blood aggregometry, which requires a smaller blood volume and measures platelet aggregation as the change in electrical impedance between electrodes. It is not widely used because it is more costly than platelet aggregation with PRP. A more recently developed multiplate analyzer is also available that operates on the principle of electrical impedance. It requires only 175 mL of blood and is therefore very useful in pediatric practice.

Preparation of Patient[1]

- The patient and control subjects should be off drugs, beverages and foods which may affect aggregation for at least 7 days prior to performing the test
- Both the patient and the control subject should fast overnight as chylomicrons may interfere with the aggregation pattern
- 20 mL of venous blood is collected in citrate tubes keeping in mind the anticoagulant to blood ratio of 1:9
- The blood should not be chilled as cold activates platelets
- PRP is obtained by centrifugation at room temperature (18–22 °C) for 10–15 minutes at 1000 rpm
- The PRP is pipetted out slowly, avoiding mixing of cells form the buffy coat or RBCs. The PRP is taken in a stoppered plastic tube filled nearly to the top to avoid changes in pH, which affect platelet aggregation and tests of nucleotide release. It is kept at room temperature till tested. It is stable for about 3 hours
- After removing the PRP, platelet poor plasma (PPP) for test and control is obtained by centrifugation of the remaining blood sample at 2000 rpm for 20 minutes
- *Standardization of PRP:* The platelet count of the PRP is adjusted to 200 – 400 × 10^9/L. If it is high, the PRP is diluted with the patient's PPP. A platelet count lower than 200 × 10^9/L gives rise to a diminished aggregation response. In the case of a low platelet count, further centrifugation of PRP is not recommended because it induces platelet activation. The control PRP should be diluted to the same count and tested as a comparison.

Aggregating Agents[1]

Five useful aggregating agents (agonists), which are sufficient for the diagnosis of most functional platelet disorders are ADP, collagen, ristocetin, epinephrine/adrenaline and arachidonic acid (AA). An extended panel of agonists can be used to characterize other defects not defined by the primary panel. This includes gamma thrombin, thrombin receptor activating peptides (TRAP), collagen-related peptide, endoperoxide analog U46619 and calcium ionophore A23187.

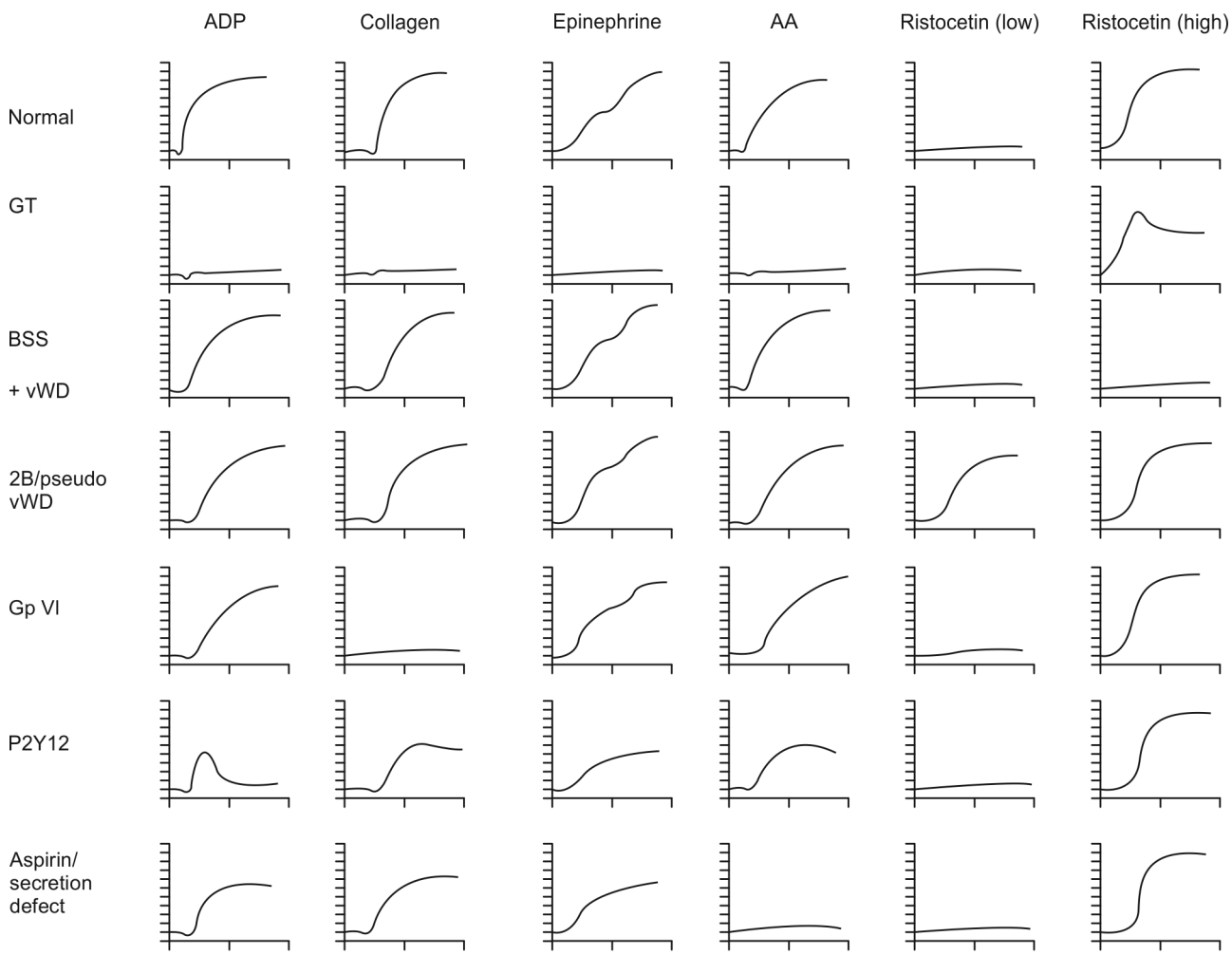
A number of pre-analytical factors can influence the results and interpretation of platelet aggregation (Table 2).

Interpretation (Fig. 6)

ADP: This is tested in two dilutions, 2.5 and 5.0 μmol/L (Figs 6 and 7). A primary or reversible aggregation curve is seen (Fig. 7) with ADP in low concentrations (<0.5–2.5 μmol/L). ADP acts by binding to a membrane

Table 2 Technical factors which may influence platelet aggregation tests[6]

Centrifugation: At room temperature, not at 4°C. Speed should be adjusted so that red cells and white cells are removed but not the larger platelets. Residual red cells in the PRP may hamper proper aggregation.
Time: Platelets are refractory to the effect of agonists for up to 30 minutes after centrifugation. Progressive increase in reactiveness occurs thereafter.
Platelet count: Slow and weak aggregation observed with platelet counts below 150 or over 400 x 10^9/L.
A pH of less than 7.7 slows down aggregation while more than 8.0 increases aggregation.
Mixing speed: <800 rpm or> 1200 rpm slows aggregation.
Hematocrit: >0.55 is associated with less aggregation, especially in the secondary phase owing to the increased concentration of citrate in PRP. It may also be difficult to obtain enough PPP. Centrifuging twice may help.
Temperature: <35°C causes decreased aggregation except to low dose ADP which may be enhanced.
Dirty cuvette may cause spontaneous platelet aggregation or interfere with the optics of the system.
Air bubbles in the cuvette cause large irregular oscillations even before the addition of agonists.
No stir bar: No response to any agonist obtained.



Note: These are illustrations and not actual tracings.

Fig. 6 Aggregometry patterns in rare platelet function disorders[1]

receptor resulting in release of Ca^{2+} ions. A complex with extracellular fibrinogen forms and the platelets change shape from discs to rounded structures. This causes a slight increase in light absorbance. After this, reversible aggregation occurs when bound fibrinogen participates in the cell-to-cell interaction. When very low concentrations of ADP are used, the platelets disaggregate after the first phase and do not show a secondary aggregation response. In the presence of higher concentrations of ADP, an irreversible secondary wave of aggregation occurs owing to activation of the AA pathway (Fig. 6) resulting in release of dense and α-granules. Therefore both concentrations of ADP should be used as If only the high dose is used primary wave defects (which measure the second pathway) will be missed.

Collagen: This is available as a 1 mg/mL stock solution. A short lag phase lasting between 10 and 60 seconds (Fig. 6) precedes aggregation with collagen. The duration of the lag phase depends on the concentration of collagen used and to the responsiveness of the platelets being tested. The lag phase is shorter with high concentrations of collagen and vice-versa. A single wave of aggregation follows the lag phase and is caused by activation of the AA pathway and release of the granules. A higher concentration of collagen (>2 μg/mL) causes a spurt in calcium concentration within the platelet and this results in a direct release reaction without causing activation of the prostaglandin pathway. Collagen responses should therefore always be measured using 1 and 4 μg/mL concentrations.

Ristocetin: It is used in a concentration of 1.4 mg/mL. If used in higher concentrations, protein precipitation might occur in plasma and give rise to false results. Ristocetin induces clumping of platelets (agglutination) by reacting with vWF and the membrane receptors. It does not act

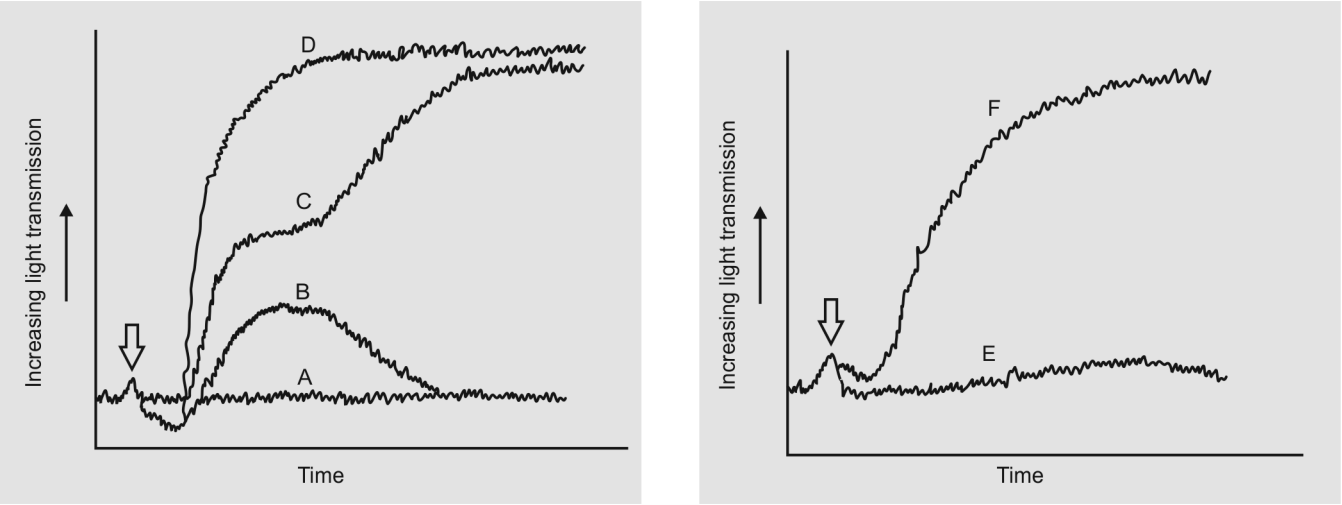


Fig. 7 Pattern of platelet aggregation with different doses of ADP[5]

A - no agonist added or thrombasthenia platelets; B – flrst wave reverse aggregation seen with low dose ADP or high dose ADP if platelets inhibited by aspirin; C - normal biphasic aggregation seen with moderate doses of ADP; D – prompt irreversible aggregation stimulated by high dose ADP and all potent agonists; E - platelets unresponsive to ristocetin as in vWD and BSS; F - normal response to ristocetin

through any of the usual aggregation pathways and does not initially cause granule release. The response is assessed on the basis of the angle of the initial slope (Fig. 6). Aggregation to ristocetin is tested in high and low doses. In Type 2B and platelet type vWD the aggregation response to high dose ristocetin is normal but response to low dose (0.5-0.7 mg/mL) ristocetin is hyper-reactive. If with high dose ristocetin the aggregation response is absent, then an external source of vWF, (e.g. cryoprecipitate, or a vWF concentrate) is added to the patient PRP and repeat testing is done to distinguish between a vWF or Gp Ib defect (BSS).

Arachidonic acid: TXA_2 generation and secretion of granule occur with AA even if there is a defect of receptors for binding AA on the membrane or of endogenous release of arachidonate induced by phospholipase (Fig. 6). In case of absence and/or inhibition of cyclo-oxygenase (e.g. aspirin effect), the AA induced aggregation is abnormal (Figs 5 and 6). Abnormal AA aggregation requires further testing with 1.0 μM U46619 to look for any thromboxane receptor abnormalities.

Epinephrine/Adrenaline: It is used in a concentration of 2.6 μmol/L. Unlike ADP there is no shape change before aggregation but the response later is the same as in ADP (Fig. 6). A severely reduced response to epinephrine is noticed in normal people due to natural variations of the adrenoreceptor numbers. These patients are clinically normal.

If abnormalities in the thrombin receptors, Gp VI, calcium mobilization and protein kinase C are suspected, an extended panel of agonists can be used for categorizing the abnormality; such as gamma thrombin (which does not cause clotting), PAR-1 (SFLLRN) and PAR-4 (AYPGKF) TRAP peptides (if gamma thrombin is abnormal), collagen-related peptide (CRP), calcium ionophore and PMA (Phorbol 12-myristate 13 acetate). These tests are available in highly specialized labs only.

Aggregation Patterns in Normal Subjects

Normal platelet aggregation response to ADP varies with the concentration used. It is usually a single reversible primary wave with 1 μmol/mL of ADP or less, a biphasic response with 2.5 μmol/mL and a single irreversible wave with 5 or 10 μmol/mL. With 1 and 4 μg/mL of collagen a single wave is seen with a lag phase of not more than 1 minute. Ristocetin shows a single phase or biphasic aggregation with 1.2 mg/mL. An abnormal response to low dose ristocetin (0.5 mg/mL) is characteristic of type 2 vWD (Figs 6 and 7). A single or biphasic response is seen with 50–100 μmol/L of AA. Both primary and secondary aggregation curves are seen with 2–10 μmol/L of epinephrine.

Every laboratory performing the test should establish its own reference intervals.

The platelet aggregation pattern for some of the most common inherited platelet function defects are shown in Table 3.

SUBSTANCES COMMONLY AFFECTING PLATELET FUNCTION[1]

- Cyclo-oxygenase inhibitors (irreversible)
  Aspirin and all drugs containing acetylsalicylic acid.

Table 3 Diagnostic criteria and LTA patterns in heritable platelet defects[1]

Plt function defect	*Plt number and morphology*	*PFA result*	*Aggregation pattern*	*Nucleotides assay*	*Flow cytometry results*	*Remarks*
vWD Type 1, 2A and 3	Within normal limits	Equally prolonged CADP/CEPi. Markedly prolonged in 2A and 3	Abnormal response to high dose ristocetin corrected by vWF addition	Within normal limits	Within normal limits	Confirm with vWF panel tests for this subtype
Type 2N VWD or platelet type	Within normal limits	Both closure times prolonged	Ristocetin aggregation normal by adding plasma or cryo. Increased response with low dose	Within normal limits	Increased vWF binding to platelets can be measured	Abnormal vWF panel in Type 2B vWD/Loss of high MW vWF in platelet type vWD
GT	Within normal limits	CADP/CEPI both very prolonged closure times	Markedly low response to all agonists except high dose ristocetin	Within normal limits	Reduced copy number of IIb/IIIa	
BSS	Mild to moderate thrombocytopenia with large platelets	CADPI/CEPI both very prolonged closure times	Low response to high dose ristocetin not corrected by adding vWF	Normal to high levels	Reduced copy number of Gp Ib (heterozygotes can also be measured)	
Defect of dense granule	Reduced or normal count Electron dense granules reduced as measured by whole mount EM	CADP normal CT CEPI sometimes prolonged	Secondary aggregation response to ADP and epinephrine is decreased	Reduced ADP Increased ATP:ADP ratio reduced ATP release	Reduced mepacrine uptake and release	Reduced serotonin release seen in Hermansky Pudlak and Chediak-Higashi syndromes which are autosomal recessive
Defect of secretion	Within normal limit	Normal CADP CT CEPI sometimes prolonged	Reduced secondary aggregation response to ADP and epinephrine	Normal but with defective release. Reduced ATP release	Normal mepacrine uptake but defective release	
Aspirin-like defect	Within normal limits	CADP normal CT CEPI normally prolonged (NB can be normal with high vWF levels)	Absent AA response but normal to U46619. Decreased secondary aggregation to ADP and epinephrine	Within normal limits		Retest or defer for 10 days if patient is on aspirin or NSAIDs
Thromboxane receptor defect	Within normal limits	CADP normal CT CEPI sometimes prolonged	Absent AA and U46619 response	Within normal limits		
Giant platelet syndrome	Macrothrombocytopenia	Sometimes normal	Normal response to ristocetin	Normal/high	Normal/ High receptor numbers per platelet	

Contd...

Contd...

Plt function defect	*Plt number and morphology*	*PFA result*	*Aggregation pattern*	*Nucleotides assay*	*Flow cytometry results*	*Remarks*
Collagen receptor defects	Within normal limits	Both prolonged	Decreased collagen aggregation	Within normal limits	Reduced Gp Ia/ IIa or Gp VI levels	
P2Y12 defect	Within normal limits	Both normal	ADP-decreased aggregation. Reversible response at high doses Reduced secondary wave	Within normal limits	Low P2Y12 number using	Retest or defer if patient taking clopidogrel or other anti-P2Y12 agents
P2Y1 defect	?	?	Decreased response to ADP-curves not reversible	Within normal limits		
Scott syndrome	Within normal limits	Within normal limits	Within normal limits	Within normal limits	Reduced expression of phosph-atidyl serine on activated platelets using Annexin-V	Reduced PCI and ETP

Abbreviations: GT: Glanzmann thrombasthenia; BSS: Bernard Soulier syndrome; PCI: Prothrombin consumption index; ETP: Endogenous thrombin potential. The result for P2Y1 defect are hypothetical as these are rare disorders and have not been completely studied.

- COX-1 and COX-2 inhibitors (reversible)
- (Nonsteroidal anti-inflammatory drugs-NSAIDs) such as ibuprofen, indomethacin, mefenamic acid
- Platelet receptor inhibitors such as abciximab, tirofiban, eptifibatide ($\alpha IIb\beta 3$), ticlopidine, clopidogrel, prasugrel (irreversible), cangrelor (revesible), ticagrelor (reversible) (P2Y12)
- Phosphodiesterase inhibitors such as dipyridamole and cilostazole
- Anticoagulants vitamin K antagonists, heparinoids and direct thrombin inhibitors.
- Cardiovascular agents which include β-adrenergic blockers (propanolol), vasodilators (nitroprusside, nitroglycerine), diuretics (furosemide) and calcium channel blockers
- *Antimicrobials:* Antibacterials such as β-lactams (penicillins, cephalosporins), antifungals (amphotericin), antimalarial (hydroxychloroquine) and nitrofurantoin
- *Chemotherapeutic drugs:* L-aspraraginase, vincristine, plicamycin
- *Psychotropics drugs:* Antidepressants (imipramine), phenothiazines (chloropromazine) and local and general anesthetic agents (halothane)
- *Thrombolytic drugs:* Streptokinase, urokinase, tissue plasminogen activator (TPA)
- Miscellaneous drugs such as clofibrate, dextrans, guaifenesin (expectorant) and radiographic contrast media
- Foods and drinks commonly consumed affecting platelet function are caffeine, alcohol, cumin, fenugreek, garlic, onion, ginger, ginseng, fish oil, tamarind, turmeric, vitamins C and E and, Chinese mushroom.

Many other substances apart from the ones mentioned above can affect platelet function. It is mandatory to take a drug and relevant dietary history from the patient. Re-testing is recommended to confirm if the defect is transient. *(Reprinted and modified with permission from Kottke-Marchant and Corcoran G. The laboratory diagnosis of platelet disorders. Arch Pathol Lab Med 2002:126:133-46 with permission from Archives of Pathology & Laboratory Medicine. Copyright 2002. College of American Pathologists.)*

FLOW CYTOMETRY[1]

Flow cytometry is used to quantify glycoproteins in GT and BSS. Flow cytometric tests are also available for measurement of dense granules using mepacrine uptake and release, and for measurement of microparticle procoagulant activity. These are used in the diagnosis of SPDs. Flow cytometry can be employed to confirm certain aggregometry findings such as (Gp Ia/IIa and Gp VI) and PAR-1 receptor densities in collagen defects. Platelet activation can be measured in response to routinely used agonists, dense granule content, and exposure of anionic phospholipids. Citrated whole blood is used for analysis.

Delay in performing the test can cause platelet activation and give false results. The test is performed by adding fluorescent-labeled antibodies to the blood, incubating the tubes at room temperature in the dark, then diluting the samples to a final volume of between 1 mL and 2 mL. The tubes should be mixed gently, by tapping to avoid platelet aggregation. Commercial antibodies/reagents are available that can quantify the various receptors. Receptor numbers may be lower in neonates. A limitation of this method is that receptors with a density of less than 500 receptors/platelet cannot be measured, so the test cannot always be used reliably to detect reduced number of receptors. It is possible to measure platelet procoagulant activity, apoptosis and microparticles by incubating samples with high affinity probes against phosphatidylserine (e.g. Annexin-V) and activating the cells with calcium ionophore, collagen-related peptide or combinations of thrombin and collagen. The diagnosis of Scott syndrome and related disorders though very rare can be made with these assays.

MEASUREMENT OF NUCLEOTIDES[1]

Measuring the adenine nucleotides is an important additional diagnostic method used along with aggregometry for detecting deficiency in dense granule numbers or content such as seen in SPDs or degranulation or release defects in granules. These defects are easily missed on platelet aggregometry alone. In spite of nucleotide measurement being an easy to perform test, in which ATP is measured by simple bioluminescent assays (using firefly luciferin/luciferase assays), it is rarely performed in a routine laboratory. Therefore, many disorders of platelet storage and secretion defects are being missed.

The lumiaggregometer is capable of performing simple assays of released platelet nucleotides in real time with whole blood or PRP. Assessment of ATP levels during platelet aggregation and release of ATP during the secondary aggregation phase can be measured by a lumi-aggregometer. However, it is not possible to distinguish between SPD and defects in release of secretory granules using this approach. Therefore, many laboratories measure the total content of ADP and ATP of the platelet by making a lysate of platelets. These assays can be performed on frozen samples which can be transported.

The platelet contains 2 nucleotide pools: the metabolic pool and the dense granule or storage pool, which makes up 60% of the total content. The ATP:ADP ratio is therefore important as there is a marked difference between the relative concentrations in the two pools. Storage defects have a decreased amount of stored and released ADP resulting in an increased ATP:ADP ratio. On the other hand, a release defect shows normal ADP levels and ATP:ADP ratio but decreased ADP release.

Serotonin (5-HT) is stored in the platelet dense granules and the uptake and release of radiolabelled serotonin into and from the platelets can be measured by ELISA tests.

WHOLE BLOOD AGGREGOMETRY[1]

A lumiaggregometer measures platelet function using electrical impedance in whole blood or optical density in plasma (LTA) while simultaneously measuring dense granule secretion (ATP release) by the luminescence method. It allows study of platelet function in whole blood in presence of other cells before decay of labile modulators. The quantity of blood required is less compared to LTA. The turbidometric features permit the performance of ristocetin cofactor assay and sticky platelet syndrome tests. It can also be used to a) monitor aspirin therapy by looking for lack of aggregation response to collagen in whole blood b) monitor plavix therapy by ADP response in whole blood c) to monitor DDAVP administration by ristocetin induced aggregation in whole blood and d) to monitor Gp IIb/IIIa receptor blockers and other anti-platelet drugs.

A study carried out by UK NEQAS surveyed 169 hemostasis centers. They found that out of 88 centers doing platelet studies, only 4 performed whole blood aggregometry. Very few studies are available which have validated this new modality clinically and in the lab with the conventional LTA.

OTHER TESTS USED FOR MEASURING PLATELET AGGREGATION

Rapid Platelet Function Assay by Ultegra–RPFA[1]

This is a turbidometric test which works on the principle of optical detection that correlates platelet aggregation with an increase in light transmittance. This test correlates well with platelet aggregometry and was originally used to monitor antiplatelet drugs by measuring the anti Gp IIb/IIIa complex.

Hemostasis Analysis System

The platelet contractile force (PCF), clot elastic modulus (CEM) and thrombin generation time (TGT) are measured in 700 µL of whole blood by this instrument. It is capable of diagnosing both thrombophilia and bleeding disorders and is also used to monitor treatment in these conditions. It is popularly used as a POC instrument in surgical settings, cardiology clinics and ICUs.

ELISA, RIA and Western Blot

Platelet α granule proteins comprising of platelet factor 4 (PF4) and β-thromboglobulin can be measured by various techniques such as ELISA, RIA or western blot. They are contributory to the diagnosis of quebec platelet disorder. However, there are problems with reproducibility and interpretation of results. Adenine nucleotide and serotonin release from the dense granules are best measured by a specialist laboratory. The release from α-granules indicates activation of platelets and thrombotic tendency. Platelet vWF is measured to diagnose some variants of vWD. If the study suggests a defect in the prostaglandin pathway, TXA_2 can be estimated quantitatively by radioimmuno assay. Highly specific assays of various steps in AA metabolism are available in specialized laboratories.[1]

Electron microscopy has made it possible to detect ultra-structural abnormalities in platelets of patients with platelet function defects. Dense granule defects can now be diagnosed by whole mount electron microscopy. Substances released from the granules can also be measured.[1]

Molecular diagnosis of inherited platelet function defects is confirmatory in affected individuals and their family members and for antenatal diagnosis. It is easily done in GT and BSS because the number of candidate genes is small. Diagnosis for clinically suspected cases of GT and BSS can be confirmed by direct sequencing of PCR-amplified genomic DNA. Individual affected genes can occasionally be identified in patients with mild defects e.g. MYH9 related disorder, CAMT, TAR, WAS, thromboxane and P2Y12 ADP receptor defects and GPVI defects. Molecular analysis of these disorders is mostly done in research laboratories. In regions having a prevalence of a specific mutation, e.g. 16bp deletion in HPS1 in Puerto Ricans with Hermansky-Pudlak syndrome, allele specific mutation detection strategies help in quick detection of the molecular defect.[1]

CONCLUSION

Nonavailability of laboratories to perform platelet function assays makes diagnosing inherited platelet disorders in children very difficult. Screening tests can be done in most routine laboratories. These include platelet count, MPV, and examination of the peripheral blood smear to exclude thrombocytopenia as a cause of bleeding in the patient.

Platelet function testing was a much-neglected area as the previous guidelines were published in the late 1980s. A variety of new tests and techniques have been added to the armamentarium of platelet function testing. These include platelet size distribution profiling and molecular markers of platelet reactivity. Lumiaggregation has added a significant new dimension to assessment of platelet function. Flow cytomertry provides a variety of specific tests that are very useful in diagnosing various defects. Reliable but simple to use whole blood tests that attempt to simulate *in vivo* hemostasis can be used to screen samples rapidly before applying the existing battery of tests. The general consensus is that the bleeding time should be replaced. Many of the simpler platelet function tests could be potentially utilized as point of care instruments for assessing bleeding risk and monitoring antiplatelet treatment. It is possible that in the near future important developments in the platelet genome will be made leading to exciting advances in this field such as platelet specific microarrays which may have a significant impact on the diagnosis and management of patients with either thrombotic or hemostatic defects.

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CHAPTER 33

Pediatric Thrombosis

Rashmi Dalvi

Thromboembolic disease represents an infrequent event in childhood, however, one associated with considerable mortality and morbidity. The incidence of venous thromboembolism (VTE) has been reported at about 0.07/10,000 children, 5.3 percent of pediatric admissions and 2.4 percent of newborns in intensive care units. Neonates are at highest risk, possibly because of physiologically lower anticoagulant levels and markedly reduced fibrinolytic activity. The incidence of vascular accidents decreases significantly after infancy, with a second peak during puberty and adolescence, again associated with reduced fibrinolytic activity. Although in the past, this was more an issue in the adult domain, thrombotic disorders are now an increasingly recognized clinical challenge. This is due partly to enhanced clinician awareness, advances in pediatric therapeutics and tertiary care, as also to specific identification and molecular characterization of a number of heritable prothrombotic defects.

PHYSIOLOGIC CONSIDERATIONS

The fluid state of blood is maintained by a delicate and dynamic balance between the coagulant, anticoagulant and fibrinolytic systems, all regulated through a series of feedback mechanisms. Despite all progress in the field, Virchow's triad of three basic risk factors for thrombosis viz:

1. Stasis, hypercoagulability and endothelial damage are still central to clinical considerations in thrombosis. In most cases, whether or not an underlying risk factor is identified, clinical thrombosis always results from a combination of two or more factors. The natural anticoagulant system includes three pathways for inhibition of procoagulant activation:
   i. Cleavage of factors V and VIII by the protein C and protein S system.
   ii. Direct inhibition of thrombin by antithrombin III (AT III), heparin cofactor II and alfa-2 macroglobulin
   iii. Inhibition of factor VIIa by the tissue factor pathway inhibitor/factor Xa complex.
2. In addition, the von Willebrand factor (vWF) cleavage protease ADAMTS13 regulates the size of vWF multimers thereby reducing its functional activity in primary hemostasis. When thrombin binds to thrombomodulin on endothelial surfaces, it no longer cleaves fibrinogen but activates protein C, which in turn complexes with protein S to proteolytically inactivate factors V and VIII.
3. Clot dissolution *in vivo* is mediated by the enzyme plasmin which is generated from its precursor plasminogen by two known activators, tissue plasminogen activator (tPA) and urokinase plasminogen activator. Inherited or acquired deficiency in these pathways may foster a hypercoagulable state.

The evolving hemostatic system in neonates and children is distinctly different from the mature adult. Neonates have a hypocoagulable state with decreased capacity to generate thrombin, which, however, is balanced by the protective effects of deficient circulating anticoagulants. The physiological immaturity may extend to variable extents through childhood, which perhaps explains the strikingly low incidence of clinical thrombosis. An elegant longitudinal study of normal children from birth to adolescence has shown that during the newborn period and infancy, although levels of AT III, protein C and S are low, thrombin generation is reduced and alfa-2 macroglobulin is increased. Likewise,

throughout childhood, protein C and heparin cofactor II are low, but alfa-2 macroglobulin is raised and prothrombin and factor VII are reduced compared to adult values. These physiologic differences bear effect on the incidence and natural history of thrombosis, as well as on pharmacokinetics and response to anti-thrombotic drugs, making clinical management a greater challenge.

Among newer concepts, is the role of the endothelium and endothelial heterogeneity in thrombosis and hemostasis. The endothelium is now not considered as merely lining vascular channels, but a highly metabolic, functional and dynamic tissue which uniquely adapts to local tissue environment. Thrombotic complications in various disorders or hypercoagulable states show a predilection to specific locations, likewise with bleeding too. Research supporting this changing paradigm has shown heterogeneous distribution of coagulation and fibrinolytic factors across organs and tissues. They also show a variable response to stimuli, e.g. cytokines (TNF-alfa), blood flow, drugs , toxins. In particular, inflammatory cytokines upregulate endothelial prothrombotic factors with a switch of homeostatic balance in favor of hemostasis, but this too varies with location.

ETIOLOGY AND RISK FACTORS IN CLINICAL THROMBOSIS

In the context of childhood thrombosis, more of venous thromboembolism than arterial is encountered in clinical practice. Arterial clotting is seen in the presence of abnormal platelet activation or endothelial dysfunction, whereas VTE is predisposed to by venous stasis, venous endothelial damage, deficiency of anticoagulant or anti-fibrinolytic factors or an excess of anticoagulant protein. As an extension of the traditional Virchow's triad, another researcher, Eberhard Mammen, postulated reduced mobility as another important factor, which albeit may be more applicable in adults. He proposed that decreased muscle contraction reduced blood flow, with stasis and accumulation of blood within intramuscular sinuses, thus triggering hypercoagulabilty due to local accumulation of activated clotting factors and simultaneous consumption of anticoagulants.

Microparticles, which are circulating small fragments of cell membranes are found to carry procoagulant and anticoagulant proteins and plasminogen system components, are mainly derived from platelets and lesser degree from leukocytes and endothelial cells. These microparticles are believed to have a thrombogenic role, as increased circulating microparticles have been associated with thromoboembolic complications in cancer, anti-phospholipid syndrome, sickle cell disease, diabetes and systemic inflammatory disorders.

In the clinical situation there may be an overlapping presence of both inherited and acquired factors. Some of the milder forms of inherited thrombophilia, develop thrombosis only in the presence of other comorbid states or therapeutic interventions, as is increasingly becoming evident, many of the so-called acquired thromboses may have an underlying thrombophilic defect.

ACQUIRED RISK FACTORS

Neonatal Thrombosis

Thrombotic complications occur five times more frequently in the newborn compared to older children. Though they may occur spontaneously, most often are associated with a known event.

Risk Factors

- Indwelling vascular catheters especially umbilical artery cannulation
- Malposition venous catheters or
- Use of hyperosmolar solutions, dehydration
- Polycythemia
- Hypoxia
- Maternal diabetes
- Intrauterine growth retardation, or
- Shock syndromes, as in asphyxia or sepsis
- Severe congenital protein C or S deficiency may present in the newborn period. Abnormal chorionic vessels occurring in a range of maternal disorders may produce chorionic thrombi which embolize to fetal pulmonary arteries or portal vein.
- Maternal antiphospholipid antibody syndrome has also been associated with neonatal arterial thrombosis.

Neonates have a propensity to large vessel thrombosis and present with limb or organ dysfunction depending on the vessel involved. Presentation may be in the form of edema, lower limb cyanosis (Inferior vena cava), scalp and facial edema (Superior vena cava, SVC), renal lump with hematuria (renal vein), or seizures. Aortic thrombosis may present with congestive heart failure, feeble femoral pulses, necrotizing enterocolitis or renal failure, peripheral artery block may have absent pulses with cold discolored skin, and cerebral artery thrombosis may have apnea and seizures. Neonates with homozygous protein C or S deficiency invariably present with purpura fulminans.

VASCULAR ACCESS DEVICES

Central venous catheters are increasingly being used in critical care settings and for prolonged vascular access in children requiring parenteral nutrition, chemotherapy, blood transfusions antibiotics. These are today

an important risk factor for pediatric thrombosis. Clinical pointers to such an event include recurrent blockage, frequent sepsis, local pain or swelling, chylothorax, SVC obstruction or appearance of chest wall collaterals. Cardiac catheterization may also be complicated by thrombosis at the site of cannulation.

DEHYDRATION AND SEPSIS

These two factors alone or in combination may promote thrombosis by causing a hyperviscosity of blood and cytokine induced endothelial damage in the course of sepsis. Thus, acute gastroenteritis with dehydration is a common clinical setting where such an event is predisposed often affecting cerebral venous sinuses. Suppurative thrombophlebitis of the internal jugular vein or Lemierre's syndrome triggered by fusobacterial sepsis, though a rare cause in children, carries a potential risk of CNS morbidity and mortality.

NEPHROTIC SYNDROME

Thromboembolic complications can occur in about 25 percent of patients with nephrotic syndrome presenting with not only renal vein thrombosis but also arterio venous thrombosis at various other sites including CNS and abdominal vessels. Factors predisposing a hypercoagulable state include AT III protein loss in urine, hyperfibrinogenemia, increased platelet activation, hyperlipidemia, increased platelet activation, hyperlipidemia, along with a propensity to intravascular volume depletion, hyperviscosity, use of diuretics, and relative immobilization.

ANTIPHOSPHOLIPID ANTIBODY SYNDROME

This condition may occur as a primary antiphospholipid antibody syndrome (APLA) or secondarily with systemic lupus erythematosus or other connective tissue disorders. APLA has most often been associated with CNS stroke in children, although arterial thrombosis elsewhere may also occur. APLA is also described in conjunction with viral diseases such as hepatitis and human immunodeficiency virus disease. APLA promotes thrombosis by platelet activation, release of endothelial platelet factor and inhibition of protein C and AT III.

THALASSEMIA

A higher than normal incidence of thromboembolic events has been observed in patients with beta thalassemia major (TM). Red blood cells in TM have been shown to facilitate thrombin formation due to altered symmetry of membrane phospholipids with enhanced exposure of phosphatidyl serine. Studies have shown elevated plasma levels of thrombin-antithrombin complexes and significantly decreased levels of protein C and S in all TM patients without other thrombophilic mutations. Thus, a chronic hypercoagulable state exists in TM, with children and adolescents presenting with CNS thrombosis, DVT, cardiac thrombosis, pulmonary embolism or recurrent thrombophlebitis. Identified risk factors for thrombosis in TM include: platelet count > 6 lacs</cumm, protein C < 50 percent, plasminogen< 50 percent, protein S < 50 percent.

MALIGNANCY

Tumor microparticles, cytokine induced endothelial changes, sepsis in neutropenic patients, vascular accesses and drugs such as L-asparaginase may promote thrombosis in cancer.

Other risk factors include trauma, surgery, immobilization, infusion of prothrombin complex concentrates, use of oral contraceptives in adolescent girls. Acquired protein C deficiency may also occur in liver disease, sepsis, disseminated intravascular coagulation (DIC) and especially in purpura fulminans and DIC with acute meningococcal infection.

Arterial thrombosis may be associated with sickle cell disease, vascular malformations/Moyamoya disease, APLA, hyperlipidemias, vasculitis.

FAMILIAL THROMBOPHILIA

Inherited prothrombotic states are usually suspected in children with an unexplained cause for thrombosis, a positive family history, recurrent thromboembolism, or thrombosis at an unusual site. Most of them present beyond adolescence unless compounded by additional risk factors. However, severe deficiencies may have spontaneous thrombosis as early as newborn period. Coinheritance of these susceptibility genes is known and the risk of thrombosis increases with multiple coinherited defects. Some of the better understood anticoagulant defects are discussed briefly here.

- Protein C deficiency in its milder phenotype is inherited as an autosomal dominant trait with a population prevalence of 0.2 percent and having two subtypes identified by quantitative and qualitative defects respectively, the latter being less common. Milder homozygotes and heterozygotes present with recurrent thromboembolism usually beyond the second decade and in association with additional risk factors. Severe homozygous protein C deficiency is usually inherited in an autosomal recessive form.
- Protein S deficiency has a very similar inheritance pattern, subtypes and clinical profile as protein C deficiency.

- Factor V Leiden is a form of inherited prothrombotic defect characterized by a mutation in factor V (factor V Arg506Gln) at exactly the site where it is spliced by activated protein C, making it resistant to the action of the latter. It is thus also called 'activated protein C resistance'. This mutation is present in about 8 percent of Caucasians, but < 1 percent Asians and Africans. Deficient individuals have a milder phenotype, and both homozygotes and heterozygotes may have recurrent thromboembolism during childhood or later, usually with an associated risk factor. It may rarely cause arterial thrombosis and has also been implicated in porencephaly, cerebral palsy and Perthe's disease.
- Prothrombin gene mutation at the nucleotide 20210A position estimated to occur with a frequency of 3 to 4 percent in the population. The mutation results in abnormally high prothrombin levels, which probably contributes to increased thrombotic risk by increased thrombin generation. Clinical manifestations are mild even in homozygotes.
- Antithrombin III deficiency displays two types of mutations, with quantitative and qualitative defects respectively. The homozygous state is incompatible with life. Heterozygotes present in early adulthood with recurrent and/or thromboembolism. Thrombosis in adulthood is rare, and its occurrence is usually associated with secondary risk factors.
- Hyperhomocysteinemia is classically due to deficiency of cystathionine biosynthase. However, another more common polymorphism in the methyltetrahydrofolate reductase (MTHFR) gene appears to reduce conversion of homocysteine into methionine. The MTHFR is not an uncommon polymorphism and its homozygosity, well documented in adults, may increase risk of thrombosis especially stroke in children.

Among other heritable prothrombotic states, yet to be clearly defined, are:

- Elevated factor VIII levels
- Raised lipoprotein associated with lipoprotein coagulation factor Lp(a)
- Dysfibrinogenemia
- Thrombomodulin mutations
- Factor XII deficiency
- Defects in plasminogen and plasminogen activator inhibitor.

Mutations causing a deficiency in the ADAMTS13 protease present as congenital thrombotic thrombocytopenic purpura, with predominantly a microangiopathic hemolytic anemia that worsens with infection, showing marked elevation in D-dimers.

Approach to Diagnosis

A high index of clinical suspicion is necessary for an early diagnosis, which is crucial so as to prevent fatal complications such as pulmonary embolism, organ dysfunction and prevent long-term morbidity. Once VTE is suspected, there are various laboratory tests and imaging modalities that help confirm the diagnosis and delineate the extent of thrombosis.

Radiologic Imaging

- Although venography is the gold standard to demonstrate thrombosis, it is used infrequently as it is invasive, needs specialized radiology, needs multiple peripheral access and is not useful for internal jugular vein as the dye cannot flow in a retrograde fashion.
- Doppler ultrasound is the most frequently used imaging, being noninvasive, easily available and lower in cost. It has a high sensitivity for lower limb and abdominal vein thrombosis. However, it may be ineffective for upper limb thrombosis due to the chest wall lung tissue and the clavicles.
- Magnetic resonance (MR) venography clearly images all large veins and is useful in cerebral sinus thrombosis, though it is expensive and needs sedation. CT angiography is also useful, but needs greater contrast, has exposure to radiation.

Likewise for arterial thromboses, though angiography is the gold standard, it is invasive, needs an interventional radiologist, and may itself cause thrombosis. Doppler ultrasound is a good screening and diagnostic modality except for the chest region.

Laboratory Evaluation

Diagnostic evaluation for pediatric acute venous thromboembolism (VTE) includes a complete hemogram, coagulation tests, comprehensive thrombophilia evaluation. Additional laboratory evaluation would depend on associated medical conditions and VTE in specific organ systems.

D-dimer assay may be useful as a screening test for massive or diffuse thrombosis, however, low levels do not exclude VTE. Other abnormal tests in VTE include circulating prothrombin fragment 1.2 and the TAT complex, however, these are not clearly adapted to the clinical setting. Prolonged activated partial thromboplastin time (APTT) with a normal prothrombin time (PT) may indicate presence of lupus anticoagulant/APLA or factor XII deficiency. Shortened APTT with normal PT may be associated with elevated factor VIII levels.

Prolongation of both PT and PTT may be seen with DIC or dysfibrinogenemia.

Hemogram may help identify sickling of red cells, thrombocytosis or thrombocytopenia in massive thrombosis or DIC. Sickling test and hemoglobin electrophoresis is recommended in arterial thrombosis.

Recommended panel for identifying thrombophilic states as per the Scientific and Standardization Subcommittee on Perinatal and Pediatric Hemostasis of the International Society on Thrombosis and Hemostasis, for laboratory evaluation of VTE in children include the following.

Acquired or Genetic

- AT III assay
- Protein C assay
- Protein S assay
- Elevated plasma factor VIII assay
- Blood homocysteine levels
- APLA (including anticardiolipin antibodies, Russell viper venom time, antibody to beta-2 glycoprotein 2, APTT–based lupus anticoagulant test).
- DIC (including platelet count, fibrinogen levels, D-dimer)
- Activated protein C resistance (APTT-based assay).

Genetic

- Factor V Leiden polymorphism
- Prothrombin G20210A polymorphism
- Elevated plasma lipoprotein(a).

Therapeutic Approach

There are few clinical trials available to guide decision making in the treatment of thrombosis. Most published guidelines are based on adult trials, uncontrolled pediatric studies, case series and reports and it is unclear how these guidelines are being used. Nevertheless patients today are being diagnosed with thrombosis and must be treated. Therapy is based on methods that are best for restoring circulation rapidly balanced by the risk of bleeding, with an aim to re-establish flow through the occluded vessel, prevent embolization, and arrest the thrombotic process.

ANTITHROMBOTIC AGENTS

The interaction of antithrombotic agents with the hemostatic system of the young differs from the adult with respect to a multitude of variables. Pharmacokinetics of these agents vary in an age-dependent manner as well as with intercurrent illnesses and medications. Limited vascular access poses problems with drug delivery and with monitoring of anticoagulant effect. With most agents, pediatric-specific formulations are not available making reproducible dosing difficult, especially so in the case of low molecular weight heparins (LMWH) and vitamin K antagonists (VKA) warfarin. Dietary differences especially in milk fed patients make VKA dosing difficult. Thus, actual clinical experience becomes important and VTE should, if possible be treated in a pediatric hematology setting, or if not by a neonatologist/pediatrician in close consultation with a pediatric hematologist.

Unfractionated Heparin

Standard heparin still remains the most common form used in pediatric patients overall and acts by enhancing AT III mediated inactivation of factor Xa and thrombin, hence needs normal ATIII levels to be effective. Its efficacy is monitored by aPTT or antifactor Xa activity. Newborns have a faster clearance of unfractionated heparin (UFH) due to a larger volume of distribution and hence need a higher dose to achieve anticoagulant effect. Its major disadvantage is the need for vascular access and repeated monitoring. Risk of bleeding with UFH for deep vein thrombosis (DVT) is low but may be as high as 24 percent in the ICU setting. Osteoporosis with UFH is rare in children. Heparin induced thrombocytopenia in various cohorts is reported in 0 to 2.3 percent, at varying ages and in UFH exposures ranging from vascular access device flushes to massive doses in cardiopulmonary bypass surgery.

Low Molecular Weight Heparin

Despite unproven efficacy, low molecular weight heparin (LMWH) have rapidly become a treatment of choice on pediatric patients, both for primary prophylaxis and treatment of VTE. Potential advantages of LMWH in children include subcutaneous route of administration, no significant need for monitoring, minimal risk of bleeding HIT or osteoporosis and no interference with diet or drug metabolism. Most available data in children is with the use of enoxaparin, although dalteparin and reviparin have been used as well. Bleeding with LMWH can be treated with protamine sulfate, although multiple doses may be required. LMWH should be withheld for 24 hours prior to any procedure, as also it needs dose adjustment in renal failure.

Thrombotic Agents

For large vessel and massive or extensive thrombosis, or pulmonary embolism, thrombolytic therapy should be considered, after weighing the potential benefits versus

risk of bleeding. It is recommended to ensure that serum fibrinogen is > 50 mg/dL and platelet count > 50,000/cumm and that efficacy be monitored by documenting increasing D-dimer levels. Thrombolysis carries significant risk in patients who have had surgery in the past 7 days, premature neonates or with cerebral sinus thrombosis when intracranial bleed may occur. Minor bleeding with thrombolysis may be treated with local pressure, but major bleeds will require stopping therapy and administration of cryoprecipitate and/or antifibrinolytics. In children there is no data showing any advantage of local over systemic administration. In fact, except for catheter related TE, catheter-directed local administration may cause more harm in view of small vessel lumen size. The thrombolytic agent of choice in pediatric patients is recombinant tPA, however, it is expensive. Streptokinase is a cheaper alternative, but may cause severe allergic reactions be less effective in the presence of plasminogen deficiency as in a newborn. Such situations will need supplementation with fresh frozen plasma to make thrombolytic agents effective. A recent study by Leary et al. has shown low-dose systemic r-tPA an effective thrombolytic agent in DVT in children, which may represent a transition between high dose r-tPA and anticoagulation.

VITAMIN K ANTAGONIST

Warfarin is the commonly used vitamin K antagonist (VKA) when long-term treatment is warranted and effects its anticoagulant action by inhibiting gamma carboxylation of vitamin K dependent proteins. Though an oral and low-cost agent, it has unpredictable pharmacokinetics, numerous drug and food interactions as well as a narrow therapeutic index. It is extremely challenging thus, to manage children under 4 years on warfarin. However, currently point of care portable devices to monitor PT and INR using capillary samples are available.

Alternative Thrombin Inhibitors

Patients with HIT may be treated with alternative agents such as danaparoid, argatroban or hirudin, though limited data is available on these children. Antifactor Xa agents such as fondaparinux act by indirect inhibition of thrombin.

ANTIPLATELET DRUGS

Aspirin is the most common antiplatelet drug used in children at an empiric dose of 1 to 5 mg/kg/day, and second commonest being dipyridamole (2–5 mg/kg/day). However, clopidogrel (1 mg/kg/day) is found to be effective and safe in children. Bleeding is uncommon except in situations with added hemostatic abnormalities or newborns also on indomethacin. Newer agents include ticlopidine and glycoprotein IIb-IIIa antagonists such as abciximab, the latter being found to induce faster regression of coronary aneurysms in Kawasaki's disease.

Venacaval Interruption

Inferior venacaval filters have been implanted in some cases to prevent pulmonary TE.

Surgical Thrombectomy

Rarely used in children, it may be required in IVC thrombosis in Wilms' tumor, blocked Blalock-Taussig shunt, massive intracardiac thrombosis following surgery, local thrombosis after vascular access.

Treatment of Venous Thromboembolism

- *Initial management:* This largely depends on the location extent and symptoms. For severe massive thrombosis, thrombolytic therapy should be considered, followed by anticoagulation. Less severe DVT, non-occlusive or small thrombi of extremities, simple catheter related thrombi can be treated with anticoagulation alone, where currently the choice is UFH or LWMH as a first line. LWMH is preferred because of its safety and convenience in children. UFH which has a shorter half life is preferred when there is a greater comorbid risk of bleeding, labile acute clinical status, impaired renal function or with cost constraints. Recommended duration of heparinization is 5 to 10 days.
- *Further treatment:* Once some flow is restored, further anticoagulation is needed to treat residual thrombi, prevent embolization and recurrence. Extended anticoagulation in a subacute phase would prefer LMWH, though warfarin is also used. For chronic anticoagulation in an older child warfarin is used.

DETAILS OF ANTITHROMBOTIC AGENT ADMINISTRATION (TABLES 1 TO 4)

Recommended Durations of Therapy

(Anti-thrombotic therapy in neonates and children: Evidence-based guidelines, P Monagle et al.)

- For idiopathic VTE as a first episode 6 months anticoagulation with LMWH or warfarin (INR 2.5).
- For secondary risk factors which have resolved, 3 months of VKA or LMWH.
- For ongoing secondary risk factors (e.g. nephrotic syndrome or L-asparaginase) continue anticoagulation till risk factor resolution.

Table 1 Administration of unfractionated heparin

Loading dose	75 u/kg over 10 minutes for all
Maintenance dose	25–30 u/kg for infants; 20 u/kg > 1 year of age
APTT monitoring	First at 4 hours, then daily and 4 hours after every change
Desired APTT	60–85 sec (antifactor Xa level 0.3–0.7 u/mL)
Dose modification	A PTT < 50 sec—increase 20% 50–59 sec—increase 10% 80–95 sec—decrease 10% 96–120 sec—withhold heparin for 30 minutes and decrease 10% >120 sec—withold heparin 60 minutes and decrease 15% Daily CBC

Table 2 Administration of LMWH (enoxaparin)

Initial treatment dose	2 mg/kg 12 hourly in neonates 1.5 mg/kg 12 hourly > 2 months age
Maintenance dose	1.5 mg/kg for neonates 1 mg/kg > 2 months age
Antifactor Xa monitoring	First at 4 hours, 24 hours, 1 week, monthly
Desired antifactor Xa	0.5–1 u/mL

Table 3 Administration of warfarin

Loading dose	0.2 mg/kg for 2–4 days
PT-INR monitoring	Daily till INR in therapeutic range, then weekly
Desired PT-INR	2.0–3.0
Dose modification	a. Loading INR 1.1-1.3—100% of loading dose INR 1.4–3 50% INR 3.1–3.5 25% INR >3.5 withhold till <3.5, restart at 50% b. Maintenance INR 1.1–1.4; 120% of previous dose INR 1.5–1.9; 110% INR 2.0–3.0; 100% INR 3.1–4.0; 90%

- For recurrent VTE with reversible risk factor, 6 to 12 months and resolution of risk.
- For idiopathic recurrent VTE, 12 months to lifelong.
- For chronic risk factor associated VTE, lifelong anticoagulation is recommended.

Table 4 Administration of systemic thrombolytic agents

r-tPA	0.1–0.6 mg/kg/hr over 6 hours—systemic high dose 0.5 mg in normal saline volume required to fill the line—local
Streptokinase	2000 u/kg bolus, then 2000 u/kg/hr for 6–12 hours
Urokinase	4400 u/kg bolus, then 4400 u/kg/hr for 6–12 hours
Monitor	PT, PTT, platelet count, fibrinogen for all

The above guidelines may be referred to for further details in management of thrombosis in a wide spectrum of clinical situations including neonates, venous access devices, arterial ischemic stroke, cardiac devices and prosthesis, cardiac procedures, cardiomyopathy, Kawasaki's disease, cancer, cerebral sinus thrombosis, APLA and purpura fulminans.

Neonatal Purpura Fulminans

This is seen with homozygous or doubly heterozygous protein C deficiency, wherein presentation is very early in the newborn period large areas of skin necrosis due to diffuse thrombosis within the skin vasculature. Clinical effects may occur even earlier, in the fetus with CNS thrombosis giving rise to neonatal seizures. Management of these babies is complicated as, neither heparin nor antiplatelet drugs are effective. Replacement therapy is necessary with a source of protein C, usually FFP 10 to 20 mL/kg, or if possible protein C concentrates, 20 to 60 u/kg, on a 12 hourly basis. However, protein C has a half life of 6 to 16 hours, and frequent administration is limited by development of hyperproteinemia, hypervolemia, hypertension, loss of venous access, potential exposure to viral agents, infections. These babies are also prone to warfarin induced skin necrosis although warfarin is required for long-term control of thrombotic diathesis. Liver transplantation may offer a cure with normalization of protein C levels.

OUTCOMES

Complications of VTE may occur acutely or over a long-term. Early adverse outcomes include bleeding associated with anti-thrombotic therapy, thrombotic hemorrhage, and organ dysfunction depending on the site/severity of thrombosis. Long-term adverse outcomes include recurrent VTE, renal dysfunction/hypertension, variceal bleeding, chronic SVC syndrome and post-thrombotic syndrome.

Recurrent TE rates are lower than adults, seen in 6 to 10 percent.

Post-thrombotic syndrome (PTS) seen in nearly 1/3rd of DVTs is characterized by edema, visibly dilated superficial collateral veins, venous stasis dermatitis and ulceration. Some patients, both neonatal VTE survivors and older children, may have persistent residual thrombosis which in the long-term may cause venous valvar insufficiency and hence PTS. Factors predicting poor long-term outcome in terms of recurrent VTE, persistent thrombosis and PTS include, complete veno-occlusion at diagnosis, and more importantly, elevated factor VIII levels >150 u/dL and D-dimer> 500 ng/mL at diagnosis and after 3 to 6 months of standard anticoagulation.

CONCLUSION

Thrombotic disorders have emerged as a serious pediatric concern and clinical challenge resulting in acute and chronic sequel. Though we have come a long way over the past decade, many questions remain regarding pathogenesis, natural history, optimal therapy. Advances in ability to predict outcomes may help risk-stratifying therapy and achieve meaningful improvements in long-term outcomes.

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CHAPTER 34

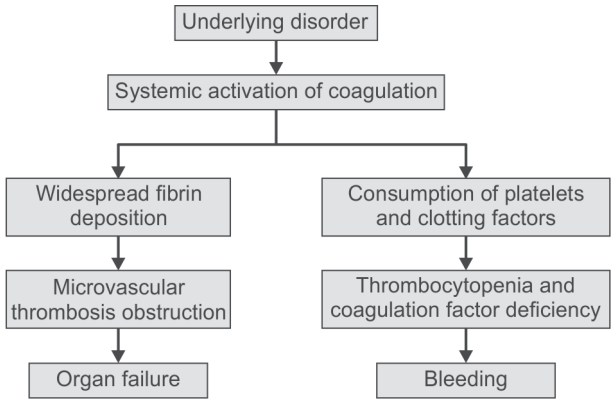
Disseminated Intravascular Coagulation in Neonates

VP Choudhary

Disseminated intravascular coagulation (DIC) is a syndrome which develops following large number of disorders (Flow chart 1). It occurs whenever there is systemic activation of coagulation system leading to generalized uncontrolled formation of fibrin with in the blood vessels leading to micro-vascular thrombosis in multiple organs along with consumption of platelets and coagulation proteins which results in variable bleeding symptoms. Therefore patients of DIC can have symptoms related to bleeding and thrombosis simultaneously. The subcommittee on DIC of the Scientific and Standardization Committee of the International Society of Thrombosis and Hemostasis (ISTH) has recently proposed the following definition of DIC. It is an acquired syndrome which presents with variable symptoms secondary to intravascular coagulation and it may spread to involve multiple organs leading to their dysfunction.[1,2]

First description of DIC appeared in the literature in 1950s.[3] Over the years the pathogenesis of DIC have been studied and with the better understanding of underlying mechanism has helped to evolve multiple diagnostic and monitoring tests and appropriate strategies for its management which have improved the survival significantly. The mortality in the neonatal period is very high. Early diagnosis and prompt management is essential. However, it is desirable that the development of DIC should be prevented by appropriate measures.

Flow chart 1 Disseminated intravascular coagulation



Systemic activation of coagulation system may occur in a large numbers of diseases. The activation of the coagulation results in clinical symptoms which may vary from decrease in platelet count, subclinical prolongation of global clotting time, mild bleeding symptoms to fulminant disease complex termed as disseminated intravascular coagulation.

PATHOGENESIS OF DIC

Many pathogenetic mechanism either singly or together play a major role in pathogenesis of DIC.[4,5] Fibrin depositions plays the key role in its pathogenesis which occurs following tissue factor mediated thrombin generation which exceeds the physiological anticoagulation mechanism (mainly antithrombin III and protein C system). In addition fibrin removal is less, as fibrinolytic activity does not increase proportional to thrombotic activity. Fibrinolytic activity infact is inhibited in DIC because of high level of PAI-1 which is a fibrinolytic inhibitor. Thus there is impairment of endogenous thrombolysis. All these processes are complex and occur simultaneously. It is for the better understanding, these factors have been described separately (Fig. 1 and Flow chart 2). There is an interaction of these factors at multiple levels.

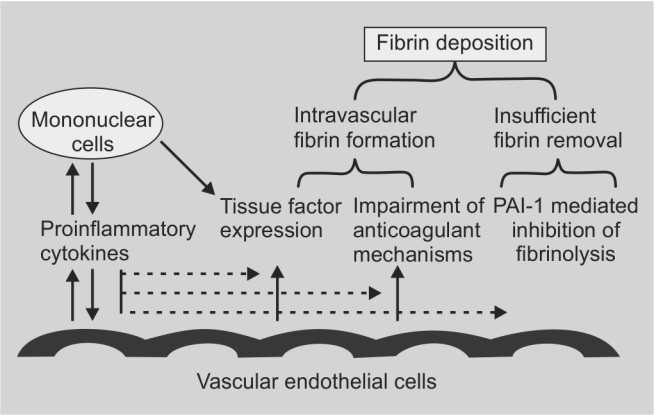
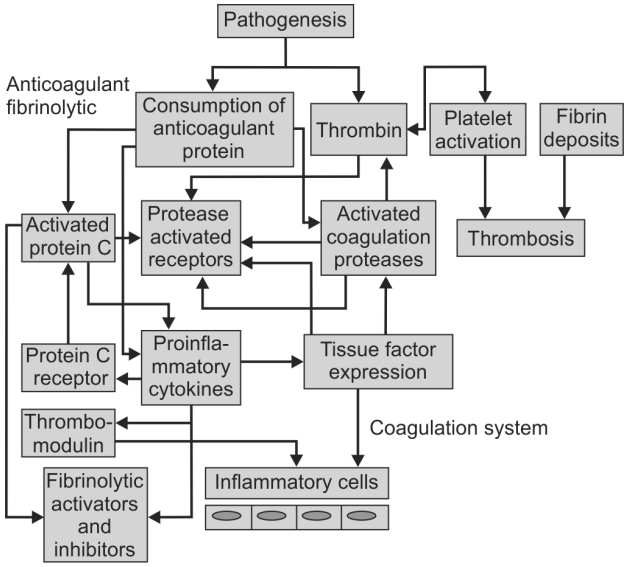


Fig. 1 Disseminated intravascular coagulation—pathogenesis

Flow chart 2 DIC—pathogenesis



Dysfunctional Physiologic Anticoagulant Pathways

Activation of coagulation system occurs through various regulatory pathways which are impaired thereby leading to amplification of thrombin generation which leads to increased thrombin formation.[6] The antithrombin III(AT-III) is an important inhibitor of thrombin whose levels are reduced in patients with severe sepsis. Multiple factors in combination, such as (a) consumption due to ongoing thrombin generation, (b) degradation by elastase, released from activated neutrophils and (c) impaired synthesis. Low levels of AT-III results in DIC with increased mortality.[7] This impaired function of the protein C pathway occurs as a result of down regulation of thrombomodulin expression on endothelial cells through various cytokines, such as TNF-α and interleukin (IL)-β.[8] These observations are further supported by improved survival following administration of activated protein C to patients with DIC. Tissue factor pathway inhibitor (TFPI) is another major inhibitor of coagulation system. There are experimental and clinical evidences which have demonstrated that administration of recombinant TFPI have blocked the inflammatory process induced by thrombin generation. In clinical studies the use of TFPI in pharmacological doses is capable of reducing the mortality significantly in systemic infection.[9]

Impaired Fibrinolysis

Presence of bacteremia and endotoxemia increase the fibrinolytic activity because of the release of plasminogen activators from the endothelial cells.[10] However, increase in plasma levels of PAI-1 results in reversal of fibrinolytic activity.[11] Rise in PAI-1 levels occurs as a result of release of cytokines and endothelial injury (Flow chart 2).

CLINICAL PRESENTATION

Based upon the onset and severity of symptoms, DIC has been subdivided into ***acute*** **and** ***chronic*** **DIC**.

Acute DIC is of sudden onset and carries very high morbidity and mortality. It is most common in neonate period and therefore has been described in detail.

- Neonates with severe acute DIC may manifest with bleeding from multiple sites such as development of purpuric and ecchymotic spots, mucosal oozing, gastrointestinal blood loss and bleeding from venous access.
- Neonates may progress to hypotension, shock and often develop fluid and electrolyte disturbances.
- The deposition of thrombin is dependent upon the organ involvement. Neonates may become lethargic and neonatal reflexes are depressed or absent, comatose, or develop seizures, become unconscious as a result of CNS involvement, may develop stroke as a result of thrombosis in cerebral vessels, which may progress to hemorrhagic infarcts.
- Micro thrombi in the renal system may progress to oliguria, renal failure as a result of acute tubular necrosis. Micro thrombi may cause acute abdominal pain simulating clinical picture of acute abdomen or bleeding in the gut as a result of gangrene.

DIAGNOSIS OF ACUTE DIC

High index of suspicion is essential. Any neonate, who starts bleeding in pressure of underlying condition should be considered to have DIC and should be investigated

periodically for diagnosis of DIC. Thrombocytopenia is an early manifestation of DIC. Patients with acute DIC are critically ill, and therefore early diagnosis is essential for improved survival. Several of sensitive and sophisticated tests are not readily available in clinical practice even in the advanced centers. Therefore the diagnosis of DIC is based upon the platelet count, examination of peripheral smear, measurement of PT and APTT , high level of fibrin degradation products (FDP) and D-dimers.

Thrombocytopenia is often attributed to consumptive processes but underproduction also plays a role in presence of severe sepsis which is often the cause of DIC in neonates. Coagulation on studies when minimally damaged, there may be difficulty in distinguishing it as abnormal as low levels in preterm are expected. Similarly there is no reliable range of D-dimers.

Fibrinogen concentration normally may increase during the first few days of life. Therefore, at time diagnosis of DIC in neonates becomes very difficult.

It has been observed that serial coagulation tests and platelet counts are usually more helpful than single laboratory results to establish the diagnosis of DIC.[12] Low fibrinogen level is detected only in severe DIC but it is not a specific marker.[3]

High levels of FDP and D-dimers help the clinicians to differentiate other disorders like chronic liver disease with low platelet count and prolonged PT and APTT.[13]

The subcommittee on DIC of the International Society of Thrombin and Hemostasis has recently published a scoring system[1] to facilitate the clinicians to establish the diagnosis of DIC. In presence of any condition known to cause DIC, algorithm suggested by the subcommittee should be used to determine the score. A score of 5 or more suggests the diagnosis of DIC. However this scoring system needs to be validated by prospective studies in neonatal DIC (Table 1).

MANAGEMENT OF DIC

The heterogeneity of the underlying disorder and clinical severity is so variable therefore, it is difficult to have a common therapeutic approach for management of DIC. Thus, the treatment of DIC should be individualized, based upon the clinical presentation, i.e. bleeding, thrombosis or both and patients conditions such as hemodynamic situation, presence of hypothermia or not, electrolyte imbalance and gas exchange along with renal, cardiac and neurological status.[14] Early diagnosis of underlying condition and their prompt appropriate management plays a major role in the outcome of DIC. The management of the underlying condition will not be discussed as the underlying conditions are many and have different specific treatment.

Replacement therapy: Platelets and coagulation factors (FFP) are administered to correct thrombocytopenia and coagulation factor to control the bleeding at multiple sites. Currently it forms the major form of therapy for treatment of DIC.[15]

Transfusion of Platelet Concentrates

One to two units of platelet concentrates per 10 kg of body weight are administered if platelet count is below 30 x 10^9/L in absence of any bleeding. Platelet therapy is essential in presence of bleeding manifestation even if the platelet count is >30 × 10^9/L. The main objective is to control the bleeding and there is no need to transfuse platelets to maintain platelet count of >50 × 10^9/L which is considered as a safe level.

Fresh Frozen Plasma

Fresh frozen plasma (FFP) should be administered at a dose of 15 to 20 mL/kg in an attempt to correct the

Table 1 Diagnostic algorithm for diagnosis of DIC

- *Risk assessment:* Does the patient have an underlying disorder known to be associated with overt DIC?
  - If 'yes' proceed
  - If 'no', then do not use this algorithm
- Order for screening coagulation tests (platelet count, PT, APTT, fibrinogen, soluble fibrin monomers or fibrin degradation products)
- Score screening coagulation tests results
  - Platelet count (>100,000=0, <100,000=1, <50000=2)
  - Prolonged PT (<3 sec = 0, >3 sec but <6 sec = 1, >6 sec = 2)
  - Fibrinogen level (≥1 g/L = 0, <1 g/L = 1)
  - Elevated fibrin related marker e.g. soluble fibrin monomers/FDP (no increase=0, moderate increase=2, strong increase=3).
- Calculate score
  - If the total score is ≥5, then patient is compatible with overt DIC, repeat scoring daily.
  - If the score is <5, it is suggestive (not affirmative) of nonovert DIC, repeat the tests at 1–2 days interval till the patient recovers or whenever the score exceeds 5, diagnosis of DIC can be made.

multiple factor deficiency and to control bleeding. Alternatively fibrinogen concentrates (total dose 2–3 g) or cryoprecipitate (1 bag/10 kg body wt) may be administered. Cryoprecipitate (5–10 mL/kg) is a better source of fibrinogen, which should be kept above 1 g/L. Dose and duration of therapy needs to be monitored with platelet counts, PT and APTT levels daily. Treatment should be directed to correct the PT and APTT levels and to prevent the further deterioration.

Anticoagulants

The role of heparin in the treatment of DIC remains still controversial.[16] However, the present data indicates that heparin is effective in treatment of neonate with acute DIC having predominant thrombotic symptoms, e.g. purpura fulminous. Presently role of heparin in chronic DIC is well established. It has been used effectively in patients with recurrent thrombosis such as hemangioma or dead fetus syndrome.[17]

Heparin is usually given at relatively low doses (5–10 U/kg of body weight per hour) by continuous infusion and may be switched to subcutaneous injection for long-term therapy. Its dose needs to be adjusted with APTT or heparin level. Alternatively low molecular weight heparin may be used. It is preferred these days as it is not essential to monitor heparin level or APTT. Secondly the incidence of heparin induced thrombocytopenia is less when compared with standard heparin.

Recombinant hirudin which is newer anticoagulant has AT III independent inhibitory activity of thrombin has been used successfully to treat DIC in experimental studies.[18]

Concentrates of Coagulation Inhibitors

In the pathogenesis of DIC it has been observed that there is deficiency of coagulation inhibitors. Therefore the normalization of anticoagulation pathway should form a part of treatment for DIC.[19] AT-III is a primary inhibitor of circulating thrombin, its use in DIC certainly is rational.[20]

It has been shown to have beneficial effects in terms of correction of coagulation parameters and organ dysfunction.[21]

Use of activated protein C and recombinant thrombomodulin has also been shown to reduce the mortality significantly in patients with severe sepsis.[22] The activation of coagulation cascade in DIC occurs exclusively through the extrinsic pathway. Thus inhibition of tissue factor should block endotoxin associated thrombin generation. De Jongeet al[23] was first to use the tissue factor pathway inhibitor (TFPI) for treatment of DIC following sepsis.

Newer Agents

Some authors have demonstrated that the administration of recombinant interleukin (IL-10), which is a potent anti-inflammatory cytokine, has been demonstrated to moderate the activation of coagulation system in humans but not in neonates. Its use completely abrogated the effects of endotoxins on coagulation pathway. Pajkrt and his colleagues[24] observed significant improvement of DIC in patients with sepsis following administration of monoclonal antibodies against TNF. While Branger et al[25]observed that p38 nitrogen activated protein kinase inhibitor modified the activation of coagulation, fibrinolysis and endothelial cells injury following administration of endotoxins in experimental studies. However, all these modalities are still experimental. There is hope that with better understanding of the pathophysiology, newer diagnostic tests and development of newer agents, the treatment of DIC will improve significantly in near future.

CONCLUSION

DIC is a syndrome characterized by systemic intravascular activation of coagulation in the circulation. It is associated with variable clinical manifestations from mild bleeding to organ failure. Better understanding of pathogenesis has lead to better clinical management strategies. Using a scoring system based on the clinical as well as laboratory tests, an early and accurate diagnosis of DIC can be made. The cornerstone of the management of DIC is the vigorous treatment of the underlying disorder. In addition, the strategies that interfere with the coagulation system, such as replacement, use of antithrombin III and activated protein C, have improved the survival greatly. However, larger studies are essential to determine their efficacy and safety of products such as antithrombin III, activated protein C, etc. Current research is likely to evolve newer novel strategies for management of DIC in near future.

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SECTION 5

Transfusion Medicine

CHAPTERS OUTLINE

CHAPTER 35

Blood Components in Pediatric Practice

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Availability of blood components has improved the outcome of various childhood hematological disorders, especially the pediatric malignancies. Every unit of whole blood collected must be subjected to components as one can satisfy the needs of more than one patient from the same unit of blood. Whole blood has a limited application in clinical practice like during massive blood loss and for exchange transfusion; and there too one can use reconstituted whole blood instead. The notable components prepared from one unit of whole blood include packed red blood cells (PRBC), platelets and fresh plasma; which can be further frozen and used as fresh frozen plasma (FFP). The PRBC can be used to improve the oxygen carrying capacity as well as volume expander in acute blood loss. Platelets are very useful to treat bleeding due to thrombocytopenia caused by decreased platelet production and also in platelet dysfunction. Single donor platelet is much more efficacious than random donor platelet but is expensive and not easily available. Platelets must be kept on a constant agitator and stored at 22°C. Platelets are often misused in clinical practice. Platelets have no role in immune causes of thrombocytopenia or as prophylaxis in chronic stable thrombocytopenia. FFP has role to play in treating patients with multiple factor deficiency classically seen in disseminated intravascular coagulation (DIC) or liver disorders. It should not be used as volume expander or as source of proteins. Leukodepleted blood products are preferred as donor lymphocytes present in the blood product can lead to severe toxicities like febrile reactions, allosensitization, increased chances of graft rejection in potential transplant recipients and transfusion associated graft versus host disease. Use of blood filters can prevent these to a large extent; however tagvhd can be only prevented by using irradiated blood products. The most important is to prevent unnecessary prescriptions of blood products and that is possible if the center has written policies which are strictly followed by the clinicians and by in-built audit.

Keywords
Blood components, pediatric, guidelines, packed red blood cells, platelets, fresh frozen plasma, leukodepletion, IAP guidelines.

INTRODUCTION

Availability of blood components has improved the outcome of children with malignancies and those in intensive care set up. It has changed the main focus in cancer therapy complications from bleeding to infections. Blood components now allow administration of high dose chemotherapy which has changed the outcome in some pediatric cancers.

Child is not a miniature adult and so also a newborn is not a miniature child. There are major differences between an adult and a child in the etiology of cytopenias, the effect of cytopenia on the homeostasis, the physiological responses by the body to the cytopenia, the need of various blood components, the choice and the dose of the blood component used. This is even more true for a newborn. Accordingly the guidelines for the use of blood components differ in a child than that for an adult and in a newborn as compared to a child. Various recent publications are available which define the guidelines for the use of blood components specific for children and newborns.

Why not Whole Blood and why Components?

Each unit of whole blood has at least four basic components which include red blood cells, white blood cells, platelets and plasma. Each of these components has specialized function. All these functions are not deranged in all the patients and hence all the components are not required all the time. Blood is always in short supply and making components from one unit of whole blood will satisfy the

needs of more than one patient from the same unit of blood. Besides, giving whole blood can lead to harmful effects like plasma overload; lymphocytes mediated toxicities or allosensitization, etc. Some components can only be given effectively as component, e.g. platelets, which are otherwise, destroyed in refrigerated stored whole blood. Some components are better given as component e.g. clotting factors as one can not achieve effective levels by using FFP alone. It is a social crime to use whole blood and waste this rare commodity!

Which Components?

From one unit of unrefrigerated whole blood one can make packed red blood cells, platelet pack (random donor platelet), granulocytes pack and fresh plasma. Fresh plasma can be further frozen at –30°C and be used as FFP in future. Pooled plasma can be converted into further components like cryoprecipitate, albumin, gamma globulins, anti-D globulins, plasma proteins, etc. One can modify and manipulate these components and obtain neocyte red cells, frozen red cells, washed red cells or platelets, filtered red cells or platelets, UV light or gamma irradiated red cells or platelets. One can select a specific donor and get CMV negative blood components, HLA matched blood components or blood products from specific minor blood group compatible donor. Lastly one can get stem cells from the umbilical cord blood of a newborn or peripheral blood of an older child for autologous or allogenic bone marrow transplant or rescue as the case may be.

Storage and Shelf Life

Whole blood is stored at 1 to 4°C. Shelf life will depend upon the type of anticoagulant and additive used. ACD is no more used. CPD or CP-2D blood can be kept for 21 days. CPDA1-A2 blood can be kept for 35 days. If one uses additives like Nutrisol or Adsol, one can keep the blood for 42 days. Packed red blood cell is stored at 1 to 40°C and should be used within 24 hrs if packed using open system. Platelets are stored at 20 to 22°C and that too on a constant agitator as resting platelets tend to aggregate. The shelf life is 3 to 7 days. Granulocytes are kept at room temperature and should be used within 24 hours of collection. FFP and cryoprecipitate have shelf life of one year and are stored at –30°C. Frozen red blood cells can be kept at –70°C and have shelf life of 5 to 7 years.

ABO and Rh Compatibility

Tables 1 and 2 describe the choice of the ABO and Rh type of the donor blood component in various recipient ABO and Rh settings.

Table 1 Choice of ABO blood group of donor components in children

Patient's ABO group	*Donor ABO group*		
	Red cells	*Platelets*	*FFP*[Δ]
O			
First choice	O	O	O
Second choice	-	A	A or B or AB
A			
First choice	A	A	A or AB
Second choice	O*	O*	
B			
First choice	B	B[#]	B or AB
Second choice	O*	A or O*	
AB			
First choice	AB	AB[#]	AB
Second choice	A or B	A	A
Third choice	O*		

* Group O component without high anti-A or anti-B titers should be selected.

[#] Platelet concentrates of B or AB group may not be easily available.

[Δ] Group O FFP should be given only to O group patients and no one else. AB group FFP may not be easily available.

Table 2 Choice of Rh blood group of donor components in children

Patient's Rh group	*Donor Rh group*		
	Red cells	*Platelets*	*FFP*[Δ]
Rh positive			
First choice	Rh +ve	Rh +ve	Rh +ve
Second choice	Rh –ve	Rh –ve	Rh –ve
Rh negative			
First choice	Rh –ve	Rh –ve	Rh –ve
Second choice	-	Rh +ve*	Rh +ve

* If Rh +ve platelets are given to an Rh negative recipient, anti-D globulin in the dose of 250 mcg should be given to the recipient, which will cover up to 5 platelet transfusion for up to next 6 weeks.

[Δ] FFP usually are not labeled as Rh positive or negative.

Whole Blood

Whole blood has all the components, but that is only in the first 6 to 8 hours that too when stored at room temperature. The platelets are the first to disappear in the first 4 to 48 hours, the labile clotting factors V and VII are the next to disappear and the other clotting factors go down thereafter. On prolonged storage the potassium levels go up whereas

the pH, the 2-3-DGP levels and the ATP levels fall. Hence for the exchange transfusion one prefers to use less than 7 days old blood. Whole blood is stored at 1 to 4°C and has a shelf life of 21 to 42 days as discussed before.

Indications

Whole blood is used only when massive transfusions are required like in exchange transfusion, massive blood loss with at least one volume blood transfused or during extracorporeal membrane oxygenation (ECMO). One can use reconstituted whole blood and one should remember that there is nothing like 'fresh' blood! 10 cc/Kg body weight of whole blood will raise HCT by 5 percent and Hb by 1 to 1.5 gm percent.

Packed Red Blood Cells

Packed red blood cells (PRBC) contains packed red cells in 22 to 50 percent of original plasma. Ideal HCT for PRBC is 70 to 75 percent and it should not be too tightly packed. For newborns, while doing exchange transfusion, HCT can be adjusted to 50 to 55 percent using additional FFP or albumin. PRBC is used for improving oxygen carrying capacity as well as volume expander. Advantage of PRBC is that it is low volume as compared to whole blood and hence does not lead to circulatory overload. It has less plasma and hence has less citrate related toxicity. It is mainly used in patients with hemorrhage or chronic anemia needing recurrent transfusions. However it contains significant amount of plasma and leukocytes to lead to toxicities related to them like allergic reactions, Nonhemolytic febrile transfusion reactions (NHFTR), allosensitization, Graft-versus-host disease (GVHD), etc. Full cross-match for ABO and Rh and screening for abnormal antibody should be done before each transfusion. 10 cc/Kg. Body weight of PRBC will raise the HCT by 10 percent and Hb by 3 to 4 gm percent.

Indications

The 'cut offs' used in various indications are shown in Table 3. It is used for replacement of volume as well as oxygen carrying capacity. It is used in acute hemorrhage where more than 15 to 20 percent blood volume is lost, monitoring vitals, blood pressure and CVP. The most common indication of PRBC is chronic transfusion dependent anemia as seen in thalassemia, sickle cell disease, congenital dyserythropoietic anemia, Diamond Blackfan syndrome, Fanconi's anemia, aplastic anemia, chronic renal failure, cancer patients, sideroblastic anemia, etc. It is also useful in episodic transfusions for acute hemolysis like in G6PD deficiency, malaria, autoimmune hemolytic anemia, etc. It is rarely, if at all, used in nutritional anemia, if patient has severe anemia with impending cardiac failure or has associated cardiorespiratory disease. Lastly it can be used before surgery, where patient is anemic with Hb less than 7 gm% and where moderate blood loss is expected during surgery.

Table 3 Indications of using PRBC in a > 4-month-old child

•	Acute blood loss of >15–20% blood volume with hypovolemia
•	Hb <8 gm% with
	– Symptomatic perioperative anemia
	– Chronic congenital/acquired transfusion dependent anemia
	– Emergency surgery with anticipated blood loss
	– Uncorrectable preoperative anemia
	– Severe infections
	– Associated severe pulmonary disease
•	Chronic transfusion dependent states, e.g.
	– Thalassemia
	– Other hemoglobinopathies
	– Bone marrow failure syndrome including Fanconi's anemia
•	Pediatric oncology
	– Hb <8 gm% with chemotherapy/radiotherapy
	– Hb <10 gm% if
	i. Intensive chemotherapy planned
	ii. Presence of febrile neutropenia
	iii. Severe lower respiratory tract infection
	iv. Thrombocytopenic bleeding
	– Hyperleukocytosis (partial exchange preferred)

It is most often misused as "top-up" in patients with nutritional anemia, or during surgery to keep Hb above "10 gm%". In such cases, it is counterproductive as it can lead to immune suppression of the recipient and delay healing.

Chronic Anemia

Special precautions are required while transfusing patients with transfusion dependant states like thalassemia. Ideally detailed blood grouping of the recipient should be done before the first transfusion so that in future one can use a specific donor if the patient develops intolerance to some minor blood group antigen. Always use Coombs' cross matched, triple saline washed PRBC in the dose of 15 cc/Kg which will raise the Hb by 3 to 4 gm%. Maintain the pre-transfusion Hb above 9.5 to 10 gm% and raise the post Hb to around 12 to 14 gm%. Keep the record of the pre- and post-transfusion Hb levels and the volume transfused every time so that one can calculate and keep

a watch on the yearly requirements. If affordable, use a WBC filter which will help reduce the nonhemolytic febrile transfusion reactions, allosensitization, etc.

Platelet Transfusions

Types of Platelets

There are two types of platelets, random donor platelet (RDP) obtained by centrifugation of a unit of whole blood, and single donor platelet (SDP) obtained by apheresis. One can use a HLA matched or CMV negative donor in specific situation. Use of WBC filters helps reduce leukocytes and hence allosensitization and febrile reactions.

Random donor platelet

Random donor platelet (RDP) is obtained by centrifugation of a unit of whole blood within 6 to 8 hours of collection. Collected unit of whole blood must be stored at room temperature till centrifugation as otherwise platelets will lose their function if stored in refrigerator. The blood bag is centrifuged first at 200 RPM for 2 to 3 minutes which will separate the whole blood in to PRBC at the top, WBCs in the buffy coat and platelet rich plasma at the bottom. The PRBC is siphoned out in another satellite bag and platelet rich plasma is then spun at 5000 RPM for another 2 to 5 minutes which will leave platelet poor plasma at the top and platelets button at the bottom. Platelet poor plasma is then siphoned out in another satellite bag and what remains is one unit of RDP.

One unit RDP of contains 5 to 6 $\times 10^{10}$ platelets in 50 to 60 mL of plasma, trace to 05 mL of RBCs or RBS stroma, and up to 10^8 leukocytes. One unit of RDP per 10 kg body weight will raise the platelet count by 20,000 to 30,000/cumm. RDP is less costly and easily available from the blood bank shelf however it is less efficacious than SDP as it contains 6 to 7 times less number of platelets. Hence patients needing repeated platelet transfusions may benefit by using SDP which will reduce the exposure to a fewer donors. This will also reduce chances of allosensitization which will reduce future platelet refractoriness as well as reduce chances of rejection in case of future stem cell transplant.

Single donor platelet

Single donor platelet (SDP) is obtained from a designate single donor using cell separator or apheresis machine. Compatible donor is screened for fitness and serology and once found to be fit is subjected to continuous or discontinuous apheresis and platelets are collected over 4 to 6 hours. SDP contains 2 to 3 $\times 10^{11}$ platelets in 250 to 300 mL of plasma, up to 5 mL of RBCs and 10^6 to 10^9 leukocytes. Thus it has 6 to 7 times more platelets than RDP (and also that much more volume). The donor should be healthy, off medicines like aspirin and should have platelet count of more than 1.5 lakhs/cumm. One can use same donor again after 2 to 3 weeks. One can select specific donor like CMV negative or HLA matched donor. But SDP is extremely costly and needs sophisticated cell separator. Also it is not a product available on the blood bank shelf and has to be preplanned.

ABO/Rh Compatibility

Whenever possible use ABO and Rh identical platelets. If not available, use ABO and Rh compatible donor. This is shown in Tables 1 and 2. Platelets though are red cells free can contain some RBCs or RBC stroma enough to lead to Rh sensitization. Hence Rh positive platelets are given to Rh negative patient only in emergency and in such cases one has to give 250 μg of anti-D globulin to recipient to prevent Rh sensitization, especially in female recipient.

Storage

Platelets are thermosensitive and become dysfunctional if stored at temperature below 20 to 22°C. Hence unlike all other blood component, platelets are not stored in refrigerator but are stored at 22°C. Shelf life of platelets is up to 5 days. Platelets have a natural tendency to aggregate when left standing still making them lose their function. Hence they need to be stored on a constant agitator. Transport the platelet quickly and infuse the same in 20-30 minutes. Again do not leave platelets in a tray lying still while awaiting transfusion. Caretaker should be told to shake gently the platelet bags periodically to prevent aggregation. Use plastic tubes and never use glassware as platelet will stick to the glass surface and get activated. Remember, platelets should never be stored in a refrigerator! In case they are put in a refrigerator, they should be discarded.

Criteria to Transfuse

Platelet transfusions are usually given to those with thrombocytopenia due to decreased production than to those with increased destruction. Platelet transfusions are given when they have significant mucosal bleeds. Only skin bleeds do not warrant platelet transfusion, but such patients should be closely monitored for any further mucosal bleeds.

It is controversial as to when to give prophylactic platelet transfusion. Child with thrombocytopenia usually does not bleed spontaneously unless the platelet count falls less than 50,000/cumm. The chances of spontaneous bleeds increase when the count drops to less than 5000 to 10,000/cumm. Hence the decision when to transfuse platelets prophylactically is based on basic disease, type of thrombocytopenia, platelet count, and presence of associated coagulation abnormalities. A well child may

be given prophylactic transfusion when the platelet count is less than 5,000-10,000/cumm. In patients with massive hemorrhage it should be given when the count is less than 50,000/cumm as most of the circulating platelets are likely to be non-functional platelets of the infused stored blood. The 'cut off' used in various indication is shown in Tables 4 and 5.

Indications

Platelet transfusions are given for thrombocytopenia or for platelet dysfunction.

1. *Decreased platelet production:* This is seen when bone marrow failure occurs like in aplastic anemia, Fanconi's anemia, thrombocytopenia with absent radius (TAR) syndrome, and other constitutional hypoplastic anemia. It is also seen when the bone marrow is infiltrated, e.g. in leukemia and other metastatic cancers or in presence of bone marrow suppression due to chemoradiotherapy or fulminant infections. Platelet transfusions have revolutionized the treatment and the outcome of pediatric cancers. The cause of mortality has shifted from bleeding to infections with better platelet support available now.
2. *Increased consumption of platelets:* It is indicated in disseminated intravascular coagulation (DIC), necrotizing enterocolitis (NEC), and Kasabach-Merritt syndrome. In these cases, there is good platelet recovery at one hour after transfusion, but not at 24 hours suggesting consumption. In cases with DIC, frequent estimation of the platelet count and coagulation screening tests should be carried out. There is no consensus on a target platelet count, but aim is to maintain the platelet count > 50,000 as in massive blood loss, would seem to be reasonable practice. In chronic DIC, or in the absence of bleeding, platelet transfusions should not be given merely to correct a low platelet count. Platelets are contraindicated in thrombotic thrombocytopenic purpura (TTP) and hemolytic uremic syndrome (HUS).
3. *Massive transfusions:* There is consensus that the platelet count should not be allowed to fall below 50,000 in patients with acute bleeding. A higher target level of 1,00,000 has been recommended for those with multiple trauma or CNS injury.

Table 4 Indications of using platelets in a >4-month-old child with thrombocytopenia

•	Prophylactic platelets (without bleeding)
	– <5–10,000/cumm in a nonsick child
	– <20,000/cumm in a sick child with
	i. Severe mucositis
	ii. Disseminated intravascular coagulation (DIC)
	iii. Platelet likely to fall <10,000/cumm before next evaluation
	iv. Thrombocytopenic bleeding
	– Hyperleukocytosis (Partial exchange preferred)
	i. Bone marrow aspiration/biopsy can be without platelet support
	ii. Lumbar puncture <30,000/cumm
	iii. Other surgeries <50,000/cumm
	iv. Surgery at critical sites like CNS, eyes <100,000/cumm
	– <50,000/cumm with acute bleeding, massive hemorrhage, head trauma, multiple trauma
•	Chronic stable thrombocytopenia only in presence of significant mucosal bleeding
•	Platelet dysfunction only in presence of significant mucosal bleeding
•	Chronic stable DIC only in presence of significant mucosal bleeding

Table 5 Platelet transfusion guidelines for neonates

A	Prophylactic platelet transfusion (nonbleeding) in the neonate:
	a. Stable preterm neonate with platelet count <20,000
	b. Stable term neonate with platelet count <10,000
	c. Sick* preterm neonate with platelet count <30,000
	d. Sick* term neonate with platelet count <20,000
	e. Preparation for invasive procedure—lumbar puncture or minor surgery (central line insertion) with platelet count <50,000
	f. Major surgery with platelet count <100,000
B	Platelet transfusion in neonate with clinically significant bleeding:
	a. Neonate with platelet count <50,000
	b. Neonate with condition that increases bleeding, e.g. DIC and platelet count <100,000
	c. Neonate with documented platelet function disorder irrespective of circulating platelet count
	d. Bleeding neonate meeting the clinical or laboratory criteria for DIC, at discretion of treating physician
	*Definition of a sick neonate:
	a. Cardiovascular instability, HR >180/min or dopamine or ionotropic infusion at >3 micrograms/kg/min b. Respiratory instability, FiO_2 requirements >0.4 or significant mechanical ventilation MAP>7 c. Central nervous system instability—within 72 hrs of seizure
A specific threshold for transfusion may not be appropriate for patients with chronic stable thrombocytopenia who are best managed on an individual basis depending on the degree of hemorrhage	

4. *Increased platelet destruction:* It can occur due to immune or nonimmune mechanisms. Non-immune destruction can occur following drugs or infections. Immune destruction can occur in post-transfusion purpura, autoimmune diseases, idiopathic thrombocytopenic purpura (ITP), and alloimmune disease of newborn. The ITP is the most common scenario in this category. Platelet transfusions are generally not effective in this group of diseases, as they will be immediately destroyed by the antibody present in recipient after transfusion. Platelet transfusions should be reserved for patients with life-threatening bleeding from the gastrointestinal or genitourinary tracts, into the central nervous system or other sites associated with severe thrombocytopenia. A large number of platelet concentrates may be required to achieve hemostasis as a result of reduced survival of the transfused platelets. Therapies such as intravenous methylprednisolone and immunoglobulin should be given at the same time to maximize the chances of stopping the hemorrhage and raising the platelet count.
5. *Hypersplenism:* Normally 1/3rd of platelets are pooled in the spleen. This proportion will increase in patients with hypersplenism due to any reason. Again platelet transfusions may not be effective in such cases, as they will be immediately removed from the circulation into the enlarged spleen.
6. *Dilutional:* Dilutional thrombocytopenia can occur following massive transfusions in patients with massive hemorrhage or following exchange transfusions. Supplemental platelet transfusions may be required in such cases to keep platelet counts of > 50,000/cumm.
7. *Platelet dysfunction:* Various congenital and acquired platelet functional disorders may present with significant bleeding. If local measures fail to control bleeding, platelet transfusions will be required. One should use platelets sparingly in such cases as allosensitization may prevent good recovery in future after a number of transfusions are given. One can use HLA matched platelets in such cases. Table 6 shows the measures to be undertaken in a case of platelet dysfunction with clinical bleeding.

Platelet transfusion efficacy: One unit of RDP per 10 kg body weight increases platelet count by 20,000 to 30,000/cumm. SDP is 5 to 7 times more effective than RDP. The efficacy of platelet transfusion depends upon various factors. Platelet factors like source of platelets, type of platelets, storage, collection and administration will affect the efficacy. Similarly, factors in recipient that affect the efficacy include pretransfusion count, fever, sepsis, size of liver and spleen, presence of antibodies or consumption coagulopathy and drugs taken by the recipient.

Table 6 Measures to be undertaken in a patient with platelet dysfunction with clinical bleeding

•	Avoid/withdraw drugs known to have antiplatelet activities
•	Correct baseline condition known to lead to platelet dysfunction
•	Correct HCT to >33% (with transfusion/EPO)
•	Use of DDAVP in a case of storage pool disorder
•	Use of alternate therapy like factor VIIa
•	Use of platelets when above measures are ineffective or inappropriate
•	Consider platelet refractoriness after repeated use of platelets

Clinically one can judge the efficacy by seeing the cessation of bleeding. One can look for the expected increments by calculating corrected count increment (CCI) ($\times 10^9$/L) as follows by doing platelet count at one hour and 24 hr after transfusion.

$$\text{CCI} = \frac{\text{Post-transfusion platelet count-Pretransfusion platelet count}}{\text{Platelets infused} \times 10^{11}} \times \text{BSA m}^2$$

Normal CCI is > 7.5×10^9/L at one hour, and > 4.5×10^9/L at 20 to 24 hrs. If CCI is normal at one hour, but less at 24 hrs, it suggests consumption coagulopathy. If CCI is less at 1 hour itself, it suggests immune destruction.

Side effects: Both RDP and SDP contain platelets, small volume of plasma, WBCs and some RBCs or RBC stroma. Hence, platelets can lead to similar reactions like other blood components including febrile reactions, urticaria, allosensitization, transfusion associated infections and rarely anaphylaxis. In a newborn, antibodies present in the small volume of plasma contained in platelets may be enough to lead to significant hemolysis if there is a mismatch of blood groups between donor and recipient. Hence it may be better to use a donor with low titer anti-A or anti-B antibodies in case of a mismatch. If not possible one can use washed platelets as saline washing will remove the antibodies to a large extent. In case of allosensitization patient will become refractory to further platelet transfusions with poor recovery of platelet counts in spite of adequate dose. Allosensitization can also increase the chances of graft rejection in case of future stem cell transplant. Such patients may benefit by using immune suppression like low dose steroids, plasmapheresis or using WBC filters while transfusing platelets. Of course one can avoid many of these complications by using WBC filters with every platelet transfusion right from 1st transfusion

in patients who are likely to need repeated platelet transfusions.

Granulocytes

Though its use in infections may sound logical, granulocytes are rarely used in current clinical practice. People have tried giving granulocyte transfusion in patients with severe uncontrollable infection in presence of congenital or acquired neutropenia or neutrophil dysfunction. It is usually reserved for neutropenic patients with fulminant sepsis not controlled by antibiotics and antifungal with ANC < 300 in newborn, ANC < 100 in infants and ANC < 500 in immune compromised host. It should always be used along with antibiotics and antifungals. As colony stimulating factors are now easily available and affordable, use of granulocytes has fallen in to disrepute.

Buffy coat preparations are not very satisfactory as the cells tend to become nonfunctional. Packs obtained by apheresis are the best. They should be used within 24 hr of collection and stored at room temperature. Each pack has 10^{11} granulocytes in 200 cc of plasma. Dose recommended is 10^9 granulocytes/kg each time. It can be repeated every 12-24 hr for 4 to 6 days. It should be given obviously without using the WBC filter. It leads to all the side-effected related to plasma and lymphocytes. One should use ABO/Rh compatible donor.

Leukodepleted Blood Components

Why Leukodepletion?

Various side effects and toxicities are associated with the presence of significant number of donor lymphocytes in the unit of blood component transfused. These include non-hemolytic febrile transfusion reactions; allosensitization; increased chances of rejection of graft in candidates for future transplant; lymphocyte mediated lung toxicity like acute respiratory distress syndrome (ARDS); transmission of viral infections like HIV, human T-lymphocyte virus (HTVL), Epstein-Barr virus (EBV), cytomegalovirus (CMV), etc. which are intracellular pathogens; transfusion associated graft vs host disease (TAGVHD) in immune compromised patients and in transfusion from first degree relatives; and immune suppression of the recipient especially in surgical patients. These donor lymphocytes ordinarily do not serve any beneficial effects and hence should be removed or depleted from the unit transfused to eliminate or reduce the chances of these side effects and toxicities.

Non-hemolytic febrile transfusion reactions (NHFTR) occur when > 5×10^6 lymphocytes are present in the unit, whereas for TAGVHD it is > 10^7 cells/kg body weight. One pack of packed red blood cells (PRBC) has 10^9 WBC, RDP has 4 to 6×10^7 WBC, SDP has 2 to 4×10^8 WBC and granulocyte pack has 10^{11} WBC. Ideally all the transfusion should be leukodepleted especially in patients needing recurrent transfusions and in immunno compromised hosts.

Methods of leukodepletion: There are various ways of leukodepletion. Each method has its own merits and demerits and efficacy.

1. *WBC filter:* Third generation WBC filters are 99.5 percent efficient in removing the donor lymphocytes. Activated lymphocytes can release cytokines like IL2, TNF-α during storage and hence it is best to remove the lymphocytes while collecting blood from the donor using in-line WBC filter, rather than using the WBC filter at bedside while giving the transfusion to the recipient. The advantage of WBC filter is its high efficacy and simplicity to use. The disadvantages include its high cost and inability to prevent TAGVHD. Each filter costs ₹ 400-500/- and is not reusable. Ideally all transfusions should be given using filters especially if patient needs recurrent transfusions and develops NHFTR.
2. *Washed cells:* 90 percent of lymphocytes and 99 percent of plasma are removed by washing the PRBC with saline or blood processor. This will help reduce NHFTR, allosensitization and other toxicities related to WBC as well as allergic reactions to plasma proteins. It is a simple technique and needs cold centrifuge but is not as effective as the WBC filter for leukodepletion. One can combine washing and use of WBC filter where the patient is prone to severe allergic reactions. Washing does not prevent TAGVHD. Lastly, washed platelets from mother are given in a baby suffering from alloimmune thrombocytopenia.
3. *Gamma irradiation:* TAGVHD can be only prevented by gamma irradiating the blood. Dosage of 2500-3500 cGy are used to irradiate the components. The only disadvantage is need for the sophisticated and expensive irradiator. There are chances of membrane leak from the irradiated cells which can result in to increased potassium levels. Hence blood should be irradiated just before infusion or else supernatant plasma should be removed before transfusion.

   Ideally all blood should be irradiated where there is risk of TAGVHD. This includes transfusion given to newborn especially preterms < 1200 g, intrauterine transfusions, patient with primary or secondary immunodeficiency, cancer patients, organ transplant recipients and transfusion given to normal person from a first degree relative donor.
4. *Frozen cells:* This is routinely available in the west but is rarely available in India. RBC frozen at –70°C has shelf life of 5 to 7 years. While freezing, deglycerolization is done to prevent intracellular ice formation. It should

be thawed gradually and once thawed should be used within 24 hours. The efficacy for leukodepletion is 90 percent and plasma depletion is 99 percent. Hence it reduces toxicities related to both lymphocytes and plasma. Advantage of frozen cells is its availability in emergency where one can use O–ve frozen cells in AB negative plasma. One can collect blood from CMV negative donors; HLA matched donor or rare blood group donor and freeze it for future use. Lastly autologous blood collected for surgery can be frozen and used in future if surgery gets postponed for some reasons. Disadvantage of frozen cell is that it needs sophisticated instruments to prepare and store it and is extremely costly. It cannot prevent TAGVHD.

Fresh Frozen Plasma

Fresh frozen plasma (FFP) is made by freezing the plasma obtained at the end of centrifugation of the whole blood unit and is stored at <–30° C. The shelf life of FFP is one year when properly stored. It should be thawed at 37°C over 30 minutes in the water bath. Thawed FFP should be used within 4 hours if used for hemophilia A or used within 24 hours if used for other conditions provided it is stored properly. The FFP contains all the plasma proteins including albumin, gamma globulins and most important clotting factors. As labile factor V and VIII tend to decrease on storage, freezing of the plasma should be done within 4 to 6 hours of collection to prevent loss of these factors. One unit of FFP has 200 to 250 mL of plasma and 1 mL of plasma contains approximately 1 unit of each clotting factor. The hemostatic content of a unit of FFP is shown in Table 7. As the maximum tolerated dose of FFP is 10 to 15 cc/kg every 12 hr, one can not achieve very high plasma level of the missing clotting factors without volume overloading the patient.

The FFP is often misused as volume expander. As FFP can lead to allergic reactions, anaphylaxis in Ig A deficient patient and can transmit all the plasma borne infections, albumin should be used as a volume expander which is much safer. Similarly albumin and not FFP should be used to replace proteins or albumin. If patient needs both volume expansion as well as clotting factors like in DIC, sepsis, NEC, etc. one can use FFP. However FFP should not be used in a case of DIC without clinical bleeding. FFP should also not be used prophylactically to prevent intracranial bleeding in neonate.

Table 8 summarizes the indications of using FFP in clinical practice. FFP is mainly used to replace clotting factors. It can be given when the patient presents with bleeding for the first time where the diagnosis is uncertain as to which factor is deficient. In known cases of hemophilia, it is better to use factor concentrates, as they are more efficient and safe. FFP is used for deficiencies of other factors like factor V, VII, etc. where factor concentrates are not available. It is also used where multiple factors need to be replaced as in case of hemorrhagic disease of newborn, liver disease, preterm with liver dysfunction, DIC, etc. FFP also contains AT III, protein C and protein S and hence is useful in the deficiency of these factors too like in the treatment of purpura fulminans; however in the west activated protein C concentrates are easily available. FFP is used for plasma exchange in patients with TTP or HUS. It can be used to reconstitute whole blood along with PRBC or to adjust HCT of PRBC for exchange transfusion in newborn. Lastly, FFP is useful to prevent and treat coagulopathy due to L-asperginase in cancer p atients.

FFP leads to all the side effects related to plasma like allergic reactions like urticaria, anaphylaxis, especially in IgA deficient patient and transmission of plasma borne infections to the recipient. In small babies, it can lead to hemolysis if it contains high levels of antibodies

Table 7 Hemostatic content of a unit of FFP

Fibrinogen	2.67 mg/mL
Factor II	80 IU/mL
Factor V	80 IU/mL
Factor VII	90 IU/mL
Factor VIII	92 IU/mL
Factor IX	100 IU/mL
Factor X	85 IU/mL
Factor XI	100 IU/mL
Factor XII	83 IU/mL
Factor XIII	100 IU/mL
AT III	100 IU/mL
VWF	80 IU/mL

Table 8 Indications of using FFP considered as appropriate

•	Inherited factor deficiency
	a. Patient with unknown clotting factor deficiency presenting for the first time
	b. Single clotting factor where factor concentrate is not available like factor V or XI
	c. Multiple clotting factor deficiency
•	DIC with clinical bleeding
•	Hemorrhagic disease of newborn
•	Liver disease with coagulopathy for prevention and control of bleeding
•	Dilutional coagulopathy as seen after massive transfusion (surgical patients) to maintain PT, aPTT to <1.5 time the control
•	Plasma exchange for TTP/HUS
•	Sick newborn with coagulopathy and bleeding

against recipient's blood group antigens. FFP has also been associated with rare but significant toxicities like transfusion related acute lung injury (TRALI).

Take Home Messages

1. It is a criminal waste to use whole blood in general as one unit of whole blood can satisfy the needs of more than one patient.
2. Whole blood is indicated only for massive blood loss and for exchange transfusion. There too one can use reconstituted whole blood.
3. Packed red blood cells can serve both the purposes, increasing the oxygen carrying capacity and volume expansion in acute blood loss.
4. Transfusions are rarely required if at all for patients with nutritional anemia.
5. Transfusions are inappropriate to raise Hb in patient with nonemergency surgery, as a 'top-up' after surgery and as a 'panacea' in a sick malnourished pale child.
6. Iatrogenic blood loses are the most common cause of anemia in a newborn. Minimize the investigations.
7. Thalassemics should ideally receive triple saline washed, Coombs' cross matched PRBC using a WBC filter.
8. Platelets are to be stored at 22°C on a constant agitator, to be used in 20 to 30 minutes and not to be kept in fridge at all. Do not use glassware while giving platelets.
9. Platelets are not indicated in immune causes of thrombocytopenia (like in ITP); and as also for prophylaxis in a case of chronic stable thrombocytopenia (like in aplastic anemia).
10. FFP is often misused as volume expander (in patient with shock), as a source of proteins (in preterms), as a source of known clotting factor 9 in a known case of Factor VIII deficiency where cryoprecipitate or factor concentrates are a better option.
11. FFP is indicated in multiple factor deficiency as seen in liver disease and DIC or as factor replacement in a patient with unknown factor deficiency coming for the first time, and for plasma exchange for TTP/HUS.
12. Leukodepleted blood products are better as the donor lymphocytes present in unit of blood product can lead to unnecessary and avoidable side effects.
13. WBC filters are an excellent method of leukodepletion and should be used more liberally.
14. One should avoid relatives as donors.

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CHAPTER 36

Nucleic Acid Amplification Testing

Anand Deshpande, Rajesh B Sawant

In today's modern healthcare, blood and blood component transfusions have a very large range of indications and are life-saving for the patients. However, with the increase in transfusions the risk of transfusion transmitted infections (TTIs) has also increased. A major challenge is to use screening assays with maximum sensitivity and specificity to make blood as safe as possible.

In India, the five tests mandatory by Food & Drugs Administration (FDA) for the donated blood units are HBsAg, HIV-Ab, HCV-Ab, VDRL and malarial parasites. Currently, testing methods carried out in India are based on serological assay detecting either antibodies or antigen. Naturally, they have a long window period as they basically detect the host immune response. The 'window period' is defined as the time period between the start of an infection to the earliest diagnostic detection. Shortening this window period has been the focus of attention in transfusion medicine for the last three to four decades.

Size of the Problem

Over two billion people worldwide are infected with HBV, which is the leading cause of liver disease. Of these, more than 350 million are chronically infected, with a higher risk for liver cancer and liver cirrhosis. Currently, available screening technologies are designed to detect core antibodies or surface antigens. However, these infection indicators do not appear until eight weeks after an infection. Thus HBV presents a higher residual risk of transmission by transfusion than HCV or HIV and the HBV infection window period is the real issue in the transfusion setting. Countries like India with a high prevalence of HBV have the highest risk of transfusion transmitted HBV and probably would be the most to gain from HBV NAT. In addition, worldwide 170 million people are infected with HCV and 40 million with HIV. These are a serious global concern.

Window Period and its Clinical Significance

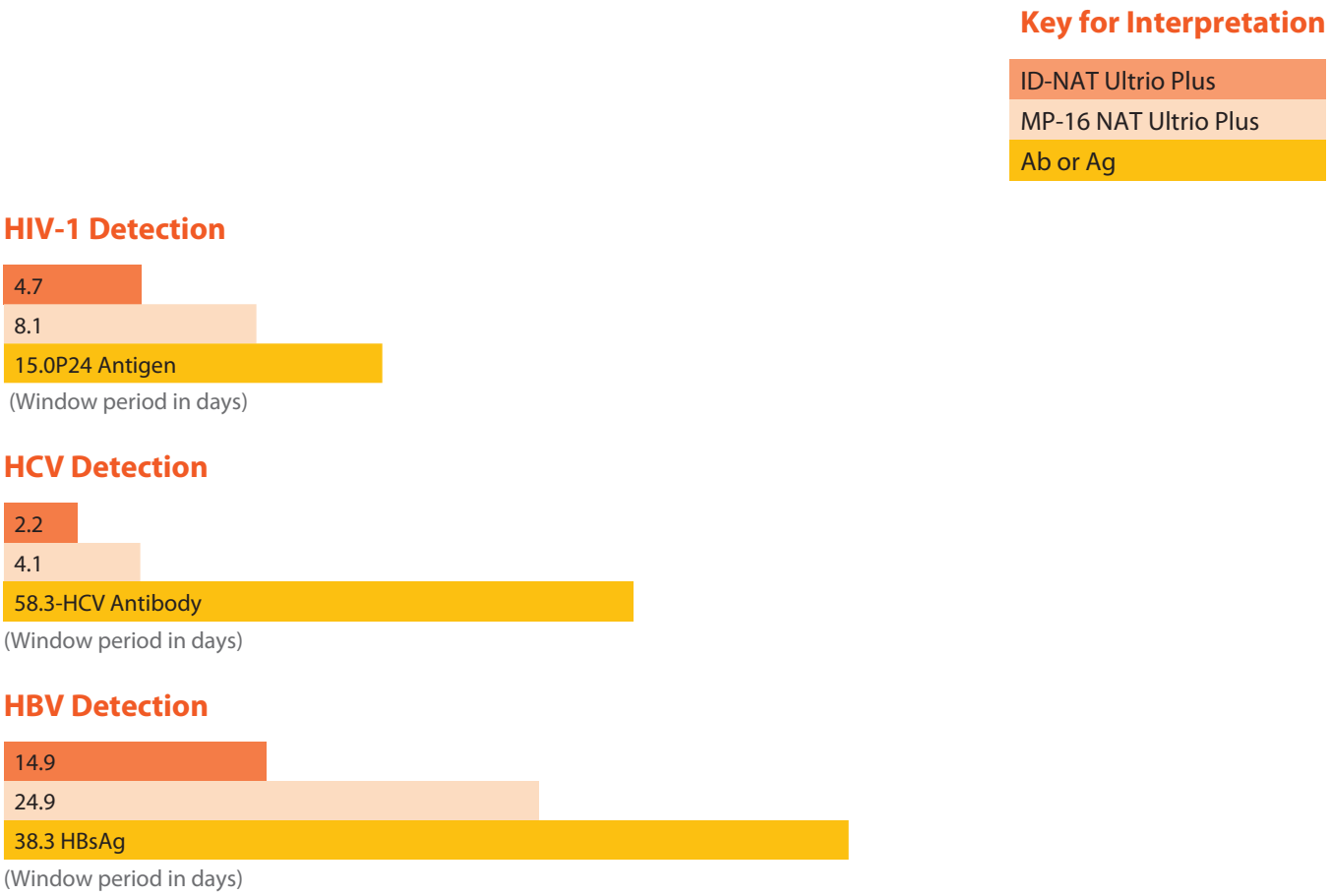
Blood is processed into blood components to enable more than one patient to benefit from a single donation. Thus, a single unit of blood collected from a donor in the window period of infection may be transfused to up to four recipients or may be added to pools of more than 1000 units to manufacture blood derived products.

The greatest threat to the safety of blood supply is donation by '*seronegative*' donors during 'window period' of initial infection and detectable seroconversion. Window period samples have a very low viral load. Detection of very low viral load samples requires highly sensitive assays.

Hence the Nucleic Acid Amplification Testing (NAT).....

With currently used ID-NAT assay the window period is shortened considerably, as it is a highly sensitive and specific test that detects very low levels of viral RNA or DNA that may be present in donated blood.

Window Period and Testing Technology: A Schematic Representation



Testing Options Available and their Principles

Genomic screening for infectious agents using NAT is performed with several *in vitro* nucleic acid amplification techniques, e.g. transcription–mediated amplification (TMA), polymerase chain reaction (PCR), ligase chain reaction and nucleic acid sequence-based amplification. All these techniques detect the presence of infectious microorganisms in donor blood by amplifying the nucleic acid sequences specific to the microorganism, giving it a much higher level of sensitivity and specificity than routine EIA test. Thus the power of NAT lies in its ability to detect viral genomic nucleic acids rather than the presence of antibodies. NAT screening is characterized by three critical processes: sample extraction, amplification and detection. NAT is used in addition to the antibody/antigen test since in some individuals theoretically the amount of virus may have fallen below detectable limits and antibodies could still be detectable as in case of HCV. In some cases with HBV again, the viral copies may be undetectable but the surface antigen is present. NAT screening can be carried out as a minipool testing or individual (ID-NAT) testing. Japan started in 1999 with a minipool of 500. All the countries currently use minipool (6/24/48/96 samples) or ID-NAT testing.

Key to interpretation

- *NAT-yield:* EIA negative, NAT positive
- *Sero-yield:* EIA positive, NAT negative

Impact of NAT: Evidence from Published Literature

A pilot project in India at Apollo Indraprastha Hospital, showed that out of 12,224 study samples 133 (1.09 %) were reactive by Ultrio assay. The 84 samples were seroreactive but NAT nonreactive. There were 8 NAT yield cases–1 HIV, 1 HIV-HCV coinfection and 6 HBV. Observed NAT yield for all three viral TTI's was 1 in 1528 (0.065 %).

However, NAT yield reported from various centers in India varies from 1 : 300 to approximately 1 : 8000 as reported at our center.

Implementation of NAT has led to a residual risk of transfusion transmitted infections of less than 1 : 1 million in case of HIV and HCV in developed countries.

Blood donor screening by NAT for at least HIV-1 and HCV has been implemented in different countries (e.g. USA, Canada, parts of Brazil, Spain, France, the UK, Denmark, Germany, the Netherlands, Belgium, Greece, Slovenia, the Czech Republic, South Africa, Ghana, Luxembourg, Switzerland, Italy, Japan, parts of China, Australia, Poland, Norway, Finland and New Zealand). One exception in Europe is Sweden. Based on the very low incidence of HIV-1 and HCV in their donor population, they decided to stop blood donor screening by NAT in 2008. In India, NAT testing is carried out for HIV, HCV and HBV also due to high prevalence of hepatitis B virus in the population.

Pathogens for which NAT Testing is Available

a. *HIV:* 1st case of HIV was reported in 1982. The virus doubling time is approximately 17 hours and the diagnostic window period is reduced to less than 5 days using ID-NAT.
b. *HCV:* The virus was first described in 1989 but it was known since 1970s that a virus other than HAV and HBV existed, it was called non-A, non-B hepatitis virus. The virus doubling time is very short (10–12 hours), and therefore the diagnostic window period has been brought down to approximately 4 days using ID NAT. In Germany, the residual TTI risk for HCV was estimated to be 1 : 200 and is currently calculated at 1 : 10.8 million.
c. *HBV:* Compared to HIV and HCV, the virus doubling time is very low (approx 2.56 days). Hence, with the current ID NAT assay the diagnostic window period has been brought down to 15 days compared to 55 days using EIA. Anti-HBc is used for blood donor screening in low endemic countries such as USA and Germany. This is not feasible in high endemic countries such as India because the percentage of anti-HBc reactive donors might cause an unacceptable loss of life-saving blood units.
d. The other viruses which can be detected using NAT are West Nile virus (WNV) infection (as in USA), hepatitis A virus infection, hepatitis E virus infection, parvovirus B_{19} virus infection, chikungunya virus infection.

HIV-2 has been found predominantly in West Africa, and cases have also been reported from India. Since EIA is always used in conjunction with NAT testing risk of finding HIV-2 yield is minimum and some NAT systems have already incorporated HIV-2 in the multiplex screening procedure.

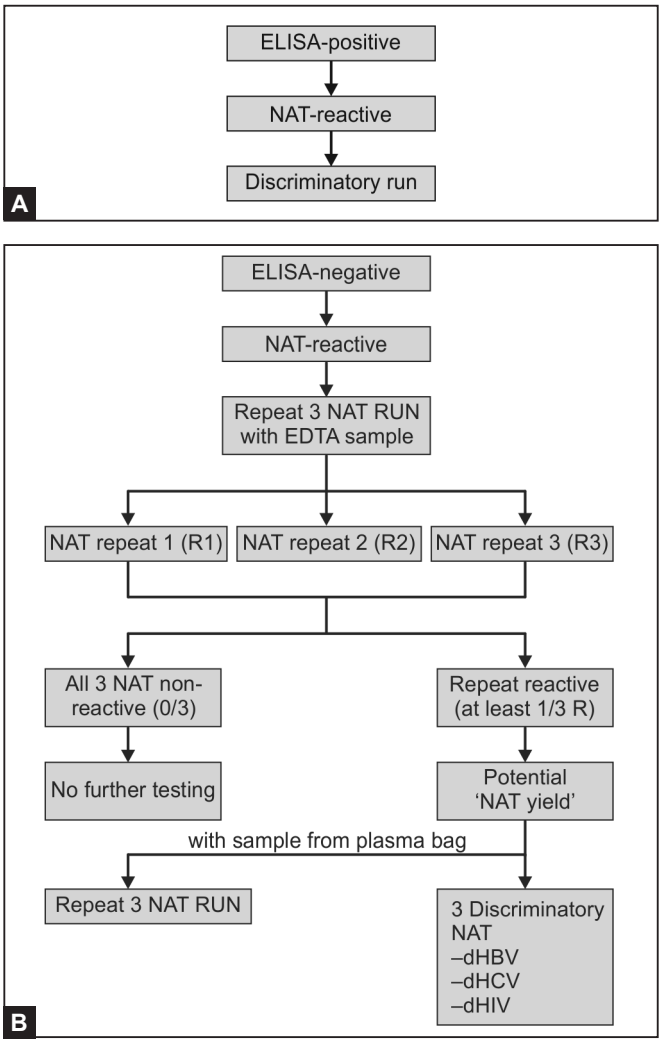
Bacterial screening poses a special challenge in transfusion medicine. With the introduction of NAT the risk of transmission of clinically relevant viral infection is far below the risk of bacterial infections. Many countries have implemented culture methods to detect very low levels of bacterial concentrations. NAT offers a good method to detect bacteria.

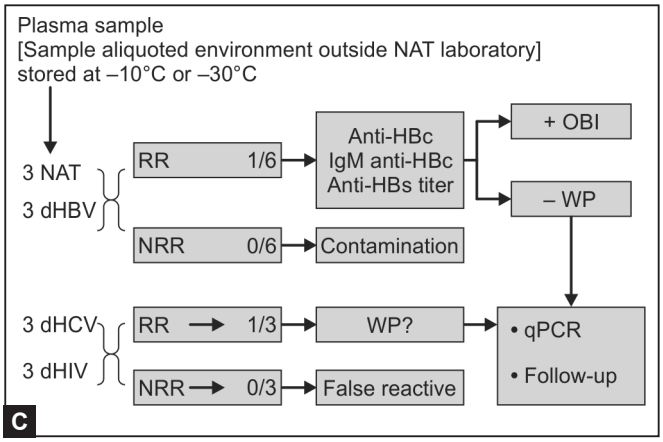
Practical Considerations

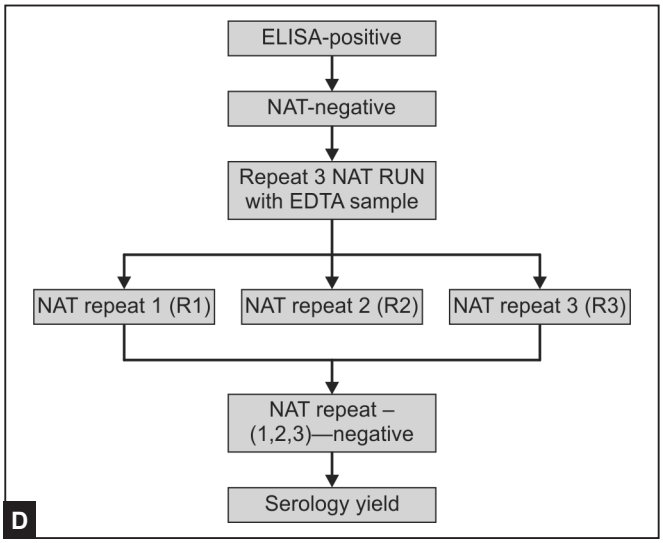
It should kept in mind that a very small number of blood donors may be infected with viral concentrations below the level of analytical sensitivity and therefore NAT can offer close to 100 percent but not 100 percent safety.

For proper interpretation of NAT results, in view of very low number of viral copies, based on Poisson distribution an algorithm was proposed in NAT users meet in India which is followed by many centers.

Proposed algorithm:







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CHAPTER 37

Transfusion Transmitted Infections

AP Dubey, Malobika Bhattacharya

Most deaths caused by blood transfusion worldwide are due to transfusion transmitted infections. The various agents (viruses, bacteria or protozoa) responsible share the following features: persistence in the donor's bloodstream giving rise to carrier states; a susceptible receptor population; the ability to cause asymptomatic infection; stability in stored blood and in many cases in plasma fractions. Infectious agents that are only present in blood cells, e.g. malarial parasite can be transmitted by all blood components except cell-free plasma. On the other hand, those viruses that are present in plasma, e.g. Hepatitis B, can be transmitted by cell-free plasma and its fractions as well as by cellular components. Screening tests are effective preventive measures but they cannot detect emerging agents such as HIV in the 1980s or West Nile fever at the beginning of this century. Presently in India, it is mandatory to test donated blood for hepatitis B and C, HIV 1 and 2, malarial parasites and syphilis.

The following sections review the various transfusion transmitted infections including their epidemiology, clinical features, management and preventive measures.

KNOWN TRANSFUSION TRANSMITTED VIRAL INFECTIONS

- Viral hepatitis
  - Hepatitis B
  - Hepatitis C
  - Hepatitis D
  - Hepatitis A
  - Hepatitis G
  - Transfusion transmitted virus (TTV) and SEN-V
- Retroviral infection
  - Human T-cell leukemia virus (HTLV) types 1 and 2
  - Human immunodeficiency virus (HIV) types 1 and 2
- Human herpes virus infection
  - Cytomegalovirus (CMV)
  - Transfusion transmitted cytomegalovirus (TT-CMV)
  - Epstein-Barr virus (EBV)
- Human herpes virus (HHV) 6 through 8.
- Parvovirus B19
- Bacterial infections
  - *Yersinia enterocolitica*
  - Other enterobacteriaceae
  - Psychrophilic pseudomonas.

Viral Hepatitis

Despite the dramatic reduction in the risk of viral transmission during the past three decades, viral hepatitis remains a serious complication of transfusions worldwide.

Hepatitis B

Hepatitis B virus (HBV) is a major human pathogen that causes acute and chronic hepatitis, cirrhosis and hepatocellular carcinoma.[1] The overall prevalence of HBV infection in the United States is about 5.6 percent as indicated by HBsAg and anti-HBc positivity rates.[2] The estimated prevalence of HBV in India is between 3 and 7 percent.[3] However, the risk of HBV infection in transfusion recipients is progressively decreasing with the use of sensitive screening tests, with an estimated risk of 1 per 205000 units in the USA.[4]

HBV is a double shelled DNA virus of the hepadnaviridae family. The virus contains various antigens that may help in distinguishing the duration of infection and infectivity of the host.

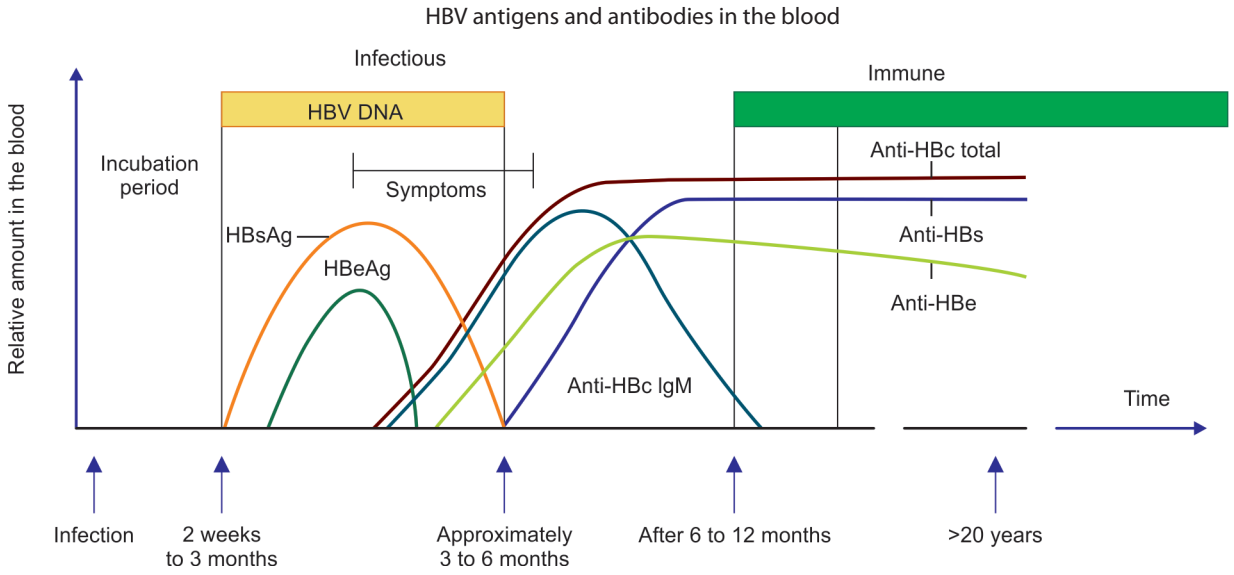

Fig. 1 Serological markers of HBV and their time of appearance during the course of infection

Hepatitis B virus is spread by transfusing infected blood, plasma or coagulation factor concentrates. The mean incubation period is 63 days (range 30–150 days) in post-transfusion hepatitis cases. Most cases of HBV infection are asymptomatic as evidenced by high carriage rate of serum markers in the absence of history of acute hepatitis. The prodrome is characterized by lethargy, malaise and anorexia and rise in liver transaminases 6 to 7 weeks after exposure. Some patients have a serum sickness-like illness during the prodrome phase. Jaundice is present in about 25 percent of the patients with onset about 8 weeks after exposure and lasts for 4 weeks.

Acute fulminant hepatitis with coagulopathy, encephalopathy and cerebral edema may occur with a mortality of 0.5 to 1 percent of all HBV infections.

Five percent develop chronic hepatitis,[5] which can lead to cirrhosis and primary hepatocellular carcinoma.

The carrier state develops most commonly after asymptomatic infection, especially if the infection is acquired during infancy.

Evaluation of an individual for HBV infection usually includes testing for serum HBsAg, antibody to HBsAg and IgM anti-HBc (antibody to hepatitis B core antigen) by enzyme immunoassays (EIA). Detection of IgM anti-HBc in serum is helpful in the diagnosis of HBV infection during "window period" prior to the appearance of HbsAg (Fig. 1). Moreover, it can detect recent HBV infection in rare HBV mutants with altered HBsAg epitopes. Although most blood centers perform screening for HBsAg, there is a convincing argument to augment it with anti-HBc testing.[6]

Hepatitis B vaccine and hepatitis B immunoglobulin (HBIG) are available for prevention of HBV infection. Universal immunization of all children with hepatitis B vaccine is recommended in both pre- and postexposure situations and provides long-term immunity. The HBIG is recommended in neonates born to HbsAg positive mothers and children with intimate contact with acute HBV infection.

Interferon-α-2b (IFN-α-2b) and lamivudine are the current two therapies available for chronic HBV infection. Long-term eradication rates of 25 percent have been reported in children. Patients most likely to respond are those with low serum HBV DNA titers, HbeAg, active inflammation and recently acquired disease. Liver transplantation also has been used to treat patients with end-stage HBV infection.

Hepatitis C

Hepatitis C virus (HCV) has now been recognized as the cause of almost all parenterally transmitted cases of what was previously called non-A non-B hepatitis. The HCV is globally distributed with a remarkably uniform prevalence rate of 1 to 2 percent.[7] The epidemiology of HCV in India is not well described, more so in children. The prevalence of HCV in blood donors in India (1–1.5%) is higher than that in developed countries (0.3–0.7%).[8-10] A high prevalence of HCV is found in many high-risk groups exposed to blood or blood-products like hemophilics (24–90% anti-HCV positive), IV drug users (70–92% anti-HCV positive), patients with pediatric hematological malignancies (55% HCV-RNA positive) and those with thalassemia (60% anti-HCV positive).[8,11,12]

Hepatitis C virus (HCV) is a single-stranded RNA virus from the Flaviviridae family. The HCV is transmitted by blood components and blood products including IVIG, anti-D Ig for IV use and factor VIII concentrate. The HCV has never been transmitted by albumin concentrates or by anti-D Ig for IM use.

- Incubation period varies from 7 to 9 weeks.
- Acute infection tends to be mild and insidious in both adults and children.
- Only 25 percent cases are icteric.
- Fulminant liver failure rarely occurs.
- About 85 percent cases develop chronic hepatitis.
- After about 20 to 30 years 25 percent ultimately progress to cirrhosis, liver failure and occasionally primary hepatocellular carcinoma.

Detection of HCV infection is based on EIA for anti-HCV antibodies or testing directly for viral RNA or DNA. The recent risk estimate of HCV is 1:103,000 per donor exposure in the US.[13] This was calculated using second generation HCV test with window period of 82 days. Screening by third generation EIA reduces the window period to 66 days and hence further decreases the risk of transmitting HCV through transfusion to 1:127,000 units transfused.[14] With the implementation of nucleic acid technology-based HCV screening (HCV-NAT) there has been a major decline in the risk of HCV transmission to 1:3,68,000 units transfused in the US. The NAT testing for HCV has shown reduction in window period for HCV from 66 to 10–30 days.[14,15] A recent study in US has shown risk of HCV infection with mini pool-NAT screening to be as low as 1 in 2 million.[16] Though NAT can significantly improve the safety of blood supply; its widespread use in developing countries like India is unlikely in the near future due to the expenditure involved.

- No vaccine is available against HCV infection. Immunoglobulin has not been found to be effective in postexposure prophylaxis.

Combination therapy with IFN-α-2b and ribavirin has resulted in sustained response (defined as normal ALT levels and negative PCR results 6 months after completion of therapy) in one-third patients and is now considered first-line therapy. Monotherapy with IFN-α-2b resulted in sustained response in 10 to 15 percent of patients.

Hepatitis D

Hepatitis D virus (HDV) is a small satellite RNA virus, originally termed the delta virus that can infect only in the presence of concurrent HBV infection:

- Its genome codes a single peptide termed the delta antigen. The infectious form of HDV is coated byHbsAg.
- The incubation period in HDV superinfection is 2 to 8 weeks; with coinfection it is the same as HBV infection. The clinical outcome of HDV infection depends on the mode of infection.
- In coinfection, acute hepatitis, which is much more severe than that caused by HBV alone, is common but risk of developing chronic hepatitis is low. In superinfection, acute illness is rare and chronic hepatitis is common. However, the risk of fulminant hepatitis is highest in superinfection.
- The diagnosis is made by detecting IgM anti-HDV, which develop 2 to 4 weeks after coinfection and 10 weeks after superinfection.
- PCR assays for viral RNA are available only as research tools.
- There is no vaccine against HDV. However, HDV has little current relevance to transfusion safety, because measures used to detect and prevent HBV infection are also effective against HDV.

Hepatitis A

Hepatitis A virus (HAV) is a RNA virus belonging to the picornaviridae family. The incidence of HAV varies significantly with age. Highest incidence rates are seen in children in the age group of 5 to 15 years accounting for 30 percent of all cases. The HAV is rarely acquired by blood transfusion with a transfusion-associated risk of less than 1 per 1,000,000 units of blood transfused.[17] Rarity of parenteral transmission of HAV has been attributed to short duration of viremia, exclusion of infectious potential blood donors on the basis of history and absence of a chronic carrier state. However, rare transmission via blood products[18] and clotting factors[19] has been reported.

Currently, no specific laboratory screening of blood donations for HAV is performed, as there is no chronic carrier state. Two inactivated safe and effective vaccines are available with 100 percent immunity after a second dose. IVIG is recommended as pre-exposure prophylaxis in susceptible travelers visiting endemic regions and in selected situations for postexposure prophylaxis within 1 week of exposure.

Hepatitis G

Hepatitis G virus (HGV) is a recently discovered RNA virus distantly related to HCV (flavivirus). Clinical data derived from studies of HGV have established its transmission by blood through donor recipient linkages and by the recovery of virus in the recipient that was not present prior to transfusion.

- The HGV is present in 1 to 2 percent of donor population.
- Detection depends on PCR technology. As yet a causal relationship has not been established between

HGV infection and hepatitis or any other disease manifestation.[20]

- Currently no method is available to prevent HGV infection.

TTV and SEN-V

These viruses were separately identified among individuals with hepatitis and were also shown to be poorly, if at all, associated with hepatitis. They were readily transmitted by transfusion. At this stage, there is little evidence that this virus pair has any pathogenic potential.

Retroviral Infection

Prior to the outbreak of the acquired immunodeficiency syndrome (AIDS) epidemic in the early 1980s, retroviruses had been identified as a cause of rare malignancies but not a threat to transfusion recipients. Presently, the clinically significant transfusion transmitted retroviruses are the human immunodeficiency virus (HIV) types 1 and 2 and the human T-cell leukemia virus (HTLV) types 1 and 2.

HIV 1 and 2

Since AIDS was first described in the USA in 1981 in young, previously healthy, homosexual men, the disease has spread worldwide. At the end of 2003, an estimated 37.8 million people, 35.7 million adults and 2.1 million children younger than 15 years, were living with HIV/AIDS (UNAIDS 2004). Approximately two-thirds of these people (25.0 million) live in sub-Saharan Africa and 20 percent (7.4 million) in Asia and the Pacific. Between 2002 and 2004, an estimated 10 million people were infected with HIV and nearly 6 million died from AIDS.[21] The HIV seroprevalence in Indian scenario has been reported between 0.2 and 1 percent.[22] As per the 2006 NACO surveillance report, 3.8 percent of the total HIV cases are less than 15 years of age.

The HIV is a member of the family Retroviridae and belongs to the genus *Lentiviridae.* The genome is a single-stranded RNA. Because HIV is both cell-associated and present in the plasma, all blood components are potentially infectious. Albumin preparations, immunoglobulins, antithrombin III and hepatitis B vaccine have not been associated with HIV infection.

For the first few days after infection, no markers of HIV can be detected in blood, an interval known as the 'eclipse' phase. Viremia follows for a period of several weeks. This stage is followed by a 'ramp up' phase at about day 10 when HIV viral copy number rises rapidly.[23]

At about day 17, p24 antigen becomes detectable in serum and at about day 22, anti-HIV seroconversion occurs. During this phase more than 40 percent patients develop a flu-like illness.[24] After 1 to 2 months of transfusion transmitted infection, more than 95 percent of HIV infected patients exhibit a wide range of antibodies to structural *env, gag* and *pol* viral proteins.[25] As soon as anti-p24 develops, p24 antigenemia disappears. A long asymptomatic period follows primary infection, which may last up to 10 years; however, disease progression continues relentlessly. Levels of all anti-HIV antibodies are very high during the asymptomatic period. Disease develops when CD4 cells are severely depleted leading to severe immunosuppression and spread of disease to multiple organs and emergence of opportunistic infections. In the absence of treatment, disease progression is relentless and almost invariably fatal.

In most countries including India, screening tests for antiHIV by EIA are now compulsory for blood donation. If reactive, additional tests are used to confirm the diagnosis of HIV infection.

Treatment of HIV infection consists of initiation of antiretroviral medications guided by patient's CD4 count and WHO clinical stage and treatment of opportunistic infections.

Risk of transfusion transmitted HIV infection can be reduced by measures introduced at the blood collection centers such as, improved donor screening, education and exclusion techniques; enlightened transfusion practice such as, judicious use of allogenic blood components, appropriate use of autologous blood, and alternative to transfusion and measures that depend on the development of new technologies, such as viral inactivation of cellular components and safe substitutes for blood. Measures such as public education, self-deferral of donors engaged in high-risk activities and confidential unit exclusion also serve a similar purpose.

Human T-cell Leukemia Virus HTLV Types 1 and 2

Human T-cell leukemia virus type I (HTLV-I) was the first human retrovirus isolated and the first to be causally associated with a malignant disease of humans, the adult T-cell leukemia.[26] It is also associated with myelopathy and tropical spastic paralysis. HTLV-II, which was described later, is known to show 60 percent homology of genetic sequences to those of HTLV-I.[26] The current incidence of HTLV infection in the United States is 1 in 6250 individuals being seropositive per year half of these being infected with HTLV-I and the rest half with HTLV-II.[27] HTLV-I infection shows geographic clustering with high endemicity in Japan, sub-Saharan Africa and Central and South America. HTLV-II shows clustering in Native American population donors. In these areas, the donors are screened using EIA screening tests.[28] The transmission rate of HTLV-I or HTLV-II in a recipient of an infective

blood unit is between 20 and 60 percent. The risk of transmission of HTLV from a screened blood unit is low (1 in 6,40,000).[13] Contact with infected viable lymphocytes can cause infection, as both the viruses are cell-associated. Transmission is by cellular components and not by cell-free plasma or its derivatives. As refrigeration of blood product over 10 days results in degradation of lymphocytes and in decrease in load of infectious viruses, plasma and plasma derivatives do not transmit the virus.[20,27,29,30] The association of infectivity with fresh cellular components raises the possibility that transmission of HTLV by transfusion requires viable T-lymphocytes and that their removal from blood donations may clear the potentially infectious cells.

With the use of combination of viral lysates from HTLV-I and II viruses, there is sensitive detection of both anti-HTLV-I and anti-HTLV-II. Such combination HTLV-I/II EIA test is being used in United States for HTLV screening as the originally licensed anti-HTLV-I EIA can miss up to 50 percent of HTLV-II infections.[30] HTLV-I and II infections have not been reported in the Indian subcontinent.

Human Herpes Virus Infection

Of the herpes viruses eight are known to infect humans. Cytomegalovirus (CMV) has the greatest clinical relevance in transfusion medicine. Other herpes viruses that may contaminate blood products are Epstein-Barr virus (EBV) and human herpes virus (HHV) 6 through 8.

Cytomegalovirus

Cytomegalovirus (CMV) is widely distributed with a seroprevalence of 30 to 80 percent in developed countries and that approaching 100 percent in developing nations.[31,32] The CMV is transmitted in a latent, particulate state only by cellular blood components (such as red cells, platelets, granulocytes), and the virus reactivates from donor leukocytes after transfusion. Fresh frozen plasma and cryoprecipitate have not been implicated.

Transfusion can lead to active CMV infection in the recipient by three mechanisms:

1. The term transfusion transmitted CMV infection (TT-CMV) is used to describe a primary CMV infection occurring in a seronegative recipient transfused with an infected blood component.
2. Reactivated CMV infection occurs when a seropositive transfusion recipient experiences reactivation of latent CMV infection after a blood transfusion from a seronegative donor. The underlying mechanism involves immunomodulatory interactions between MHC mismatched leukocytes of the donor and recipients.
3. Finally, CMV superinfection occurs when a seropositive recipient is transfused a new strain of virus.

The clinical significance of TT-CMV is significantly more than CMV reactivation and superinfection, because in TT-CMV, the recipient has no immunological memory, while the two former conditions are unlikely to cause morbidity. Around 1.2 percent of immunocompetent recipients experience TT-CMV.[33]

In contrast, TT-CMV causes significant morbidity and mortality in immunocompromised recipients with 13 to 37 percent acquiring infection from infected and unfiltered blood.[34]

The at-risk groups include premature newborns born to seronegative mothers, seronegative recipients of seronegative bone marrow transplant and solid organ transplant and seronegative patients with AIDS.[35] These patients first have a flu-like illness followed by disseminated disease including hepatitis, retinitis, encephalitis, pneumonitis and gastroenteritis.

CMV Infection can be Detected by Serologic Assays for AntiCMV Antibodies

The TT-CMV can be prevented by using seronegative blood and filtered blood components. In comparison to unscreened blood, the use of seronegative units can reduce the incidence of TT-CMV from 13 to 37 percent to 2.5 percent in at-risk individuals.[36] No effective vaccine is available at present. Ganciclovir and IVIG are used in the treatment of severe CMV infection immunocompromised patients.

Epstein-Barr Virus

Epstein-Barr virus (EBV) has been implicated in endemic Burkitt's lymphoma, AIDS-related lymphoma, post-transfusion lymphoproliferative disease and nasopharyngeal carcinoma.

Transmission of EBV by blood transfusion can manifest in a similar manner to classic infectious mononucleosis. Although EBV-seronegative blood components reduce the incidence of TT-EBV, they are difficult to obtain given the high seroprevalence of EBV.

Human Herpes Viruses 6 and 8

With ubiquity of (HHV-6) antibodies and absence of disease associations after transfusion, no recommendations have been made for protection of seronegative blood recipients from transmission by blood components.[37] HHV-8 (Kaposi's sarcoma associated herpes virus) has been found in apparently healthy blood donors

but transfusion transmission of HHV-8 has not been demonstrated.[38]

Parvovirus B19

Parvovirus B19 was discovered incidentally during the screening of blood samples for hepatitis B surface antigen. About 30 to 60 percent of blood donors have antibodies to parvovirus B19. This is indicative of immunity rather than chronic persistent infection.[39] The virus has been found regularly in clotting factor concentrates and has been transmitted to persons with hemophilia. It is also transmitted by cellular blood components and plasma, but not intravenous immunoglobulin and albumin.[40]

Parvovirus B19 can infect and lyse red cell progenitors in the bone marrow[41] resulting in sudden and severe anemia in patients with underlying chronic hemolytic disorders. Patients with cellular immunodeficiency, including those infected with HIV, are at risk for chronic viremia and associated hypoplastic anemia.

However, parvovirus B19 screening of whole blood donations has not been a high priority because of the benign and/or transient nature of most parvovirus diseases, the availability of effective treatment for chronic hematologic sequelae and the extreme rarity of reports of parvovirus B19 transmission by individual components.

Bacterial Infections

Septic shock was one of the earliest recognized complications of blood transfusion. Prospective studies have indicated that the clinical presentation is wide and milder reactions are often misdiagnosed as febrile non-hemolytic transfusion reactions.

Transfusion reactions associated with contaminated red cell concentrates are extremely severe with mortality of 70 percent.[42] Fever, rigors, hypotension, nausea, vomiting and diarrhea are the usual presenting features. Septic shock, oliguria and disseminated intravascular coagulation are frequent complications. Majority of the reactions are caused by infusion of endotoxins of gram-negative organisms such as *Yersinia enterocolitica,* other Enterobacteriaceae and psychrophilic pseudomonas.

Because platelet concentrates are stored at room temperature, they offer the most favorable media for bacterial growth thus limiting their permissible duration of storage to more than 3 days. Coagulase negative staphylococci are the most frequent pathogens. *Pseudomonas* species have been isolated from plasma and cryoprecipitate thawed in contaminated water baths.

Possible mechanisms of blood component contamination include donor bacteremia, inadequate skin disinfection and use of contaminated equipment during blood collection storage and processing.

Possible measures to prevent transfusion associated bacterial sepsis include extension of donor screening, improved donor skin disinfection, removal of first aliquot of donor blood, limiting storage time, pretransfusion detection, altered blood processing and chemical and photochemical decontamination.

Syphilis

Transfusion transmitted syphilis is not a major hazard of modern blood transfusion therapy. *Treponema pallidum*, the infectious agent causing syphilis survives at the most for 5 days in blood stored at 4°C.[43] Only rare cases of transfusion transmitted syphilis have been documented and the causes of this decline are universal donor screening and the overall decline in the incidence of syphilis with the advent of penicillin. The rapid plasma reagin (RPR) test is commonly used for screening the blood products for syphilis. Blood donations from individuals who have had or been treated for syphilis should be deferred for at least 12 months after successful completion of treatment. As per the AABB standards, blood donations from any person with a positive serological test result for syphilis should be deferred for 12 months.[44] It is not the transmission of syphilis that is worrisome. Being a sexually transmitted disease, its presence points towards donor's indulgence in "high risk" behavior and consequent higher risk of exposure to infections like HIV and hepatitis. However, the RPR test used for screening is not specific and a large portion of positive tests in healthy donor population may represent a biological false positive reaction.

Malaria

Malaria can be transmitted by the transfusion of any blood component likely to contain even small number of red blood cells; platelet and granulocyte concentrates, fresh plasma and cryoprecipitate have all been implicated. Plasma that has been frozen or fractionated does not transmit malaria. Malaria parasite of all species can remain viable in stored blood for at least a week and longer in adenine containing solutions. The incubation period of transfusion malaria depends on the number and strain of plasmodia transfused, on the host and on the use of antimalarial prophylaxis. With *P. falciparum* and *P. vivax* it is between 1 week and 1 month, but with *P. malariae* it may be many months.[45] When blood smear are examined by simple microscopy, a density of less than 100 parasites per microliter of blood cannot be detected. Since most apparently healthy donors have very low parasitemia, serological tests are useful in detecting latent malarial infection. In endemic areas, it is recommended that chemoprophylaxis should be given to all recipients. In nonendemic areas, screening donors by travel history can exclude the asymptomatic carriers.

Transfusion transmitted malaria responds to conventional anti-malarials.

Babesiosis

As with malaria, asymptomatic individuals infected with Babesiosis may present as prospective blood donors. Babesiosis has been transmitted following the transfusion of infected packed red cells, frozen-thawed-deglycerolized red blood cells and platelet concentrates. The parasite can survive at 4°C in a unit of RBCs for up to 35 days. No test is currently available for mass screening to detect asymptomatic carriers of *Babesia* species.

Trypanosomal Infection

Only a few cases of transfusion-transmitted *T. cruzi* infection have been diagnosed. The parasite is viable for at least 21 days in the whole blood and RBC units that have been stored at 4°C.

Leishmaniasis

Transfusion transmission of *Leishmania* species is a rare risk in countries where such organisms are endemic.

Toxoplasmosis

Toxoplasma gondii is a WBC-associated parasite that can survive for several weeks in stored whole blood. Toxoplasmosis is caused by the ubiquitous parasite *Toxoplasma gondii* and infection has been reported as a rare transfusion complication in immunocompromised patients. Given the high risk of symptomatic transfusion-transmitted toxoplasmosis, the option of using leukocyte-reduced blood may be considered while providing packed cell or platelet transfusions to the immunocompromised individuals.

Microfilariasis

Filarial infections are usually transmitted by vectors but if blood from a microfilaremic individual is transfused, the transfused microfilaria may persist in the recipient's circulation for more than 2 years. Transfusion-acquired microfilaremia is self-limited because transfused microfilariae do not develop into adult filarial worms. Routine testing of donor blood is, therefore, not warranted.

In conclusion, there has been a substantial decline in the incidence of transfusion-transmitted infections due to improvement in donor screening, testing and viral inactivation of blood products, particularly in developed nations. However, in developing nations, blood safety continues to be a major problem due to the high prevalence of infections markers among blood donors compounded with the problem of limited resources that preclude the use of sophisticated, sensitive but expensive technologies for screening of blood products. The last two decades have also witnessed surfacing of new and re-emerging infections. Hence, despite stringent donor eligibility criteria, improved donor screening and introduction of sophisticated technology, transfusion-transmitted infection continues as a challenge for transfusion experts.

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CHAPTER 38

Noninfectious Hazards of Blood Transfusion

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The risks of blood transfusion should always be borne in mind whilst considering benefits of transfusing red cells, platelets, and plasma. With the advances in infectious disease testing, noninfectious complications are now many-fold more likely to cause serious morbidity or death following transfusion. Some of the more common noninfectious hazards of transfusion include transfusion reactions (hemolytic, febrile, allergic/urticarial/anaphylactic). Other noninfectious hazards include transfusion related acute lung injury, post-transfusion purpura, transfusion-associated graft versus host disease, transfusion related immunomodulation, alloimmunization, metabolic derangements, transfusion-associated circulatory overload and iron overload. Individuals who administer blood transfusions should recognize these complications in order to be able to quickly provide appropriate treatment.

Though transfusion has many benefits it also involves risks to the recipient. Any unfavorable event occurring in a recipient due to blood transfusion, either during or after transfusion, is considered an adverse effect of transfusion.

Transfusion can lead to serious adverse effects including infectious and noninfectious complications. With the more stringent donor selection criteria and use of sensitive screening methods for transfusion transmissible infectious marker testing, the risk of infectious complications has decreased. The term NISHOT (Noninfectious serious hazards of transfusion) was first described in a 2000 AABB bulletin to broadly encompass all noninfectious transfusion complications. Currently, a patient is up to 1000-fold more likely to experience a NISHOT than an infectious complication of transfusion.[1] The noninfectious complications can be acute (within 24 hours) or delayed.

Children are a unique patient group and many have special transfusion requirements. Neonates in particular are often intensively transfused and are especially vulnerable to the potential infective and toxic effects of transfusion. They have immature immune system and metabolism, and are still undergoing rapid neurodevelopment. The chances for acute side effects of transfusion may be greater for small children than for adults, as a single unit of transfused blood forms a greater proportion of their blood volume than that in an adult. Problems may occur due to lack of knowledge of special requirements in the neonatal age group or human error such as overtransfusion. It is estimated that the incidence of adverse outcome is 18:100,000 red blood cells issued for children aged less than 18 years and 37:100,000 for infants. The comparable adult incidence is 13:100,000.[2] The health care personnel involved in the transfusion process should therefore be aware of correct recognition, prevention and appropriate management of the adverse effects of transfusion.

In most instances of transfusion reactions, the pathophysiology, clinical diagnosis and management are similar as the transfusion reactions in adults.

CATEGORIES OF NONINFECTIOUS HAZARDS OF BLOOD TRANSFUSION

Acute

- Allergic
- Acute intravascular hemolysis
- Febrile nonhemolytic transfusion reaction (FNHTR)
- Transfusion related acute lung injury (TRALI)
- Metabolic complications.

Delayed

- Delayed hemolytic transfusion reaction
- Alloimmunization
- Immunomodulation
- Post-transfusion purpura (PTP)
- Graft versus host disease (GvHD)
- Circulatory overload
- Iron overload.

ACUTE HAZARDS OF TRANSFUSION

Transfusion reactions can occur rapidly after transfusion e.g. hemolytic reaction, or many days or weeks after transfusion, e.g. graft versus host disease (GvHD). By definition, acute transfusion reactions (ATRs) occur within 24 hours of transfusion administration[3] although most occur during or within four hours after the end of a transfusion. There are only few studies which have reported the incidence and type of acute reactions in pediatric patients. In one prospective study of acute transfusion reactions in pediatric intensive care units, the incidence of acute transfusion reactions was 1.6 percent.[4]

In that study, however, the only blood product transfused was red blood cells (RBCs) and the only ATR reported was febrile nonhemolytic transfusion reaction (FNHTR). Another prospective study of platelet transfusions in children by Couban et al[5] reported seven ATRs associated with sixty six leucoreduced platelet transfusions (11%). ATRs consisted of two FNHTRs, four allergic reactions, and one mixed reaction. According to the Quebec Hemovigilance System (QHS) 2001 report, the most frequent immediate transfusion reaction for all components transfused was FNHTR (12:10,000 transfused units), followed by minor allergic reactions (10:10,000).[6]

Allergic Reaction

Allergic reactions associated with transfusion may vary from mild uncomplicated allergic reaction to anaphylactoid and severe anaphylactic reactions. Allergic reactions are the most common cause of adverse transfusion reactions. Uncomplicated mild allergic reactions consists of localized or diffuse urticaria characterized by erythematous circumscribe raised lesions present over the upper trunk and neck often associated with itching. Mild urticarial reactions constitute 1 to 3 percent of adverse transfusion reactions.[3] Anaphylaxis is the severe form of this type of reaction characterized by hypotension, and is often associated with bronchospasm, dyspnea, and in rare cases death. These severe reactions may occur after infusion of few mL of blood.

The anaphylactoid reaction is placed in between mild urticaria and anaphylaxis and is characterized by one or more of the symptoms like urticaria, flushing, respiratory tract obstruction, cough, chest pain, dyspnea, nausea, vomiting, diarrhea, tachycardia and arrhythmias. Absence of fever helps in differentiating these reactions from hypotension due to hemolytic reaction or bacterial contamination.[3]

Allergic reactions occur due to exposure to soluble substances present in donor plasma which binds with IgE antibodies resulting in release of histamine. This assumption is based on the facts that these reactions recur in an affected recipient and can be prevented by removal of plasma. Anaphylactic and anaphylactoid reactions are sometimes associated with anti-IgA, particularly in IgA deficient recipient. First description of anaphylactic reaction associated with anti-IgA was reported in 1968 and approximately 30 additional cases have been reported by 1995.[7]

Treatment and Prevention

Anaphylaxis or anaphylactoid reactions should be recognized promptly when the recipient shows symptoms and the transfusion should be immediately stopped. Treatment of transfusion related anaphylaxis or anaphylactoid reaction is same as that of anaphylaxis due to other reasons. Hypotension needs to be managed by administering fluids and if required administration of epinephrine along with supportive care.

If the recipient is known to be IgA deficient or there is a previous history of life-threatening anaphylactic reaction blood components which lacks IgA, either by washing or obtained from IgA deficient donor, should be administered. Availability of washed red cells is not so difficult but washed platelets are not generally easily available as washing of platelets may result in low platelet recovery.

Treatment of uncomplicated urticaria requires discontinuation of transfusion and administration of antihistaminics. If symptoms are resolved the transfusion may be restarted.

Immune Mediated Hemolysis

Immune mediated lysis of donor red cells can result in hemolytic transfusion reaction. It is the most severe hemolytic transfusion reaction and occurs due to destruction of transfused donor red cells by recipient's antibodies, e.g. in case of ABO incompatible red blood cell transfusion. Depending on the nature of the antibody the hemolytic transfusion reaction may be acute or delayed and may result in intravascular or extravascular hemolysis. Incidence of hemolytic reaction varies from 1:38,000 to 1:70,000.[3]

Transfusion of an ABO incompatible unit causes rapid destruction of red cells with the release of free hemoglobin and RBC stroma in the circulation. ABO incompatible transfusion may be life-threatening as it can lead to acute renal failure, shock, and disseminated intravascular coagulation.

In intravascular hemolysis the interaction of red cell antigen-antibody causes complement activation and release of cytokines resulting in clinical manifestations. Complement activation depends on the specificity and class of antibody, and number of antigen sites. The complement activation results in formation of membrane attack complex causing intravascular red cell lysis which leads to hemoglobinemia and, if it exceeds renal threshold, to hemoglobinuria. The free hemoglobin impairs renal functions. The factors responsible for renal failure in severe cases are hypotension, renal vasoconstriction, antigen-antibody complex deposition, and formation of thrombi in renal vasculature, all of which affect the renal cortical blood supply.

If the antibody involved in immune reaction does not fix the complement, there will be acute extravascular hemolysis. Usually extravascular hemolytic reactions are not associated with severe clinical symptoms. They may present with fever. On investigation the direct anti-globulin test (DAT) may be positive due to binding of antibodies to incompatible donor red cells.

Infants less than four months of age generally do not have developed anti-A and anti-B antibodies, and therefore are not usually susceptible to these reactions. However, maternal IgG antibodies can cross the placenta and may cause hemolysis of transfused red cells. Considering this possibility it is recommended that the compatibility testing should be performed using mothers serum up to four months of age. RBCs units lacking corresponding antigens should be selected for transfusion.

The symptoms of ABO incompatible transfusion are chills, rigors, fever, abdominal or back pain, pain at the infusion site, nausea, vomiting, hemoglobinuria, oliguria. These clinical manifestations may be observed after transfusion of even a few mL of blood. Therefore, the initial period of transfusion is very important, and the rate of transfusion during the initial 15 to 20 minutes should be slow and can be increased later. Similarly, the recipient should be under continuous medical monitoring during transfusion so that any adverse reaction can be identified and managed immediately. Information to the recipient or attendants of recipient about the symptoms of adverse reaction is necessary.

The severity of hemolytic reaction due to an ABO incompatible transfusion varies and depends on the rate and the volume of red cells transfused. In a 10 years study of analysis of transfusion errors it was found that nearly half (47%) of the recipients of ABO incompatible red cells suffered no ill effects even after receiving a full unit. Half (50%) exhibited an acute hemolytic transfusion reaction and about 4 percent died as a result.[8]

Treatment and Prevention

Treatment of acute hemolytic reaction depends on its severity. The aim is to maintain adequate renal perfusion in order to prevent renal failure. Adequate renal perfusion can be monitored by measurement of urine output for at least 24 hours. The usual initial step is infusion of intravenous normal saline but clinical monitoring is necessary to avoid fluid overload. Furosemide is considered a better diuretic as it improves renal cortical blood flow. If the intravascular hemolysis is also complicated with coagulopathy, administration of platelets, fresh frozen plasma, and cryoprecipitated antihemophilic factor (AHF) may be necessary.

The most common cause of transfusion of incompatible units is clerical error such as incorrect labeling of blood sample, blood bag or request form. Failure to follow the identification procedure during sample collection and just prior to transfusion may result in wrong blood transfusion and hemolytic episode following transfusion. For bed side transfusions each institute should develop policies and procedures to ensure proper patient identification, sample collection and labeling and unit identification. In addition to this continuous monitoring and training of the staff responsible for collection and administration of blood is also required.

Hemolysis can also occur with the transfusion of ABO incompatible platelet and plasma products. There have been several reports of severe hemolytic reactions after transfusion of minor ABO mismatched platelet transfusion.[9]

Nonimmune Mediated Hemolysis

There are chances of *in vitro* hemolysis due to improper handling during transportation or at the time of administration. Malfunctioning of blood warmer or accidental freezing can cause temperature related damage to red cells. Addition of drugs or hypotonic solutions may result in osmotic lysis of red cells. Transfusion of such units can cause nonimmune mediated hemolysis in the recipient. Treatment of such cases depends on the severity of the reaction. If the patient develops shock, renal failure intensive medical management is needed.

Such cases are preventable. All the staff involved in processing, issuing and administration of blood should be trained for storage and correct handling of blood and blood components. The equipment used for warming and

for administration of blood should be properly maintained and staff should be adequately trained for use of such equipment.

Febrile Nonhemolytic Transfusion Reaction

A febrile nonhemolytic transfusion reaction (FNHTR) is defined as temperature rise of more than 1°C associated with transfusion without any other demonstrable cause. FNHTRs are caused by antibodies present in recipient's plasma that interact with white cells in the transfused product and are most frequently HLA antibodies or sometimes granulocyte antibodies. This mechanism appears to be the primary cause of FNHTR after transfusion of red cells but for FNHTRs consequent to platelet transfusions the leukocyte derived cytokines, generated during the warmer room temperature storage of platelets, have been implicated.

FNHTRs present with fever with or without chills. Most FNHTRs are mild and benign but they cause discomfort to the recipient. Severe reactions may be accompanied by hypotension, cyanosis, tachycardia, tachypnoea, dyspnea, cough, transient leukopenia. Febrile reactions should be differentiated from hemolytic transfusion reaction and reactions due to a bacterial contaminated product. If the patient has received multiple units it becomes difficult to find out which unit has caused the febrile reaction. In such instances all units transfused within four hours prior to transfusion should be included for transfusion reaction workup.

Treatment and Prevention

FNHTRs need prompt management and exclusion of other causes of fever. Like any other adverse reactions the transfusion should be stopped immediately and evaluation done to rule out other causes of fever. Generally, fever responds to antipyretic agents such as acetaminophen.

At some centers pretransfusion medication is given specially in recurrent FNHTRs. But this practice is not recommended as it may mask the fever associated with other types of reactions like hemolytic transfusion and bacterial contamination.

Febrile reactions to red cells can be prevented in most of the cases by removal of leukocytes by either prestorage or poststorage leukoreduction or administering saline washed red cells because leukocyte antibodies are the primary reason. Cytokines related febrile reactions may be most effectively prevented by prestorage leukoreduction.

Transfusion Related Acute Lung Injury

Transfusion related acute lung injury (TRALI) is an important cause for transfusion related morbidity and mortality. The incidence of TRALI is estimated to be between 0.08 percent and 15 percent of patients receiving a blood transfusion.[10] TRALI can occur after transfusion of whole blood, red blood cells, fresh frozen plasma, platelet concentrate (random donor and apheresis), cryoprecipitated antihemophilic factor VIII.

Transfusion related acute lung injury is characterized by acute noncardiogenic edema and respiratory compromise associated with transfusion. The National Heart, Lung and Blood Institute (NHLBI) working group has defined TRALI as 'new acute lung injury occurring during or within six hours of transfusion, with a clear temporal relationship to transfusion in the patient without alternate risk factor for lung injury'.[11] Clinically, TRALI should be considered whenever the recipient experiences acute respiratory insufficiency and or chest X-ray shows bilateral pulmonary edema. It is accompanied by other symptoms like fever, chills, and hypotension. The severity of TRALI is not related to the volume of blood transfused.

The exact mechanism of TRALI is not known but it may result from multiple factors. Antibodies against HLA class I and II or neutrophils antigens present in donor units are thought to cause increased permeability of pulmonary microcirculation resulting in collection of fluid in interstitial and alveolar spaces.

Treatment and Prevention

The transfusion should be stopped when there is respiratory distress and the same unit should not be started even if the symptoms have subsided. Management of TRALI is mainly supportive with oxygen therapy and ventilation. Most of the patients recover within 48 to 72 hours.

No specific precautions are needed if the reaction has occurred due to antibodies in donor plasma, and components from other donors are available. In many centers in the UK the current practice is not to transfuse plasma sourced from female donors.

Metabolic Complications

Hypothermia

Hypothermia may be caused by rapid infusion of large quantities of cold blood (2–10°C) or RBC units. Due to their large body surface area-to-weight ratio infants and children are predisposed to hypothermia. Hypothermia during massive transfusion can induce cardiac arrhythmia and arrest.[12] Transfusion of cold blood in neonates has been associated with apnea and hypoglycemia.[13] Blood does not have to be warmed for transfusions administered at a standard rate. For rapid infusion, generally considered

to be more than 50 mL/kg/hour for an adult and more than 15 mL/kg/hour for a child, blood should be warmed using a monitored blood warming device.

Hyperkalemia

During storage of red cell products potassium leaks from the cells, increasing the potassium concentration. Risk of hyperkalemia from a blood transfusion depends on the patient's size, clinical condition, type and amount of component transfused, concentration of potassium in the plasma. Hyperkalemia resulting from massive transfusion of older RBC units containing elevated amount of extracellular potassium can cause significant cardiac complications or possibly death in some patients.[14] In one case of neonatal mortality following transfusion of red cells with high plasma potassium levels reported by Hall et al[15] it was observed that the patient's cardiac arrest was probably related to rapid transfusion of RBCs with high plasma potassium levels.

In neonatal transfusions, hyperkalemia can be avoided by use of RBC units less than seven days old or older units that have been saline washed.[16]

Citrate Toxicity

Blood collected for transfusion is anticoagulated with citrate, which chelates calcium ions. Plasma and whole blood are the blood components most likely to cause hypocalcemia because they contain the most citrate per unit volume. Rapid blood transfusion can cause a transient decrease in ionized calcium and a hypocalcemic state.[17] Symptoms of hypocalcemia include peripheral and perioral paraesthesia, muscle spasm, cardiac arrhythmia and hypotension. Symptomatic hypocalcemia is rare and calcium supplementation is usually not required. Mild citrate toxicity is managed by slowing the rate of transfusion. If severe, parenteral calcium may be required.

DELAYED HAZARDS OF TRANSFUSION

Delayed Hemolytic Transfusion Reaction

Delayed hemolytic transfusion reaction (DHTR) occurs when the antigen on donor red cells elicit immune response in the recipient. Secondary immune response occurs usually four to seven days after transfusion. Most of the antibodies associated in DHTR are IgG and the resulting hemolysis is of extravascular type. Secondary immune response may not result in hemolysis always. In some patients clinically significant delayed hemolytic reaction occurs. Clinically, it is detected by falling hemoglobin, fever, mild jaundice and positive antibody screen. If DHTR is suspected, tests for detection of red cell antibodies should be done along with other investigations like direct antiglobulin test (DAT) and serum bilirubin. Detection of red cell alloantibody with the history of recent transfusion indicates DHTR in such cases. Delayed intravascular hemolysis is uncommon and is often associated with antibodies to Duffy or Kidd blood group system.[18]

Treatment and Prevention

In most of the cases no specific treatment is required. The patient should be monitored for renal function and coagulation.

For future transfusion requirements the donor unit should lack the corresponding antigen for the alloantibody.

Alloimmunization

Alloimmunization is one of the clinically significant adverse effects of blood transfusion. Development of antibodies against donor antigen present in the blood component may pose difficulties in finding compatible red cell units or result in platelet refractoriness or adverse transfusion reaction. The immune system of the recipient reacts to donor antigens as they are foreign to recipient. The first exposure generally sensitizes the immune system of recipient. With subsequent exposure the secondary immune response results in rapid production of large amount of IgG type of antibodies. These antibodies attach to the surface of the antigen carrying cells and causes destruction of cells by complement system or reticulo-endothelial system. Primary alloimmunization to red cell or platelet antigens with transfusion is rare in the first few months of life.[19] Beyond newborn period, pediatric patients with clinical conditions like sickle cell disease or thalassemia requiring repeated transfusions may develop alloimmunization to red cell antigens.

Alloimmunization to platelet antigens and refracto-riness to platelet transfusions may be a problem in oncology patients or other clinical conditions dependent on platelets. HLA alloimmunization is the most common immune cause of platelet refractoriness and can be confirmed by demonstration of HLA class I antibodies.[20]

Clinically the severity varies from mild symptom of fever and falling hematocrit to severe effects like platelet refractoriness and bleeding.

Laboratory work up to detect clinically significant red cell antibodies needs to be done. For HLA antibodies lymphocytic panels and lymphocytotoxic antibody can be done using patient's serum.

Treatment and Prevention

Treatment depends on the severity of reaction. Mild reaction in case of a single time need of transfusion may

not need any active treatment. Patients requiring multiple transfusions need to undergo antibody identification and should be transfused preferably with antigen negative blood to prevent further alloimmunization. However, it is very difficult to prevent alloimmunization completely. Selection of phenotype matched blood is recommended specially in patients requiring multiple transfusions to prevent alloimmunization. Use of leukoreduced products for transfusion also prevents alloimmunization up to certain extent. Several strategies have been evaluated to prevent alloimmunization to platelets. They include reduction in numbers of leukocytes in the platelets and use of ultraviolet B irradiation.[20] The report of Trial to Reduce Alloimmunization to Platelets (TRAP) study group indicated that use of either leukocyte filtered or UVB irradiated blood components reduced the incidence of HLA antibody generation from 45 percent to 17 and 21 percent respectively.[21]

Transfusion Related Immunomodulation

Blood transfusion can modulate the immune response of the recipient. This phenomenon was reported by Opelz and coworkers in 1973, who observed the improved renal allograft survival in transfused patients.[22] However, there may be other adverse effects of transfusion in different clinical situations, such as increased risk of bacterial infection, activation of latent infections and tumor recurrence. In one controlled study on patients with hematologic malignancies, a history of allogeneic blood transfusion was associated with an increased risk for lymphoplasmacytic and marginal zone lymphomas.[23] The exact mechanism is not clear but the soluble mediators released from WBCs are postulated as a potential cause for the immunomodulatory effect. Several studies have been conducted to understand the effect of blood transfusion on immune response and to develop preventive strategies. Leukoreduction as a preventive strategy remains controversial as the only clinical situation where the findings of randomized clinical trials of adverse transfusion related immunomodulation (TRIM) effects have been consistent is in cardiac surgery patients. In that setting, the use of leukoreduced allogeneic RBCs has been shown to reduce the short-term (up to three months post-transfusion) mortality from all causes. Based on the results of the cardiac surgery RCTs, leukoreduction of all cellular blood components transfused in cardiac surgery patients should be recommended. In clinical settings other than cardiac surgery, the available evidence does not yet justify implementation of universal leukoreduction for the specific prevention of adverse TRIM effects, but universal leukoreduction may be justified on the basis of other WBC-related adverse effects.[24]

Post-transfusion Purpura

Post-transfusion purpura (PTP) manifests as sudden and severe thrombocytopenia occurring one to two weeks after red cells, plasma or platelet transfusions. The platelet count may fall up to below 10×10^9/L. The recipient may have hematuria, melena and vaginal bleeding.

Post-transfusion purpura is generally thought to be due to the anamnestic development of antibodies against donor platelets. These antibodies also start destroying autologous platelets leading to severe thrombocytopenia and purpura. Most frequently found antibody is anti-HPA-1a. Platelet antibodies attach to platelet surface resulting in extravascular destruction of platelets. The exact mechanism of platelet destruction is not fully known. Multiparous female patients are more likely to develop PTP after transfusion because of sensitization during previous pregnancies.

Treatment and Prevention

The thrombocytopenia is usually self limiting. This condition can be treated by corticosteroids, plasmapheresis and exchange transfusions. Intravenous immunoglobulin can also be given. Platelet transfusions should be avoided during the treatment of PTP. There are no preventive measures for PTP. History of transfusion and any adverse reaction should be asked before blood transfusion. Patients with positive history should be tested for presence of antibodies.

Transfusion Associated Graft versus Host Disease

Transfusion associated graft versus host disease (TA-GvHD) is a severe immunological reaction in a susceptible host resulting from engraftment and proliferation of donor lymphocytes present in the transfused product. The engrafted lymphocytes elicit immunogenic response against recipient tissues leading to the clinical presentation of pancytopenia, bleeding, diarrhea and skin rash.

Transfusion associated graft versus host disease occurs in an immunocompromised host or in immucompetent recipient when the donor cells are homozygous for an HLA type for which the recipient is heterozygous. The pathophysiology is complex. The donor lymphocytes are not recognized by recipient as foreign and are not cleared by the host immune system resulting in engraftment and proliferation in the host. Later these donor cells start attacking host tissue. Clinically, GvHD presents as skin rash, blanching and maculopapular erythema of upper trunk, neck, palms and soles that may develop into blistering lesions. Skin biopsy shows infiltration of upper

dermis by mononuclear cells and damage to the basal layer. Involvement of liver is manifested as hepatitis, raised bilirubin and enzyme levels. Enterocolitis causes anorexia, nausea and severe diarrhea.

Treatment and Prevention

The clinical effects of TA-GvHD are difficult to treat even with immunosuppressive drugs. Irradiation of cellular blood components is accepted as method for prevention of TA-GvHD. The recommended dose is 25 Gy to the center of the blood container and at least 15 Gy to the periphery of the blood container. This dose renders the T-lymphocyte inactive without affecting functional status of red cells, platelets and granulocytes. These blood components, given to recipients from donors homozygous for an HLA haplotype shared with the recipient, pose a specific risk for TA-GvHD. This circumstance can occur when first and second degree relatives serve as directed donors and when HLA matched platelet components donated by related or unrelated individuals are being transfused. Irradiation of blood components has been recommended in these situations. Other recipients who should receive irradiated cellular blood component are recipients of hematopoietic progenitor cells transplant and intrauterine transfusions, and patients with congenital immunodeficiency.

Blood transfusion of irradiated blood product in pediatric patients requires more attention. Irradiated red cells undergo an enhanced efflux of potassium during storage at 2 to 6°C.[25] When irradiated red cells are used for neonatal exchange transfusion or the equivalent of a whole blood exchange is anticipated, red cell washing should be considered to prevent the possible adverse effects caused by hyperkalemia associated with irradiation and storage.[26]

Circulatory Overload

Transfusion therapy sometime may result in circulatory overload especially in young children and the elderly. Rapid increase in blood volume due to transfusion is not tolerated by some patients with compromised cardiac and pulmonary functions. Circulatory overload presents as dyspnea, cyanosis, severe headache and hypertension. Congestive cardiac failure may occur during or after transfusion.

Treatment

The condition can revert if the transfusion is stopped with the onset of symptoms. Diuretics and oxygen support may be needed in some patients, even if the symptoms are not resolved phlebotomy may be required.

Iron Overload

This complication is particularly common in multiple transfusion recipients especially those with hemoglobinopathies since every red blood unit contains approximately 200 mg of iron. These patients are multiply transfused at regular intervals to maintain hemoglobin levels, resulting in accumulation of excess iron. During the initial phase iron is stored in reticuloendothelial sites and later in parenchymal cells. Iron deposition hampers the function of heart, liver, endocrinal glands. Cardiac involvement causes most of the morbidity and mortality.

Treatment

Treatment is to remove excess iron without affecting hemoglobin levels. Use of iron chelating agents like desferrioxamine can reduce iron stores.

CONCLUSION

Though the list of noninfectious hazards of transfusion is long and diverse and although blood transfusion will never be absolutely safe, tremendous progress has been made in the understanding of the complications of transfusion and their prevention and management. To prevent hemolytic reactions, advanced identification systems that link donor and recipient with greater precision minimize human error. Febrile non hemolytic transfusion reactions (FNHTR) and platelet refractoriness due to HLA alloimmunization can be prevented by transfusion of leukoreduced blood. Though TA-GVHD can be prevented by selective irradiation concerns about transfusion related immunomodulation (TRIM) still need to be resolved. Blood centers and transfusion services should provide education for creating clinical awareness of the complications of transfusion so that potential transfusion risks are identified and managed while they continue to occur, until, ultimately they are reduced to a negligible concern for transfusing physicians and their patients.

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SECTION 6

Hemato-Oncology

CHAPTERS OUTLINE

CHAPTER 39

Pediatric Acute Lymphoblastic Leukemia

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Acute lymphoblastic leukemia (ALL) is a neoplasm of precursor hematopoietic cells (B- and T-lymphoblasts) involving bone marrow (BM) or other tissues like lymph node, thymus with or without peripheral blood involvement. ALL is clinically, morphologically, immunophenotypically, and genetically a heterogeneous disease. It is the most common childhood malignancy and accounts for nearly 30 percent of all childhood cancers and approximately 75 percent of all cases of childhood leukemia. The treatment of childhood ALL is one of the true success stories of modern clinical oncology. Before the advent of effective chemotherapy in the 1960's, ALL usually was a fatal disease. In the developed countries, with modern intensive protocols approximately 83 to 93 percent of children with ALL are now long-term survivors.[1] However, this is not the case in developing countries where 80 percent of the children with ALL reside.

INTRODUCTION

Though as per the cancer registry data, it has been noted that incidence of ALL is less in India as compared to the developed countries, it is estimated that approximately 8000 new cases of childhood ALL diagnosed each year in India.[2] Unfortunately of these only 25 percent receive appropriate treatment. Studies from India have shown that 40 to 60 percent of patients treated in a pediatric oncology center on an affordable protocol, with manageable toxicity can be cured.[2,3] There are several reasons for these poor results including poverty, lack of awareness, lack of access to adequate medical care, lack of adequately trained personnel and competent oncology units. These factors lead to delayed and sometimes improper diagnosis, delayed referrals, poor compliance, inadequate or inappropriate therapy and poor outcome.

CLINICAL PRESENTATION

Suspected case of acute leukemia should be examined meticulously. Evaluation starts with a detailed history and clinical examination.

- Patients commonly present with symptoms including weakness, fatigue, fever, bleeding, bone pains, either gradually or abruptly. Children with ALL often present with signs and symptoms that reflect bone marrow infiltration and/or extramedullary disease (Table 1).
- Bone marrow failure due to infiltration by leukemic blast manifests as anemia, thrombocytopenia, and neutropenia.
- History of prior treatment in the form of transfusion or drugs prescribed especially steroids should be taken as these factors may interfere in diagnosis.
- Respiratory distress and orthopnea secondary to a mediastinal mass may be a presenting symptom in patients with T-lineage ALL; B-lineage ALL may present with mass in abdomen, head and neck or in central nervous system.
- Very frequently patients present with life threatening complications like congestive cardiac failure, acute bleeding, tumor lysis syndrome (TLS) (hyperuricemia, hyperphosphatemia, hyperkalemia hypocalcemia and/or azotemia)[4] and superior vena cava syndrome.

Clinical examination and investigations (pathological, biochemical and imaging) helps to find out need for urgent intervention and management of these life threatening consequences of leukemia.

- *Differential diagnosis (Table 2):* ALL should be differentiated from benign conditions like juvenile rheumatoid arthritis, infectious mononucleosis, idiopathic

Table 1 Symptomatology of acute lymphoblastic leukemia

• Fever
• Bleeding (e.g. petechiae or purpura)
• Bone pain
• Lymphadenopathy
• Splenomegaly
• Hepatosplenomegaly
• Testicular swelling
• Central nervous system symptoms (cranial nerve palsies, intracranial bleed, seizures)

Table 2 Differential diagnosis of acute lymphoblastic leukemia in children

• *Nonmalignant conditions:*
– Juvenile rheumatoid arthritis
– Infectious mononucleosis
– Idiopathic thrombocytopenic purpura
– Pertussis; parapertussis
– Aplastic anemia
– Acute infectious lymphocytosis
• *Malignancies:*
– Hematolymphoid malignancies—Acute myeloid leukemia, Hodgkin lymphoma, non-Hodgkin lymphoma (NHL)
– Neuroblastoma
– Retinoblastoma
– Rhabdomyosarcoma

thrombocytopenic purpura, pertussis, parapertussis, aplastic anemia, and acute infectious lymphocytosis. These diseases have overlapping symptomatology.

Hematolymphoid malignancies especially non-Hodgkin lymphomas with marrow involvement have a common spectrum of signs and symptoms and should be differentiated with ALL. Clinical symptomatology like abdominal mass in Burkitt's lymphoma, bony involvement in anaplastic large cell lymphoma helps in making working diagnosis. Clinical features, morphology and immunohistochemistry are helpful tool to distinguish ALL from acute myeloid leukemia (AML). Some features are given in Tables 3 and 4.

Solid tumors with advanced stage (i.e. bone marrow infiltration) may mimic ALL. Neuroblastoma with bone marrow involvement may have features of anemia, body pain, abdominal distension, though the early age of presentation, ecchymosis, primarily abdominal mass may help it to distinguish it from ALL. Other solid tumors like retinoblastoma, rhabdomyosarcoma, also having morphology of small round cell tumor, should be kept as differential diagnosis though the site of origin and immunohistochemistry is helpful to diagnose these solid tumors.

Table 3 Usual cytological features of AML and ALL

Parameters	*AML*	*ALL*
Blast size	Large and uniform	Small to medium: variable
Chromatin	Finely dispersed	Coarse
Nucleoli	1-4 often prominent	Absent or 1–2, indistinct
Cytoplasm	Granules often present	Granules lacking
Aur rods	60-70% cases; diagnostic	Absent
Myelodysplasia	Often present	Absent

Table 4 Commonly used immunophenotypic markers

Myeloid or Monocytic	CD13, CD33, CD117, CD15, CD16, MPO At least 2 of the following: NSE, CD14, CD64, CD11c, lysozyme
B-lymphoid	CD19, CD20, CD22, Cyto CD79a Cyto CD22
T-lymphoid	CD1a, CD2, CD3, CD4, CD5, CD7, CD8, Cyto CD3
Other markers	CD34, Tdt, HLADR, CD41, CD61

Laboratory confirmation is mandatory for the diagnosis and management of ALL.

LABORATORY DIAGNOSIS OF ACUTE LYMPHOBLASTIC LEUKEMIA

Laboratory investigations should be directed for confirming the diagnosis, assessment of organ function, and to rule out need for urgent intervention like tumor lysis syndrome.

Confirmation of Diagnosis

Laboratory work up starts with complete blood counts (CBC), peripheral blood and bone marrow evaluation. CBC generally shows anemia and thrombocytopenia. However patients may have leukopenia, normal leukocyte count or leukocytosis. Peripheral blood examination is usually followed by bone marrow (BM) examination. BM aspiration is preferably done from the posterior superior

Table 5 Immunological markers in B-ALL and T-ALL[5]

Lineage	*Immunological subgroup*	*Frequency*	*Immunophenotypic profile*	*Remarks*
B-ALL	Early pre-B cell	60–70%	CD10, CD19, CyCD22, CD34, and Tdt positive. CD24 strongly positive, CD45 dim/-ve	Good except CD10-ve ALL, especially in <1 year age group, infantile leukemia associated with translocation of 11q23 and have poor prognosis
	Pre-B cell	20%	Cyu heavy chain, CD10, CD19, cyCD79a, and CD22 +ve	Poor compared to early pre-B. Expression of t(1:19) is the primary determinant of adverse prognosis in pre B-ALL
	Mature B-Cell	2–5%	Surface immunoglobulin (bright), CD19, CD20, CD22, CD24 +ve CD34. Tdt –ve.	Poor with standard ALL therapeutic regimen, but dose intensive chemotherapy leads to cure rate of 75%.
T-ALL	Pro-T-cell		CyCD3 and/or CD7 +ve	With more intensive chemotherapeutic approach outcome reaching to the level of non-T-cell ALL.
	Pre-T-cell		CyCD3, CD7, CD2 and/or CD5 +ve	
	Cortical T-cell		CyCD3, CD7, CD2 and/or CD5 and CD1a +ve, co-expression of CD4/CD8	
	Mature T-cell		CyCD3, CD7, CD2 and/or CD5 and CD3 positive, segregated CD4 or CD8 +ve	

iliac bone and sample is aliquoted for morphology, flow cytometry, molecular studies (EDTA vacutainer), cytogenetics (Heparin vacutainer) and trephine biopsy (formalin fixed). The diagnosis of ALL should be established by cytomorphological examination of May-Grunwald Giemsa (MGG) or Wright-stained smears of peripheral blood and/or a BM aspirate.

FAB criteria and scoring system have become generally accepted for morphological classification. The myeloperoxidase (MPO) or Sudan-Black (SB) reaction and the non-specific esterase (NSE) reactions are recommended for differentiation from AML. In ALL, the MPO reaction is negative. A positive PAS and/or acid phosphatase reaction may support the diagnosis of ALL; these reactions, however, are not positive in all cases of ALL.

Flow Cytometry

Leukemic cells demonstrate particular subsets of surface and intracellular molecules defined as cluster of differentiation (CD) antigens (Table 5). Identification of which by flow cytometry (FCM) helps in lineage identification and sub-categorization. Diagnosis of acute leukemia by immunophenotyping is based on the fact that leukemic cells frequently disclose aberrant phenotypes compared to normal hematopoietic cells, known as the leukemia-associated phenotypes (LAP).

Cytogenetics of Acute Lymphoblastic Leukemia

Blasts in ALL contain somatically acquired genetic abnormalities that help in understanding pathogenesis and strongly influence prognosis. This includes changes in chromosome number (hyperdiploid/hypodiploid) and chromosomal translocations.

Numerical Abnormalities

- *High hyperdiploidy:* It is defined as 51 to 65 chromosomes per cell or a DNA index larger than 1.16.
- *Hypodiploidy:* Patients with fewer than 45 chromosomes defined as hypodiploidy.

Structural Abnormalities (Chromosomal Translocations)

- *TEL-AML1, (t [12; 21]):* It is seen in 20 to 25 percent of cases of B-precursor ALL at least in the developed countries. The t (12; 21) occurs most commonly in children aged 2 to 9 years.
- *Philadelphia chromosome, (t [9; 22] translocation):* It is present in three percent of children with ALL, and is more common in older patients with precursor B-cell ALL and high WBC count.

- *MLL gene rearrangements:* It is seen in five percent of childhood ALL cases. The t(4; 11) is the most common translocation involving the MLL gene in children with ALL. Patients with t (4; 11) are usually infants with high WBC counts; usually have CNS disease.
- *E2A-PBX1, (t[1; 19] translocation)*: It occurs in five percent of childhood ALL cases. The t(1;19) translocation has higher risk of CNS relapses.

Role of Trephine Biopsy

It is especially required in cases wherein the BM aspirate smears are acellular or dry tap, diluted with peripheral blood, in partially treated cases where blood transfusion/treatment by steroids and other drugs alter the morphological picture or in cases where the sample is degenerated during transport to a referral laboratory for ancillary investigations. It is recommended to obtain a trephine biopsy upfront in all new cases of hematolymphoid malignancies as the paraffin block is an invaluable diagnostic archival material for immunophenotyping.

Organ Function Assessment

Renal function test, liver function test, serum electrolyte, serum lactate dehydrogenase (LDH) with serology especially of hepatitis (B&C), HIV should be done routinely. Total leukocyte count, serum LDH, and extramedullary involvement (hepatosplenomegaly) are the indicators for tumor burden and helps to find patients who need urgent intervention.

Other Tests

Lumbar puncture with cytospin morphologic analysis is performed before systemic chemotherapy is administered to assess for central nervous system (CNS) involvement and to administer intrathecal chemotherapy. In males, USG of testis is done to rule out testicular involvement, if clinically indicated.

PROGNOSTIC FACTORS (TABLE 6)

Outcome of patients with ALL has improved over time though the prognosis depends on multiple factors, still type of treatment and its response is the strongest point for predicting the outcome.

Prognosis depends on various factors:

- The host genotype (host biology)
- The leukemic cell genotype (tumor biology)
- Response to therapy.

A. Host Biology

- Age
- Gender.

Age

The age at diagnosis correlates with clinical outcome. In childhood ALL, infants and adolescents have a worse prognosis than patients aged 1 to 10 years[6]. The improved outcome in patients between 1 to 10 years is due to more frequent occurrence of favorable cytogenetic features in the leukemic blasts including hyperdiploidy, or translocation t(12;21).[7] Infants with ALL have high-risk of treatment failure as they have high presenting leukocyte counts, increased frequency of central nervous system leukemia at presentation and a very high incidence (~80%) of rearrangement of the MLL gene on chromosome 11q23. Amongst infants with MLL gene rearrangements, those

Table 6 Prognostic factors in childhood ALL

		Adverse	*Favorable*
Clinical	Age	<1, >10	1–9.99
	WBC	>50,000	<50,000
	Sex	Boy	Girl
Laboratory	DNA index	<1 (Hypodiploidy)	>1.16 (Hyperdiploidy)
	Immunophenotype	T-ALL, EPB	CALLA+
	Cytogenetics	t (4;11)	TEL-AML
		t (9;22)	Trisomy 4,10,17
Treatment		Inappropriate treatment	Appropriate treatment
In vivo response		Poor ESR*	Good ESR*
(Most important)		MRD** +	MRD** –

*ESR: Early steroid response; **MRD: Minimal residual disease.

presenting at a young age (< 6 months) or with extremely high leukocyte counts (> 300,000/μL) have the worst prognosis.[8]

Adolescents (ages 16-21 years) with ALL have a less favorable outcome than children aged 1 to 10 years, as they frequently present with T-cell immunophenotype, high leukocyte counts, and a higher incidence of the Philadelphia chromosome [t(9;22)].[9] Adolescents are also at higher risk for certain treatment-related complications, such as hyperglycemia, osteonecrosis, pancreatitis and deep vein thromboses, which may also impact prognosis.[10]

Gender

The prognosis for boys with ALL is slightly worse than girls.[11] Potential reason for the better prognosis for girls is the occurrence of testicular relapses among boys. Also, boys appear to be at increased risk of bone marrow and CNS relapse for reasons that are not well understood. With current treatment regimens, there is no difference in outcome between males and females.[12]

B. Tumor Biology

- White blood cell (WBC)
- CNS status at diagnosis
- Testicular involvement at diagnosis
- Immunophenotype
- Cytogenetics
- Numerical abnormalities
- Structural abnormalities (Chromosomal translocations)
- Early response to therapy.

White Blood Cell Count at Diagnosis

White blood cell count reflects tumor burden. Although the relationship between WBC count and prognosis is a continuous rather than a step function, the National Cancer Institute (NCI) stratifies patients into two subsets based on WBC counts; standard risk (WBC count < 50,000) or high-risk (WBC count > 50,000). High WBC count is usually associated with unfavourable chromosomal translocations such as t(4; 11), t(9; 22) and T cell immunophenotype.[13]

Central Nervous System Status at Diagnosis

The presence of CNS disease at diagnosis is an adverse prognostic factor in spite of intensification of therapy. Patients are divided into three categories based on the number of WBC/μL and the presence/absence of blasts on cytospin (Table 7).

The adverse prognostic significance associated with CNS2 status can be overcome by the application of more intensive intrathecal therapy, especially during the induction phase.[12] Furthermore, a traumatic lumbar puncture (more than 10 erythrocytes/μL) that includes blasts at diagnosis appears to be associated with increased risk of CNS relapse and requires intensification of therapy.

Table 7 Definition for central nervous system involvement by leukemia

• CNS-1 No lymphoblasts
• CNS-2 <5 WBCs/μL with definable blasts on cytocentrifuge examination
• CNS-3 ≥5 WBCs/μL with blasts or cranial palsy

Testicular Involvement at Diagnosis

Overt testicular involvement at the time of diagnosis occurs in approximately two percent of males. Historically, testicular involvement at diagnosis was identified as an adverse prognostic factor, but with aggressive therapy it has lost its prognostic significance.[14] Overt testicular involvement is not an independent prognostic factor, despite association with high-risk features.[15]

Immunophenotype

The World Health Organization (WHO) classifies ALL as either "B-lymphoblastic leukemia"(B-ALL) or "T-lineage lymphoblastic leukemia" (T-ALL), based on its cell of origin detected by surface or cytoplasmic expression of B or T-cell antigens. Precursor B-cell ALL is defined by the expression of cytoplasmic CD79a, CD19, HLA-DR, and other B cell-associated antigens. It accounts for 80 to 85 percent of childhood ALL and has a better prognosis compared to T-ALL. Precursor B-cell ALL patients are further divided into immunologic subtypes, of which Pro-B ALL (CD10 negative and no surface or cytoplasmic Ig) is commonly seen in young infants with a t (4; 11) translocation and has a poor outcome. T-cell ALL is defined by expression of the cytoplasmic CD3, with CD7 plus CD2 or CD5 on leukemic blasts. High-risk features at presentation were significantly more frequent in T-ALL as compared to B-lineage ALL.[16]

T-ALL is further divided into immunologic subtypes, of which early T-progenitor (ETP)-ALL (CD1a and CD8 negative, CD5 weak, at least one stem-cell-associated or myeloid-associated antigen) has stem-cell-like features with high-risk of induction failure or relapse.[17]

Myeloid antigen expression: Myeloid-associated antigen expression is associated with specific ALL subgroups (MLL gene and TEL-AML1 gene rearrangement). No independent adverse prognostic significance exists for myeloid-surface antigen expression.[18]

Cytogenetics

Blasts in ALL contain somatically acquired genetic abnormalities that help in understanding pathogenesis and strongly influence prognosis. This includes changes in chromosome number (hyperdiploid/hypodiploid) and chromosomal translocations

Numerical Abnormalities

High hyperdiploidy: High hyperdiploidy generally occurs with clinically favorable prognostic factors (patients aged 1–9 years with a low WBC count) and is itself an independent favorable prognostic factor. Hyperdiploid leukemia cells are particularly susceptible to undergoing apoptosis and accumulate higher levels of methotrexate and its active polyglutamate metabolites which may explain the favorable outcome commonly observed for these cases[19]. Among specific trisomies, patients with triple/trisomies (4, 10, and 17) have been shown to have an improved outcome.[19]

Hypodiploidy: Patients with fewer than 44 chromosomes have a worse outcome than patients with 44 or more chromosomes in their leukemic cells. Cases with 24 to 28 chromosomes (near haploidy) have the worst outcome.[20]

Structural Abnormalities (Chromosomal Translocations)

- *TEL-AML1, (t[12; 21]):* The *t*(12; 21) occurs most commonly in children aged 2 to 9 years.[21] It has good prognosis. However, its impact may be modified by factors such as early response to treatment, NCI risk category, and treatment regimen. There is a higher frequency of late relapses in patients with TEL-AML1 fusion compared with other B-precursor ALL.[22]
- *Philadelphia chromosome, (t[9; 22] translocation):* It is associated with poor prognosis especially in those who present with a high WBC count or have a slow early response to initial therapy. However, its prognosis seems to have improved by incorporation of tyrosine kinase inhibitors, such as imatinib, in the treatment. A COG study, using intensive chemotherapy and concurrent imatinib given daily, demonstrated a 3-year EFS rate of 80.5 percent.[23]
- *MLL gene rearrangements:* The *t*(4; 11) is the most common translocation involving the MLL gene in children with ALL. Patients with *t*(4; 11) are usually infants with high WBC counts; usually have CNS disease and respond poorly to initial therapy. Children with MLL rearrangement have a better prognosis than infants.[24]

  The *t*(11; 19) occurs in one percent of cases and occurs in both early B-lineage and T-cell ALL. Outcome for infants with *t*(11; 19) is poor, but outcome appears favorable in older children with T-cell ALL.
- *E2A-PBX1, t(1;19):* It is associated with pre-B ALL immunophenotype. It was associated with poor prognosis in the context of less intensive anti-metabolite-based therapy in the past, but with most current treatment protocols, the t(1;19) translocation has no adverse prognostic significance except higher risk of CNS relapses.[25]

C. Early Response to Therapy

The kinetics of the reduction in tumor burden in response to treatment has been shown to be highly prognostic of event free survival. Response to therapy is the most reliable prognostic factor, as it reflects leukemic cell drug sensitivity, intensity of therapy, and pharmacogenomic as well as pharmacodynamic features of the host.

Peripheral Blood Response to Steroid Prophase

Patients with a reduction in peripheral blast count to less than 1,000/μL after a 7-day induction prophase with prednisone and one dose of intrathecal methotrexate (good prednisone response) have a more favourable prognosis than patients whose peripheral blast counts remain above 1,000/μL (a poor prednisone response).[26] German Berlin-Frankfurt-Munster (BFM) clinical trials group stratifies its treatment based on early response to the 7-day prednisone prophase.

Day 7 and Day 14 Bone Marrow Responses

Patients who have a rapid reduction in leukemia cells to less than 5% (M1 marrow) in their bone marrow within 7 or 14 days following initiation of multiagent chemotherapy have a more favorable prognosis than do patients who have slower clearance of leukemia cells from the bone marrow.[27]

Peripheral Blood Response to Multiagent Induction Therapy

Patients with persistent circulating leukemic cells at 7 to 10 days after the initiation of multiagent chemotherapy are at increased risk of relapse compared with patients who have clearance of peripheral blasts within 1 week of therapy initiation.[28]

Induction Failure

Five percent of patients do not achieve complete morphologic remission by the end of induction therapy. A cut-off of five percent blasts in the bone marrow is used to determine the remission status. Patients at highest risk of induction failure include T-cell phenotype and patients

with B-precursor ALL with very high presenting leukocyte counts or the Philadelphia chromosome. Induction failure portends a very poor outcome.[29]

Minimal Residual Disease

Bone marrow morphology cannot discriminate well between patients at high-risk of relapse and patients with excellent prognosis. Therefore, more sensitive techniques have been developed for detection of submicroscopic levels (<5%) of malignant cells during and after treatment, i.e. MRD. Minimal residual disease (MRD) is defined as the detection of the clones of cells resistant to the chemotherapy given.

Three types of techniques allow detection of MRD of 10^{-3} to 10^{-6} (1 leukemic cell in 1000 to 1 million cells): Multiparametric flow cytometry (MPFC) for surface phenotype of leukemia cell (sensitivity of 10^{-3}–10^{-4}) can be used in up to 80 to 90 percent patients with ALL; PCR for T-cell receptor or immunoglobulin gene rearrangement or fusion transcripts (sensitivity 10^{-3}– 10^{-5}) can be performed in 90 to 95 percent patients with ALL; PCR Analysis of breakpoint fusion regions of chromosomal aberrations can be performed in 30 to 45 percent patients MRD is the most robust and strongest independent predictor of outcome in children and adolescents with ALL, which is independent of age, sex, immunophenotype, WBC count and treatment group.

MRD discriminates outcome even in subsets of patients defined by cytogenetic abnormalities and other prognostic factors. Patients with higher levels of end-induction MRD (>0.01%) have a poorer prognosis than those with lower or undetectable levels. Therefore, post-induction MRD is utilized as a factor determining the intensity of post-induction treatment. MRD levels at earlier (e.g. day 8 and day 15 of induction) and later time points (e.g. week 12 of therapy) also predict outcome.[30]

Newer Factors

Molecular Genetic Abnormalities

IKAROS/IKZF1 deletions, JAK mutations and kinase expression signatures have been associated with poor prognosis in B cell acute lymphoblastic leukemia.[31-33] Based on combination of gene expression profile and flow cytometric measures of minimal residual disease (MRD), children with high-risk B-precursor ALL can be classified as low, intermediate, and high-risk and thus allow prospective identification of children who respond or fail current treatment regimens. Furthermore, integrated use of both MRD and IKZF1 status allows prediction of 79% of all the relapses with 93% specificity in MRD-medium risk group as compared to 46 and 54 percent of the relapses respectively predicted by use of MRD or IKZF1 alone. The above findings signify that the use of combined parameters enhances the risk stratification, particularly for patients originally classified as nonhigh-risk.[34]

Pharmacogenetics

It is the study of genetic variations in drug-processing genes and individual responses to drugs which enables improved identification of patients at higher risk for either disease relapse or chemotherapy-associated side effects. Patients with ALL who are homozygous for TMPT mutant alleles experience severe or fatal myelotoxicity and increased relapse because of long delays in therapy.[35]

Studies from St Jude Children's Research Hospital (SJCRH) have shown that when patients are treated pharmacologically according to phenotype or genotype, carriers of variant TMPT alleles experience outcomes as good as, or better than, those with wild-type TPMT.[36]

The reduced folate carrier (RFC) is the primary transporter of MTX into cells. RFC expression in leukemic blasts is linked to MTX sensitivity, while defective transport associated with reduced RFC expression is a common mechanism of acquired methotrexate resistance. Increased copies of RFC are present in hyperdiploid blasts and MTX-polyglutamate accumulation in blasts correlates with better outcome in hyperdiploid ALL. The null genotype of glutathione S-transferases, enzymes that catalyze the inactivation of many antileukemic agents, has been associated with a reduced risk of relapse.

RISK STRATIFICATION OF CHILDHOOD ALL

NCI Risk-grouping

Age and WBC count at diagnosis strongly correlates with outcome in B-Precursor ALL. The high predictive value of age and WBC among all studies, and the fact that these variables can be easily and reliably measured by all investigators worldwide, make them one of the most important prognostic factors based on which the patients with ALL are divided into two risk groups:

1. Standard risk	Age 1–9.99 years
	WBC <50000/cumm
2. High-risk	Age >10 years
	WBC >50000/cumm

Children's Oncology Group Risk Stratification

In the current children's oncology group (COG) classification system and treatment algorithm, patients with precursor B-cell ALL are initially assigned to a

standard-risk or high-risk group based on NCI grouping. All children with T-cell phenotype are considered high-risk regardless of age and initial WBC count. Early treatment response, assessed by day 7 or day 14 marrow morphology along with end-induction MRD assessment and cytogenetics is subsequently used to determine the intensity of post-induction therapy. Patients are classified as very high-risk if they have very high-risk cytogenetics with poor response or induction failure as detailed here.

In developing countries outcome of disease is also affected adversely by inadequate supportive care, delay in diagnosis, and poor access to acute care.[2]

MANAGEMENT OF ACUTE LYMPHOBLASTIC LEUKEMIA IN CHILDREN

- Induction chemotherapy for ALL
- Consolidation/Intensification therapy
- Maintenance therapy
- Central nervous system (CNS) therapy

The treatment of childhood acute lymphoblastic leukemia (ALL) has advanced significantly over the past 3 decades, with overall survival rates progressing from 20 to 95 percent.[1,37,38] The standard "backbone" of treatment for ALL has remained unchanged for over 25 years and includes remission induction, consolidation, treatment to prevent overt leukemic infiltration of the central nervous system (CNS directed therapy), and continuing (maintenance) therapy. The steady improvement in survival of children with ALL is a result of a number of modifications of this treatment, the value of which have been confirmed by randomized clinical trials (Tables 8 and 9).

In an effort to appropriately balance the risks and benefits of therapy, "risk-adapted therapy" has been adopted. The NCI criteria risk stratified in to standard and high-risk based on: age, initial white blood cell (WBC) count, and the presence of extramedullary disease at diagnosis.[39]

Currently most cooperative groups use additional risk factors that have been shown to have an impact on patient outcomes (e.g. ploidy, blast karyotype/cytogenetics, and early morphologic response). The resulting classification system thus incorporates the strongest prognostic indicators predictive of outcome, and stratifies the treatment based on their risk of relapse.

Table 8 Principles of childhood ALL therapy

• Treat patients on cooperative group based clinical trials, if possible
• *Adopt effective treatment components of successful clinical trials:* – Reinduction therapy: BFM, CCG – Intensive asparaginase: DFCI – Augmented therapy: BFM – Intensive intrathecal therapy: SJCRH
• *Individualized therapy:* – Risk assessment based mainly on MRD studies – Targeted HD-MTX dose – Mercaptopurine dose based on TPMT, 6TGN and ANC
• *Risk-adapted therapy to decrease late complications:* – Omit cranial irradiation in all patients – Decrease dose of anthracyclines to bare minimum – Avoid use of etoposide

Table 9 Key components of treatment in ALL

• Protocol based therapy
• Risk-directed therapy
• Empiric multiagent induction therapy
• Presymptomatic CNS therapy
• Early intensification of chemotherapy
• Systemic and IT
• Consolidation/intensification
• Early reinduction
• Augmented therapy only in high-risk patients
Late intensification where indicated:
• Delayed intensification
• Double delayed intensification
Extended continuation of treatment:
• Dose intensity of antimetabolites
• Dexa/VCR pulses

Induction Chemotherapy for ALL

Three-drug induction therapy using vincristine, corticosteroid (prednisone or dexamethasone), and L-asparaginase in conjunction with intrathecal (IT) therapy, results in complete remission (CR) rates of greater than 95%. For patients who are at standard risk or low risk of treatment failure, four-drug induction therapy does not appear necessary for favorable outcome provided that adequate post remission intensification therapy is administered. Because of the likelihood of increased toxicity with four-drug induction therapy, many co-operative groups including the Children's Oncology Group (COG), National Cancer Institute (NCI), protocols for standard-risk precursor B-cell acute lymphoblastic leukemia (ALL) utilize a three-drug induction consisting of dexamethasone, vincristine, and PEG-L-asparaginase.

For patients presenting with high-risk features, a more intensive induction regimen (four or five agents)

may result in improved event-free survival (EFS), and such patients generally receive induction therapy that includes an anthracycline (e.g. daunorubicin) in addition to vincristine, prednisone/dexamethasone, plus L-asparaginase.

Consolidation/Intensification Therapy

Once remission has been achieved, systemic treatment in conjunction with central nervous system (CNS) sanctuary therapy follows. The intensity of the post induction chemotherapy varies considerably depending on risk group assignment, but all patients receive some form of intensification following achievement of remission and before beginning maintenance therapy. Intensification may involve use of the following:

Intermediate-dose or high-dose methotrexate with leucovorin rescue or escalating-dose methotrexate without rescue;[40] drugs similar to those used to achieve remission (re-induction or delayed intensification);[41] different drug combinations with little known cross-resistance to the induction therapy drug combination; L-asparaginase for an extended period of time; or combinations of the above.[42]

In children with standard-risk acute lymphoblastic leukemia (ALL), regimens utilizing a limited number of courses of intermediate-dose or high-dose methotrexate as consolidation followed by maintenance therapy (without a re-induction phase) have been used with good results. Similarly favorable results for standard-risk patients have been achieved with regimens utilizing multiple doses of L-asparaginase (20–30 weeks) as consolidation, without any post induction exposure to alkylation agents or anthracyclines.[43]

Post induction consolidation for regimens using a German Berlin-Frankfurt-Munster "BFM-backbone," such as those of the Children's Oncology Group (COG), include a delayed intensification phase, during which patients receive a 4-week re-induction (including anthracycline) and reconsolidation containing cyclophosphamide, cytarabine, and 6-thioguanine given approximately 3 months after remission is achieved. In a Children's Cancer Group (CCG) study, which included a three-drug induction and utilized prednisone as the corticosteroid throughout all treatment phases, two blocks of delayed intensification produced a small event-free survival (EFS) benefit compared with one block of delayed intensification in intermediate-risk patients.[44,45]

In high-risk patients, a number of different approaches have been used with comparable efficacy. Treatment for high-risk patients generally is more intensive than that for standard-risk patients, and typically includes higher cumulative doses of multiple agents, including anthracyclines and/or alkylating agents. The former CCG developed an augmented BFM treatment regimen featuring repeated courses of escalating-dose intravenous methotrexate (without leucovorin rescue) given with vincristine and asparaginase during interim maintenance and additional vincristine/L-asparaginase pulses during initial consolidation and delayed intensification. Augmented therapy also included a second interim maintenance and delayed intensification phase. Of note, there is a significant incidence of osteonecrosis of bone in teenaged patients who receive the augmented BFM regimen.

The augmented BFM regimen has also been evaluated in children with high-risk ALL and a rapid early response to induction therapy. For these children, augmented intensity during consolidation, interim maintenance, and delayed intensification resulted in a higher EFS rate than that achieved with standard-intensity treatment. Increased duration of intensive therapy was not beneficial, and a single application of delayed intensification was as effective as two applications.[46]

For children with Ph+ ALL, imatinib mesylate in conjunction with chemotherapy during post induction therapy has produced a 3-year EFS of 87.7 ± 10.9 percent. These patients fared better than historic controls treated with chemotherapy alone (without imatinib), and at least as well as the other patients on the trial who underwent allogeneic transplantation. Longer follow-up is necessary to determine if this novel treatment improves cure rate or merely prolongs DFS.

Infant ALL is uncommon, representing approximately 2 to 4 percent of cases of childhood ALL. Despite the inclusion of post induction intensification courses with high doses of cytarabine and methotrexate. Long-term EFS rates remain below 50 percent, and for those infants with MLL gene rearrangement, the EFS rates continue to be in the 17 to 40 percent range.[47]

Factors predicting poor outcome for MLL-rearranged infants include a very young age (<6 months), extremely high presenting leukocyte count (300,000/μL), and high levels of MRD at the end of induction and consolidation phases of treatment.[48]

The role of bone marrow transplantation in infants with MLL-rearranged ALL remains controversial.

Maintenance Therapy

(Standard risk and high-risk ALL) The backbone of maintenance therapy in most protocols includes daily oral mercaptopurine and weekly oral methotrexate. On many protocols, intrathecal chemotherapy for CNS sanctuary therapy is continued during maintenance therapy. The use of continuous 6-thioguanine (6-TG) instead of 6-mercaptopurine (6-MP) during the maintenance phase is associated with an increased risk of

hepatic complications, including veno occlusive disease and portal hypertension. Because of the risk of hepatic complications, 6-TG is no longer utilized in maintenance therapy in current protocols.

Pulses of vincristine and corticosteroid are often added to the standard maintenance backbone, A CCG randomized trial demonstrated improved outcome in patients receiving monthly vincristine/prednisone pulses,[49] and a meta-analysis combining data from six clinical trials showed an EFS advantage for vincristine/prednisone pulses. Maintenance chemotherapy generally continues until 2 to 3 years of continuous complete remission. On some studies, boys are treated longer than girls; on others, there is no difference in the duration of treatment based on gender. Extending the duration of maintenance therapy beyond 3 years does not improve outcome.[50]

Central Nervous System Therapy

Options for central nervous system (CNS)-directed therapy include IT chemotherapy, high dose systemic chemotherapy, and cranial radiation. The type of CNS-therapy, i.e. used, is based on a patient's risk of CNS-relapse, with higher-risk patients receiving more intensive treatments. The proportion of patients receiving cranial radiation has decreased significantly over time, with those receiving cranial radiation, the dose has been significantly reduced. IT chemotherapy is usually started at the beginning of induction, intensified during consolidation and, in certain protocols, continued throughout the maintenance phase. IT chemotherapy typically consists of either methotrexate alone or methotrexate with cytarabine and hydrocortisone. Unlike IT cytarabine, IT methotrexate has a significant systemic effect, which may contribute to prevention of marrow relapse.

Systemically administered drugs, such as dexamethasone, L-asparaginase, high-dose methotrexate with leucovorin rescue, and high-dose cytarabine, provide some degree of CNS protection. For example, in a randomized CCG study of standard-risk patients who all received the same dose and schedule of IT methotrexate without cranial irradiation, oral dexamethasone was associated with a 50 percent decrease in the rate of CNS relapse compared with oral prednisone.[51]

RELAPSE OF DISEASE AND ITS MANAGEMENT

- Diagnosing relapse of ALL
- Risk stratification of relapsed ALL
- Treatment of marrow relapse
  - Reinduction therapies after marrow relapse
  - Postremission therapy
  - Continuation chemotherapy
  - Hematopoietic stem cell transplantation (HSCT)
- Treatment of central nervous system relapse
- Isolated testicular relapse.

Diagnosing Relapse of ALL

Suspected patients should undergo bone marrow aspiration with morphology, surface marker studies and cytogenetic studies. Examination of extramedullary sites like CNS, testis, eyes, and skin should be done at the time of diagnosis of relapse.

Risk Stratification of Relapsed ALL

It is important to have risk stratification of relapse for counseling and tailoring the salvage treatment.

- *Length of 1st CR:* Patients with very early (< 18 months from start of therapy) BM relapse have < 10 percent long-term survival (LTS) compared to approximately 50 percent LTS for patients who have late BM relapse.
- *Immunophenotype:* Childhood ALL patients with T-cell ALL fare more poorly than those with CALLA+ ALL.
- *Site of relapse:* Historically, isolated marrow relapse has had the worst prognosis; isolated CNS, testicular, or other extramedullary relapse carried a significantly better prognosis, and combined marrow and extramedullary relapse, an intermediate prognosis
- *The nature and intensity of previous therapy:* Patients previously treated with lower intensity primary therapy have a higher reinduction rate.
- *Minimal residual disease (MRD) studies:* MRD is an important tool even for patients with ALL at time of relapse. The level of minimal residual disease after achieving second remission or before transplant may predict outcomes.[52]

Treatment of Marrow Relapse

Reinduction Therapies after Marrow Relapse

Typical treatment of first relapse involves a combination of vincristine, a glucocorticoid (prednisone, prednisolone, or dexamethasone), and asparaginase, plus an anthracycline, methotrexate, or cytarabine in varying doses and schedules. Only a few randomised trials have investigated reinduction therapy.[53]

In the absence of randomized trials and consistent risk-stratified reporting of patient's outcomes, with possibly exception of mitoxantrone, no reinduction combination is significantly superior to the others.

Postremission Therapy

Therapeutic options after CR2 include further chemotherapy and HSCT. Most pursue HSCT options for patients with early relapses, although outcomes remain poor for most patients with measurable MRD after reinduction. Similar outcomes are reported for matched related donor and matched unrelated donor transplants.

For late marrow relapse, outcomes are similar with chemotherapy and HSCT options. Some recommend HSCT options for patients with late marrow relapse and an MRD-positive CR2. Chemotherapy options may be pursued for isolated extramedullary relapse with success in most patients.

Continuation Chemotherapy

All patients who achieve a second remission receive additional chemotherapy, even if hematopoietic stem cell transplantation is planned. To maintain control of disease, higher dose intensity is used, and higher regimen-related toxicity is tolerated than in first-line treatment.

Most reports describe single-arm studies with combinations of vincristine, glucocorticoids, methotrexate, cytarabine, etoposide, cyclophosphamide or ifosfamide, and thiopurines, with or without maintenance therapy for up to 2 years.

CNS prophylaxis includes high-dose methotrexate or cytarabine, intrathecal chemotherapy, and in more recent BFM group trials, 1200–1800 cGy cranial irradiation.[54]

Hematopoietic Stem Cell Transplantation

The only strategy that has shown to improve outcome of patients with relapsed ALL with high-risk features is to give intensified consolidation chemotherapy regimen, followed by hematopoietic stem cell transplantation (HSCT). Recent data has shown that outcome of HSCT is better than chemotherapy alone even in patients with intermediate risk relapsed ALL and HSCT is currently being offered to these patients if they have an HLA matched sibling donor.

Treatment of Central Nervous System Relapse

A common approach is first to induce a CSF remission with IT chemotherapy, reinstitute systemic therapy, and then later administer craniospinal irradiation, at doses of 2,400 to 3,000 cGy to the cranial vault and 1,200 to 1,800 cGy to the spinal axis.[55]

Isolated Testicular Relapse

Isolated testicular relapse, again, is very rare with modern aggressive protocols. The incidence of testicular relapse is around 2 to 5 percent with current protocols. Effective treatment includes systemic chemotherapy and administration of local radiotherapy. Doses of 2400 cGy to both testes is optimal.

LATE EFFECTS OF THERAPY

Prolonged consequences of ALL therapy should be kept in mind while following these patients and need special after therapy clinic to diagnose and manage these effects.

- *Central nervous system:*
  - Cortical atrophy
  - Necrotizing leukoencephalopathy
  - Subacute leukoencephalopathy
  - Mineralizing microangiopathy
- *Neuroendocrine abnormalities:*
  - Growth hormone deficiency
  - Obesity
- *Cardiac abnormalities:*
  - Cardiomyopathy
  - Late onset congestive heart failure
- *Others toxicities:*
  - Avascular necrosis
  - Primary gonadal failure
  - Second malignancies (SMN)
  - Post-traumatic stress disorder (PTSD)

SUMMARY

Acute lymphoblastic leukemia is the most common malignancy in pediatric age group. With current chemotherapy agents high cure rate can be achieved. Detailed work-up and sophisticated management is required while dealing with child of acute lymphoblastic leukemia. Type of treatment and minimal residual disease (MRD) measurement are promising tool to predict the prognosis. Treatment is based on risk stratification or response evaluation. Though the treatment is prolonged with significant toxicities, still outcome is quite satisfactory. Unlike relapse of other malignant condition pediatric acute lymphoblastic leukemia can be treated with intensive chemotherapy and hematopoietic stem cell transplant with reasonable outcome. Major worry after completion of treatment is post treatment consequence of chemotherapy and radiotherapy which needs special attention.

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CHAPTER 40

Pediatric Acute Myeloid Leukemia

Maya Prasad, Shripad Banavali

Acute myeloid leukemia (AML) is a heterogeneous disease. AML accounts for 15 to 20 percent of all acute leukemias in children.[1] Five-year survival rates for children in the US younger than age 15 years with AML increased from < 20 percent in 1975 to 1978 to 58 percent in 1999-2002.[2] Although there are no systematic statistics for pediatric AML from India, survival outcomes range from 23 to 53.8 percent.[3] The understanding of disease biology has resulted in changes in classification and risk stratification. This, along with improvements in supportive care which allow for more intensive treatment, risk-adapted treatment approaches, and the selective use of hematopoietic stem cell transplant (HSCT) have all contributed to improvements in outcome. However, Pediatric AML has unfortunately not replicated the success story of pediatric acute lymphoblastic leukemia (ALL), and still remains a challenging disease to treat, especially so in resource-poor settings.

BIOLOGY AND PATHOGENESIS

A number of inherited and acquired disorders have been associated with development of AML in children, although the cause is unknown in the vast majority of patients. Inherited conditions like Down syndrome, Fanconi anemia, congenital neutropenia, inherited bone marrow failure syndromes; acquired conditions like aplastic anemia, paroxysmal nocturnal hemoglobinuria, myelodysplastic syndrome as well as environmental exposures to drugs (alkylating agents and topoisomerase inhibitors) and ionizing radiation have been found to have an increased association.

The cancer stem cell model is increasingly being accepted in the pathogenesis of AML—it proposes that AML cells, like normal hematopoietic progenitors, are hierarchically organized into compartments that contain leukemic stem cells (LSCs) that have unlimited self-renewal capacity and are capable of propagating leukemia.[4,5] Multiple genetic mutations have also been implicated in the pathogenesis of AML. These have been broadly classified as type/class 1 and 2 mutations. Class 1 mutations include lesions that confer a proliferative and/or survival advantage by aberrant activation of protein kinases, such as FLT3, cKIT, and RAS, whereas Class 2 mutations impair cell differentiation and confer self-renewal properties and are gene fusions commonly generated by chromosomal translocations, such as t(8;21)(q22;q22)/RUNX1-RUNX1T1 and inv(16)(p13.1;q22)/CBFB-MYH11.[6] Data suggest that class 1 and 2 mutations co-operate to induce leukemia. Currently, more than 90 percent of pediatric AML cases are identified to have at least one known genomic alteration.[7]

CLASSIFICATION

Although, the French-American-British (FAB) classification[8] (based on morphological features) continues to be widely used, the most comprehensive classification of AML is the WHO 2008[9] classification, which includes clinical features, morphological findings, immunophenotyping and cytogenetics, to define specific disease entities. (Table 1). Although, the present classification is for adult population, it is being incorporated slowly in the practice of pediatric oncology.

CLINICAL FEATURES AND DIAGNOSIS

Pediatric AML can have a varied presentation. As in other acute leukemias, patients can present with fever, pallor, bleeding, hepatosplenomegaly and infections.

Children presenting with high leukocyte counts may have symptoms due to CNS or pulmonary leukostasis. Approximately 10 to 20 percent of AML may present with extramedullary myeloid tumors (EMMT, granulocytic sarcoma/chloroma) due to infiltration of tissues with leukemic blasts.[5] Common sites include lymph nodes, skin (leukemia cutis), gums and orbits. These are usually associated with t (8; 21), inv (16) or 11q23 translocation.

Central nervous system (CNS) involvement in the form of CSF involvement or CNS chloromas can occur in approximately 11 percent[10] of children with AML, and is more common in subtypes M4/5, and hyperleukocytosis. Children with AML have a lower number of functional neutrophils and thus may present with infections (including florid), even at diagnosis.

An initial step in diagnosis is examination of complete blood count and peripheral smear, which typically show pancytopenia with circulating blasts. Twenty percent of children may have a WBC count > 100,000/mm^3 at diagnosis.[5]

Table 1 Current classification of myeloid neoplasms (WHO 2008)[9]

1. AML with recurrent genetic abnormalities:
• AML with t (8; 21) (q22; q22), RUNX1-RUNX1T1 (CBFA/ETO). • AML with inv (16) (p13; q22) or t (16; 16) (p13; q22), CBFB-MYH11. • Acute promyelocytic leukemia with t (15; 17) (q22; q11-12), PML-RARA. • AML with t (9; 11) (p22; q23), MLLT3-MLL. • AML with t (6; 9) (p23; q34); DEK-NUP214. • AML (megakaryoblastic) with t (1; 22) (p13; q13), RBM15-MKL1. • AML with mutated NPM1. • AML with mutated CEBPA.
2. AML with myelodysplasia-related features.
3. Therapy-related myeloid neoplasms.
4. AML, not otherwise specified:
• AML with minimal differentiation. • AML without maturation. • AML with maturation. • Acute myelomonocytic leukemia. • Acute monoblastic and monocytic leukemia. • Acute erythroid leukemia. • Acute megakaryoblastic leukemia. • Acute basophilic leukemia. • Acute panmyelosis with myelofibrosis
5. Myeloid sarcoma
6. Myeloid proliferations related to Down syndrome:
• Transient abnormal myelopoiesis. • Myeloid leukemia associated with Down syndrome
7. Blastic plasmacytoid dendritic cell neoplasm.

- Other laboratory features may include electrolyte abnormalities suggestive of tumor lysis (hyperuricemia, hyperkalemia, hyperphosphatemia), although less commonly than in acute lymphoblastic leukemia (ALL). A small proportion of patients also may present with coagulopathies—a DIC like picture may be seen most commonly in acute promyelocytic leukemia (APML) but also in AML M4/5 and in the presence of infection.
- The definitive diagnosis can be made by bone marrow aspirate and trephine biopsy. The differential count is to be done on at 500 bone marrow cells (or 200 cells in peripheral blood). For the diagnosis of AML, there should be 20 percent or more myeloblasts in the peripheral blood or bone marrow. Certain types of translocations, such as t (8;21),t(15;17) and inv 16, are also diagnostic of AML irrespective of blast percentage.[9] On morphology, myeloblasts are classically described as being large and uniform with finely dispersed chromatin, with 1 to 4 nucleoli, granular cytoplasm and the presence of Auer Rods in 60 to 70 percent of cases. Myelodysplastic changes may be seen in cases of secondary AML.
- A bone marrow trephine biopsy is especially required in cases wherein the BM aspirate smears are acellular or dry tap, diluted with peripheral blood, in partially treated cases where blood transfusion/treatment by steroids and other drugs alter the morphological picture or in cases where the sample is degenerated. The paraffin block is an invaluable diagnostic archival material for immunophenotyping and other studies.
- Myeloperoxidase (MPO) stain is specific for the diagnosis of AML and positivity in > 3 percent of blasts is considered diagnostic. Blasts of AML-M0,

Table 2 Initial evaluation of a patient with suspected acute myeloid leukemia

Diagnostic tests/procedures
• Complete blood count with differential count
• Bone marrow aspirate and trephine biopsy
• Lumbar puncture
• Immunophenotyping
• Cytogenetics
• Molecular genetics/translocations/mutations
Additional evaluation
• Physical examination
• Syndromes/constitutional anomalies
• Biochemistry, coagulation
• Chest X-ray, echocardiography

M6 (erythroblasts), M7 (megakaryoblasts) and M5 (monoblasts) are MPO negative. Nonspecific esterase (NSE), alpha naphthyl butyrate (ANB) and alpha naphthyl acetate (ANA), show diffuse cytoplasmic activity in monoblasts and monocytes.

- Immunophenotyping utilizes various lineage-specific monoclonal antibodies that detect antigens on AML cells, and should be used at the time of initial diagnostic workup. The common myeloid markers are CD13, CD33, CD117, CD15, CD16, and MPO. For the diagnosis of monocytic lineage, at least 2 of the following are required: NSE, CD14, CD64, CD11c, and lysozyme Table 2.

The basic work-up of a patient with suspected acute myeloid leukemia is given in Table 2.

RISK STRATIFICATION AND PROGNOSTIC FACTORS

As with ALL, multiple variables have been associated with outcomes in pediatric AML. Host factors such as younger age[11] and constitutional abnormalities (Down syndrome)[12,13] have been found to be favorable. Other factors include disease related features—FAB morphology (M3-favorable; M0 and M7-unfavorable), cytogenetics and molecular markers. The presence of CNS disease has not been found to affect overall survival.[10]

The current focus is on genetic/molecular abnormalities and Minimal residual disease.

- *Cytogenetic abnormalities:* Multiple recurrent cytogenetic abnormalities are described in AML, some of which have a consistent clinical and immunophenotypic profile. These are summarized in Table 3.
- Favorable cytogenetic Abnormalities include t(8; 21), inv(16), and t(16;16). This group is classically described as core binding factor (CBF) leukemias, and found to have a favorable prognosis in many studies[13-18] with overall survival > 90 percent.
- Intermediate risk cytogenetic abnormalities have classically included those with normal cytogenetics, trisomy 8 and a few abnormalities associated with 11q23 translocations – t (1; 11) and t (10; 11).
- Poor risk cytogenetics include monosomy 5 and 7, deletion 5q and 7q, and other less common abnormalities like inv(3), t(3;3), t(6;9) and t(9;22). t(9;11) has been found to be favorable[19] in some studies and unfavorable[16,18] in others.
- *Molecular genetics:* The widespread use of molecular markers has helped further refine the risk stratification of AML. The commonly used mutations/abnormalities include:

Table 3 Risk status based on validated cytogenetic and molecular abnormalities

Risk status	*Cytogenetics*	*Molecular abnormalities*
Better risk	Inv (16) or t (16;16), t (8;21) t 915;17	Normal cytogenetics—with NPM1 mutation or isolated CEBPA mutation in the absence of FLT3-ITD
Intermediate risk	Normal cytogenetics +8 t(9;11) Other nondefined	t (8;21), inv (16), t (16;16); with c-KIT mutation
Poor risk	Complex (>= 3 clonal chromosomal abnormalities) -5, del 5q, -7, del 7q 11q23-non t (9;11) Inv(3), t (3;3), t(6;9).t(9;22)	Normal cytogenetics: with FLT3–ITD mutation

Adapted from the National Comprehensive Clinical Network (NCCN) Clinical Practice Guidelines in Oncology Acute Myeloid Leukaemia v 2.2013[28]

  - *Nucleophosfomin (NPM1) mutations:* These are usually seen associated with normal karyotypes, and carry a favourable prognosis,[20-22] with OS > 80 percent.
  - *FMS-like tyrosine kinase (FLT3)-ITD mutations:* There is strong evidence that these mutations confer a higher risk of relapse in children with AML.[23,24] The ratio of FLT3-ITD to wild type allele > 0.4 is an independent predictor of adverse outcome with a PFS of <10 percent.[27] Outcomes might be improved by HSCT.[25]
  - *c-KIT mutations:* Usually seen in CBF leukemias. Although they confer inferior prognosis in adults, their role in children is not clear.
  - *CEBPA mutations:* Like NPM mutations, they are associated with a normal karyotype and favorable outcome.[26]

Other molecular markers with adverse prognostic significance include WT1 mutations and high expression of BAALC.[23]

- *Minimal residual disease (MRD):* A recent advance in risk adapted approach to AML has been monitoring of MRD, and is currently the most important predictor of outcome. The presence of MRD (with varying cut-offs) at the end of induction has been found to consistently predict a higher rate of relapse, and have been found to be a better predictor of outcome compared to a risk stratification schema based on FAB, cytogenetics and

Day 15 blast percentage.[14,27] MRD levels at later time points do not appear to have any significance.

TREATMENT OF PEDIATRIC AML

Although treatment approaches to pediatric AML have evolved over the past few decades, the outcomes have not paralleled that of pediatric ALL. Improvements in risk stratification (including monitoring of MRD) and supportive care, as well as intensive treatment regimens and the selective use of hematopoietic stem cell transplant (HSCT), have brought up the 5-year event-free survival (EFS) of children with AML to only 40 to 55 percent (Table 4).[29]

Induction Therapy

The primary goal of induction therapy is to achieve a significant reduction of leukemia burden, i.e. achievement of remission defined as a normal peripheral blood cell count (absolute neutrophil count >1,000/mm^3 and platelet count >100,000/mm^3), normocellular marrow with less than 5 percent blasts in the marrow and no signs or symptoms of the disease.[30]

Most treatment regimens use an intensive combination of anthracycline and cytarabine. The classic '3 + 7' regimen (daunorubicin 45 mg/m^2 per day for 3 d; and cytarabine 100 mg/m^2 per day for as continuous infusion for 7 d) was demonstrated to induce remission in 60 to 70 percent of AML patients and became the standard of care for induction therapy in the 1980s.[1]

There have been several attempts to improve on this regimen—these include:

- *Higher doses:* Intensification of cytarabine dose has been most recently studied in the AML02 trial, where the introduction of high-dose (18 g/m^2) versus low-dose cytarabine (2 g/m^2) did not significantly lower the rate of MRD-positivity after induction 1.[14] Similarly, POG 9421 study which compared different doses—high dose cytarabine (1 g/m^2) and standard dose cytarabine in 3+7 regimen failed to show any improvement in remission rates; however 3 y EFS was higher in those who received two courses of high-dose cytarabine.[31]

  Randomized trials in adults comparing high and standard doses of daunorubicin, have had conflicting results, and there is no clear data in pediatric population. Also, dose intensification of anthracyclines has an increased risk of cardiotoxicity, especially in children.
- *Addition of other agents:* Various groups have tried to add other agents in an attempt to improve outcomes, but there has been no convincing effect of benefit. Examples include 6-thioguanine,[32] etoposide,[33] cladribine,[34] fludarabine and gemtuzumab ozogamicin.[35]
- *Dose intensification:* The classic example of dose intensification by compression of timing was demonstrated by the CCG-2891 study[36] where 4-day treatment courses were separated by only 6 days rather than the standard timing of 2 weeks or longer; EFS in the former group was better. Similarly, in the MRC-10 trial,[37] the duration of cytarabine infusion was prolonged to 10 days in order to intensify dose with improved outcomes.

The choice of anthracycline in the treatment of AML has been a topic of debate.[38-40] Certain anthracyclines (idarubcin and mitoxantrone) are favored for their perceived greater antileukemic effect and/or their lower cardiotoxicity, but no anthracycline agent has been demonstrated to be superior.

Postremission Therapy

Most protocols for pediatric AML (including CCG,[33] BFM,[41] POG[42] and MRC[37] groups) consolidate therapy with a backbone of high-dose cytarabine), although the timing, dose and accompanying agents vary considerably. Other strategies used in consolidation include continuous delivery of multiagent low-dose chemotherapy (thioguanine, vincristine, cyclophosphamide, fludarabine), delivery of repeated cycles of myelosuppressive therapy with or without stem cell rescue and allogeneic stem cell transplantation.[1] The optimum number of cycles for post-remission chemotherapy has yet to be determined and probably depends on the therapy used for induction. In the MRC AML 12 trial,[39] children randomly assigned to receive four cycles vs. five had the same EFS and OS rates.

Maintenance Therapy

In the background of more intensive induction and post-remission therapies, most studies[33,43] have shown no additional benefit of additional maintenance therapy in non-M3 AML. However, some groups[44,45] continue to use either oral or parenteral maintenance chemotherapy to improve outcome.

Current Status of Stem Cell Transplantation in Pediatric AML

Although allogeneic SCT has improved outcomes in some subsets of pediatric AML, the role in other subsets as well as the timing, remain a controversial issue. Studies have varied in recommending HSCT in first complete remission (CR1), 2nd complete remission (CR2) or at relapse.

Studies from the North American CCG trials (251, 213 and 2891)[46,47] demonstrated the superiority of allogeneic SCT over autologous SCT, and also that there was no benefit of HSCT in favorable risk cytogenetics. However,

European studies[32,40] have not demonstrated the survival advantage of allogeneic HSCT on outcomes as compared to intensive chemotherapy. This variation in outcomes at different centers has led to varying recommendations. In general, study groups in the United States recommend HSCT for a larger proportion of patients than do the European groups. A meta analysis of co-operative trial groups[48] (POG, CCG and MRC) indicated that HLA matched related donor HSCT is an effective treatment of intermediate risk AML in first CR, but that patients with high risk AML fare poorly even with HSCT.

There is emerging data to suggest that among children with high risk AML, the 5-year OS does not differ according to donor source.[49]

In summary, most pediatric co-operative groups agree that there is no role for HSCT in patients with favorable risk cytogenetics. There is some evidence of benefit for those with intermediate risk disease. However, outcomes remain dismal in high risk disease, and HSCT is of unknown benefit in these patients.[5] The potential benefits of SCT need to be weighed against the risk of transplant related mortality and morbidity. Autologous transplantation is not recommended in the management of pediatric AML.

SUPPORTIVE CARE

Infectious complications remain a major cause of morbidity and mortality in children with AML, both at presentation, as well as following intensive chemotherapy/SCT.[50,51] Both bacterial and fungal infections can be life-threatening. Randomized, controlled trials which demonstrate the role of prophylactic antibiotics in reducing the rates of

Table 4 Result from recent pediatric AML trials (*Source:* Reference 2)

Study	*Years of enrollment*	*Eligible age (years)*	*Number of patients*	*CR rate* (%)*	*Outcome*	*Reference*
MRC AML 10	1988–1995	≤14	341	92	7-year EFS: 48% 7-year OS: 56%	Stevens et al (1998)
LAME 89/91	1988–1996	<20	268	90	6-year EFS: 48% 6-year OS: 56%	Perel et al (2002) Perel et al (2005)
TCCSG M91-13, M96-14	1991–1998	NA	192	89	5-year EFS: 54% 5-year OS: 60%	Tomizawa et al (2007)
AML-BFM93	1933–1998	<18	471	82	5-year EFS: 50% 5-year OS: 58%	Creutzig et al (2001a) Creutzig et al (2001b)
NOPHO-AML93	1993–2000	<18	219	91	7-year EFS: 49% 7-year OS: 64%	Lie et al (2003)
POG9421	1995–1999	≤21	565	89	3-year EFS: 36% 3-year OS: 54%	Gale et al (2005)
MRC AML12	1995–2002	<16	529	92	10-year EFS: 54% 10-year OS: 64%	Gibson et al (2011)
CCG2961	1996–2002	≤21	901	88	5-year EFS: 42% 5-year OS: 52%	Lange et al (2008)
AML-BFM 98	1998–2003	<18	473	88	5-year EFS: 49% 5-year OS: 62%	Creutzig et al (2006) Lehrnbecher et al (2007)
AML99	2000–2002	≤18	240	95	5-year EFS: 62% 5-year OS: 76%	Tsukimoto et al (2009)
SJCRH AML02	2002–2008	≤21	216	94	3-year EFS: 63% 3-year OS: 71%	Rubnitz et al (2010a)
COG AAML0391	2003–2005	≤21	350	87	3-year EFS: 53% 3-year OS: 66%	Cooper et al (2012)
NOPHO-AML 2004	2004–2009	≤18	151	92	3-year EFS: 57% 3-year OS: 69%	Abrahamsson et al (2011)

Abbreviations: BFM: Berlin-Frankfurt-Münster study group; CCG: Children's cancer group; LAME: Leucamie aique myeloide enfant (The French Cooperative AML Group); MRC: Medical research council; NOPHO: Nordic society of paediatric haematology and oncology; POG: Pediatric oncology group; SJCRH: St Jude children's research hospital; TCCSG: Tokyo children' cancer study group; CR: Complete remission; EFS: Even-free survival; OS: Overall survival.
*CR rate after two courses of induction therapy.

bacterial infection in children are lacking.[50] Prophylaxis with intravenous cefepime or a vancomycin regimen, and voriconazole, reduced morbidity in children with AML, and resulted in dramatic decreases in the incidence of septicemia and hospitalization days in a retrospective single center analysis.[52] A current COG open-label, randomized, controlled trial (ACCL0934) is designed to evaluate whether prophylactic therapy with levofloxacin will decrease the incidence of bacteremia in children undergoing intensive chemotherapy/HSCT. On the basis of randomized, controlled trials of prophylactic antifungal therapy in adults with cancer studies, many pediatric oncologists recommend antifungal prophylaxis with voriconazole, posaconazole, micafungin, or caspofungin, which have a broad-spectrum of activity and good tolerability. A recent survey conducted by COG[53] found that Antibacterial and G-CSF prophylaxis reduced infection rates while mandatory hospitalization did not reduce infection or significantly affect nonrelapse mortality.

Novel Therapeutic Agents

With outcomes remaining poor in many subtypes of AML, there is a constant search for newer therapeutic agents. Many new agents (Table 5) with diverse mechanisms of action are currently being studied in relapsed/refractory disease, but only a few are being used in children.

- *Gemtuzumab ozagamicin (GO):* GO is a humanized anti-CD33 antibody conjugated to cytotoxin calicheamicin. It has been evaluated both singly,[54] as well as in combination with chemotherapy, (in previously untreated as well as relapsed/refractory patients) with encouraging results.[14,35] Although, it was approved for use in AML by the US Federal Drug Administration in 2000, it was withdrawn in 2010 when an interim analysis of a randomized trial [SWOG (South Western Oncology Group) 106] in adults demonstrated an increased early death rate in the GO arm of the trial. Judicious use of GO in combination with chemotherapy may improve clinical outcome in selected subgroups of newly diagnosed patients and may also be a useful agent in the treatment of relapsed AML.
- *FLT3 inhibitors:* FLT3 is a trans-membrane enzyme that promotes proliferation after activation by ligand binding and plays an important role in the survival and proliferation of AML blasts. The signaling inhibitors lestaurtinib, midostaurin, quizartinib, and sorafenib are being tested in adult and pediatric trials.
- *Farnesyltransferase inhibitors (FTI):* Farnesylated protein products of the Ras gene family are frequently activated in MDS and AML. Two farnesyltransferase inhibitors, tipifarnib and lonafarnib, are currently under development for the treatment of AML.
- *Epigenetic agents:* Histone deacetylase inhibitors (HDAC) inhibitors

  HDAC inhibitors are a class of agents that modulate the expression of genes by causing an increase in histone acetylation, thereby regulating chromatin structure and transcription. There is increasing evidence to show that HDAC inhibitors and demethylating agents may have a prominent role in the treatment of AML.[55] HDAC inhibitors evaluated in clinical trials in adults with AML/MDS are depsipeptide, vorinostat and valproic acid.[56] A drug which holds promise in resource poor settings is sodium valproate (VPA), which has recently been identified as an antitumor agent with HDAC inhibition. Pediatric phase I studies of decitabine, valproic acid, and SAHA are underway.[57]

TREATMENT OF RELAPSED ACUTE MYELOID LEUKEMIA

Acute myeloid leukemia recurs in 30 to 40 percent of patients and is associated with a poor prognosis; less than one-third of children with relapsed AML survive. The length of first remission (CR1) is an important factor affecting both remission rates, as well as survival; those with CR1 < 1 year have substantially lower rates of both than those with CR1 >1 year.[58] Other prognostic factors following relapse include not having undergone HSCT in CR1, and favorable cytogenetics (t(8;21), t(15;17), and inv(16).[58] The standard approach is to use intensive chemotherapy regimens to achieve remission, followed by consolidation with allogeneic SCT. It is often necessary to administer non-anthracycline based chemotherapy as most patients are heavily pretreated. Various remission induction regimens, including fludarabine plus cytarabine and mitoxantrone plus etoposide,[58,59] have been used in children. The targeted immunotherapy agents such as gemtuzumab ozogamicin and clofarabine along with newer chemotherapeutics as mentioned earlier in this chapter have shown some activity in clinical trials.

ACUTE MYELOID LEUKEMIA IN CHILDREN WITH DOWN SYNDROME

Children with Down syndrome (DS) have a ten-fold to twenty-fold increased risk of leukemia compared to children without DS. The ratio of ALL to AML incidence is similar to that in normal children, except in the first 3 years of life, where AML, particularly the M7 (megakaryoblastic subtype dominate. Typically, AML in DS exhibits a distinctive biology characterized by *GATA1* mutations. Ten percent of neonates with Down syndrome may also develop a transient myeloproliferative disorder (TMD), which typically improves spontaneously within the first 3 months of life. Although, TMD is usually a self-resolving

Table 5 Novel therapeutic agents in the treatment of AML

Class	*Agent(s)*	*Target*
Deoxyadenosine analog	Clofarabine	Ribonucleotide reductase, DNA polymerase, mitochondria
Tyrosine kinase inhibitor	Sorafenib, quizartinib, lestaurtinib, midostaurin, sunitinib	Tyrosine kinase (e.g. FLT3-ITD)
Demethylating agent	Azacitidine, decitabine	DNA methyltransferase
Proteosome inhibitor	Bortezomib	Proteasome
Histone deacetylase inhibitor	Valproic acid, vorinostat, panobinostat, depsipeptide	Histone deacetylase
Farnesyltransferase inhibitor	Tipifarnib, lonafarnib	Ras, lamin A
Janus kinase (JAK) inhibitor	Ruxolitinib, TG101348	JAK
Chemokine receptor (CXCR4) antagonist	Plerixafor	CXCL12/CXCR4 axis
Apoptosis inducer	Obatoclax, oblimersen	BCL2
Angiogenesis inhibitor	Bevacizumab	Vascular endothelial growth factor (VEGF)
Multidrug resistance inhibitor	Cyclosporine	P-glycoprotein
Lineage-specific antibody	Anti-CD33 (mylotarg), -CD45, -CD66	Lineage-specific antigen
Immune therapy	NK cells, T cells	Leukemia cells

condition, it can be associated with significant morbidity and may be fatal in 10 to 20 percent of affected infants. DS children with leukemia frequently experience higher levels of treatment-related toxicity, including cardiac and infectious complications; with an increased sensitivity to cytarabine and daunorubicin. The outcome is generally favourable (EFS > 80%), especially in children aged 4 years or younger at diagnosis. Appropriate therapy for these children is less intensive than current AML therapy, and hematopoietic stem cell transplant is not indicated in first remission. Since, this is an exhaustive topic beyond the scope of this chapter, interested readers may refer to these publications.[12,13,60-62]

Treatment of AML in children with Down syndrome is a large topic in itself, which is why we have given the references of standard review articles on this topic.

Therapy-related Acute Myeloid Leukemia

The development of AML following treatment with ionizing radiation or chemotherapy, particularly alkylating agents and topoisomerase inhibitors, is termed therapy-related AML (t-AML). The risk of t-AML/t-MDS is related to the cumulative doses of chemotherapy agents received, as well as the dose and field of radiation administered. High cumulative doses of either epipodophyllotoxins (e.g. etoposide or teniposide) or alkylating agents (e.g. mechlorethamine, melphalan, busulfan, and cyclophosphamide) are implicated. T-AML (and t-MDS) resulting from epipodophyllotoxins and other topoisomerase II inhibitors (e.g. anthracyclines) usually occur within 2 years of exposure and are commonly associated with chromosome 11q23 abnormalities whereas t-AML following exposure to alkylating agents or ionizing radiation often occurs 5 to 7 years later and is commonly associated with monosomies or deletions of chromosomes 5 and 7.

Treatment of t-AML is challenging due to the following due to previous treatment, and poor remission rates resulting from adverse cytogenetics. HSCT is the only curative option, although rates of transplant related morbidity and mortality are high. Studies describing treatment outcomes in t-AML have not been encouraging.[63,64]

ACUTE PROMYELOCYTIC LEUKEMIA

Although acute promyelocytic leukemia (APML) was conventionally classified as AML FAB M3, it is considered to be a separate entity in view of its unique morphology, cytogenetics and molecular characteristics. Cytogenetically, it is characterized by a balanced translocation between the PML gene on chromosome 15 and the RARA gene on chromosome 17, i.e. t(15;17). This translocation leads to the PML-RARA transcript which leads to maturation arrest at the promyelocyte stage. This translocation is also a target for therapy, and the discovery of all transretinoic acid (ATRA, which induces differentiation of

leukemic blasts into mature granulocytes) has dramatically improved outcomes in APML.[65]

Acute promyelocytic leukemia in children behaves similarly to that in adults, and commonly presents with coagulopathy and bleeding manifestations. Notable differences include a higher incidence of hyperleukocytosis (WBC count higher than 10×10^9/L) and microgranular (M3v) variant. Coagulopathy and hemorrhage are more common in M3v.[6]

The risk stratification of APML is based on WBC count and platelet count at presentation.[66] High-risk patients have presenting WBC count $>10 \times 10^9$/L, intermediate risk WBC count $<10 \times 10^9$/L and platelet count $<40 \times 10^9$/L; and low-risk patients WBC count $<10 \times 10^9$/L with a platelet count of $>40 \times 10^9$/L. *FLT3* mutations are observed in 40 to 50 percent of APL cases, and are associated with both hyperleukocytosis and M3v, and an increased risk of induction death, and of treatment failure.[67]

The initial management in APML includes stabilization and correction of coagulopathy with platelets and fresh frozen plasma +/– cryoprecipitate The standard of care for treatment of APML is induction with sequential combination of ATRA followed by chemotherapy (usually anthracycline), which has been found superior to either agent used singly. This is followed by 2 to 3 cycles of anthracycline based consolidation therapy.[65-68] In contrast to other subtypes of AML, patients with APML have been found to benefit from (ATRA based) maintenance therapy.

Another drug which is being widely used in the management of APML is arsenic trioxide, either singly or in combination with ATRA in newly diagnosed and relapsed patients.[69-71] HSCT is an option in persistent MRD as well as relapsed disease. Interestingly in APML; autologous transplant also has satisfactory outcomes in the absence of a suitable donor.

For detailed guidelines on the management, please look up references.[72,73]

CONCLUSION AND FUTURE DIRECTIONS

In spite of remarkable advances in the field of diagnosis and treatment of pediatric AML, the survival of these patients is not more that 50 to 60 percent. Better understanding of the biology of disease will allow more personalized and risk-adapted treatment, thus improving outcomes while minimizing toxicities. In resource poor settings such as ours, early detection and appropriate emergency management at primary/secondary care level is woefully lacking. Very few centers are equipped to manage AML. Late presentations, financial and other logistical constraints, treatment abandonment and refusal all contribute to poor outcomes. Improvements in existing infrastructure, as well as scrupulous supportive care measures, along with a risk-adapted approach, will hopefully improve outcomes in children with AML.[74]

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CHAPTER 41

Chronic Myeloid Leukemia

Nirav Thacker, Brijesh Arora

Chronic myeloid leukemia (CML) is clonal hematopoietic stem cell disorder involving the entire myeloid lineage and at least some of the lymphoid lines. It is characterized by myeloid hyperplasia of the bone marrow, extramedullary hematopoiesis, leukocytosis (presence of complete range of granulocyte precursors in peripheral blood) and a specific cytogenetic signature.

CML was a model disease from its discovery: the word "leukemia" (white blood) was coined to describe the neoplastic nature of colorless corpuscles or leukocythemia seen in the blood of these patients by Virchow in 1845.[1] CML usually progresses from a chronic phase through an accelerated phase to a myeloid/lymphoid blast crisis (BC), which depicts the molecular "multi-hit" theory of oncogenesis. CML was the first neoplasm associated with a chromosomal aberration, known as the Philadelphia chromosome (Ph).[2] The elucidation of molecular pathogenesis of this disease led to development of specific therapy, making CML a model of targeted therapy for human malignancies.

EPIDEMIOLOGY

CML is primarily a disease of middle age; peak incidence being in the 4th and 5th decade of life with annual incidence of 1–2 cases per 100,000 population/year. In children, CML comprises 3 percent of newly diagnosed leukemia. It is even rarer in children younger than 4 years with 80 percent of cases of pediatric CML diagnosed after 4 years and 60 percent after 6 years of age.[3] As per the population-based registry of Mumbai, proportion of CML is 5.1 percent of all newly diagnosed leukemias in females and 2.5 percent in males.[4]

There is no ethnic or genetic predisposition. Ionizing radiation is a risk factor for development of the disease with an increased incidence in radiologists, survivors of atomic explosions and in persons exposed to therapeutic radiation. However, radiation and other environmental exposures have not been demonstrated to be causal in children.

PATHOPHYSIOLOGY

CML is disease of hematopoietic stem cells, having cytogenetic hallmark in form of Ph chromosome, which results from t(9:22) (q34;q11) balanced reciprocal translocation. This translocation leads to juxtaposition of the *ABL1* gene from chromosome 9 and *BCR* gene from chromosome 22, resulting in formation of the *BCR-ABL* fusion oncogene that encodes a chimeric protein with a constitutive tyrosine kinase activity (Fig. 1).[1]

ABL1: The wild type of *ABL1 gene* encodes a 145-kd protein that is predominantly located in the nucleus and is universally active in hematopoietic cells at all stages of differentiation. The ABL1 protein participates in signal transduction and regulation of gene transcription, also acting as a tyrosine kinase.

BCR: The BCR region is a part of a much larger gene known as BCR, which encodes for a 160-kd protein.

BCR-ABL1: This fusion gene encodes tumor specific 210-kd hybrid protein that differs from normal ABL1 kinase in following aspects:

- It has higher and constitutive TK activity
- It has ability to autophosphorylate
- It is translocated to cytoplasm, thereby exposing it to new spectrum of substrates
- It binds to F-actin.

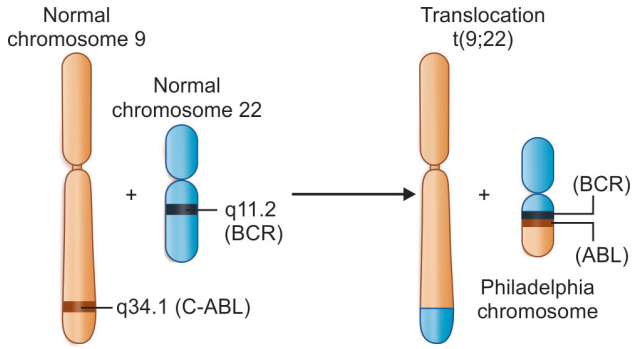


Fig. 1 The Philadelphia chromosome

Formation of BCR-ABL1 fusion protein in CML activates numerous downstream signaling pathways including RAS pathway leading to increased transcription of *MYC* and *BCL-X* genes, activation of BCL-2 by abrogation of inhibitory effect of interferon consensus sequence-binding protein and activation of RAC family kinases such as CRKL, ERK as well as c-Jun N-terminal kinase, which leads to increased proliferation and survival of myeloid progenitors.

There are 3 main breakpoint cluster regions (m-bcr, M-bcr, and µ-bcr) in BCR and ABL1 contains 2 alternative first exons (1b and 1a) with different breakpoints. The combination of breakpoints within *BCR* and *ABL1* genes generates at least 3 different fusion transcripts encoding proteins with distinct molecular weights (p210, p190, p230) that are associated with different neoplastic phenotypes (Table 1). p190 is found to be associated with Ph+ acute leukemia in almost 80 percent of pediatric patients and 50 percent of adults; and has got five times higher tyrosine kinase activity as compared to p210 protein.

Biology of CML

CML is an acquired clonal disorder of unicellular origin, where the target of neoplastic transformation is multilineage stem cell with potential for generating all hemic cell lines and in some instances lymphoid lineages. An initial triggering event (possibly rearrangement of C-ABL) may occur prior to the formation of BCR-ABL fusion protein, which is acquired at some point in the disease evolution. The cytogenetic changes in CML precursor cells are expressed in its progeny as multiple cytologic abnormalities that confer a distinct survival advantage, in turn producing a neoplastic clone (Table 2). Overtime approximately 80 percent of patients with CML develop additional nonrandom cytogenetic abnormalities in Ph cell, i.e. "clonal evolution", which reflects the genetic instability and transformation to advanced-phase CML.[5] The most common of these are trisomy 8, isochromosome 17, and duplicate Ph chromosome. At the molecular level, most common mutations associated with CML progression are p53 (25%–30% of patients with myeloid BC) and INK4A/ARF exon 2 (50% of cases of lymphoid BC).[6]

Table 1 BCR-ABL1: Alternate splicing patterns

Fusion gene	*Region*	*Clinical phenotype*
p190 kd*	Minor BCR	Ph positive ALL
p210 kd	Major BCR	Positive CML
p230 kd	µ-BCR	Chronic neutrophilic leukemia
*Has 5-fold higher TK activity than p 210		

Clinical Features

The natural history of CML is triphasic progressing through 3 phases, from a predominantly mature hyperproliferative phase called 'chronic phase' (CP) through an advanced 'accelerated phase' (AP) to a predominantly immature 'blast crisis' (BC), as defined in Table 3. Approximately, 95 percent of children with CML present in CP and only 5 percent in advanced phases.[7] Chronic phase usually lasts for 3 years. Sudden onset of blastic phase, i.e. within 3 months of previously documented complete hematological response can rarely occur at a rate of 0.4 to 2.6 percent in first 3 years with interferon[8] and 0.7 percent on imatinib.

Table 2 Cytologic abnormalities in CML

Abnormality	*Consequence*
Distorted adhesive kinetics	Reduced stromal/stem cell interaction and abrogation of normal cell surface signal maturation
Discordant nucleocytoplasmic maturation	Extension of late progenitor proliferative phase
Resistance to apoptosis	Increased survival and accumulation
Abnormalities in feedback regulation • Production of inhibitory molecules • Insensitivity to feedback inhibition	 Suppression of normal HSC Selective growth advantage
Altered cell kinetics	Prolonged survival/increased accumulation

Table 3 WHO criteria for chronic, accelerated and blast phase[9]

CML–CP	*CML–AP*	*CML–BC*
Documentation of t(9,22) or BCR-ABL fusion gene	Blasts 10–19% of peripheral blood WBC or bone marrow (BM)	Blasts >20% of WBC or BM cells
Bone marrow blast <10%	Peripheral blood basophils at least 20%	Extramedullary blast proliferation
Does not meet criteria for AP or BC	Persistent thrombocytopenia unrelated to therapy	Large foci/clusters of blasts in BM biopsy
	Increasing spleen, or WBC count or high platelets unresponsive to therapy Cytogenetic evidence of clonal evolution	
	Megakaryocytic in sheets/clusters, with marked fibrosis, and/or severe granulocytic dysplasia	

Symptoms and Signs

Patients in CML-CP usually present with complains of fever, night sweats, fatigue, asthenia, and left upper quadrant pain. Complications in form of priapism, neurologic dysfunction or respiratory distress can be seen with hyperleukocytosis.

On examination, patient would usually have pallor, massive splenomegaly, and hepatomegaly. Papilledema, retinal hemorrhages, visual loss, and tachypnea can be seen in patients presenting with hyperleukocytosis. The onset of accelerated phase is marked by progressive systemic symptoms and increasing splenomegaly despite therapy. Occasionally, the first manifestation of progression could be extramedullary, i.e. meningeal leukemia or a chloroma. In patients with extreme basophilia, histaminemic symptoms would be present in form of cold urticaria, pruritis and gastric ulceration. Blast crisis usually presents with signs and symptoms of acute leukemia.

Investigations

- *Complete blood counts and peripheral smear (Fig. 2):* Chronic phase CML is generally characterized by a normocytic normochromic anemia, hyperleukocytosis (mean WBC count of 2.5 lac/mm^3 which is usually higher than in adults) with predominant neutrophilia with or without thrombocytosis (mean: 5,00,000/cumm). Peripheral smear shows myeloid cells in all stages of differentiation with predominant neutrophils, increased absolute basophil and eosinophil count; however, myeloblasts and promyelocytes account for less than 15 percent of differential count. Accelerated phase manifests as increasing leukocyte/platelet counts with higher proportion of blasts between 10 to 19 percent and more than 20 percent basophils. Blast crisis usually manifests as pancytopenia and more than 20 percent blasts. The characteristic biochemical abnormality of the granulocytes in CML is reduced leukocyte alkaline phosphatase (LAP) score.
- *Bone marrow examination (Fig. 3):* In CML-CP, bone marrow is hypercellular with granulocytic and megakaryocytic hyperplasia and less than 10 percent blasts. Accelerated phase has 10 to 19 percent blasts and blast crisis is characterized by >20 percent blasts. Myelofibrosis may be seen in 30 to 40 percent of cases during the course of disease.

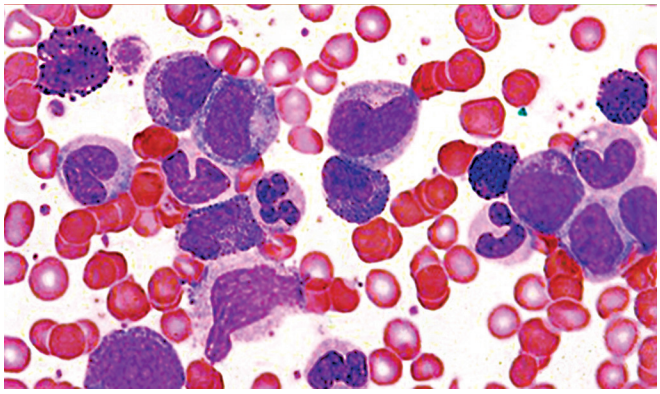


Fig. 2 Peripheral blood picture of CML-CP

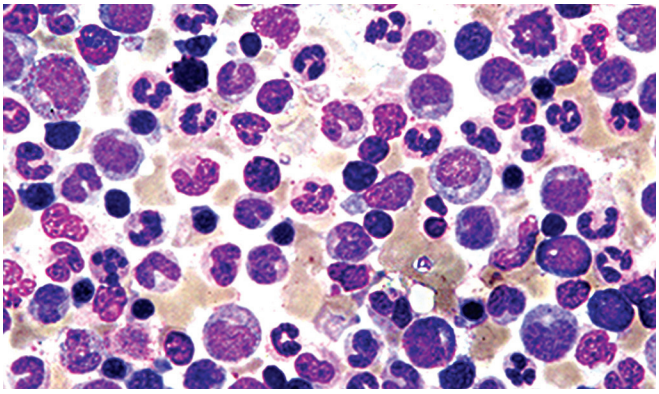
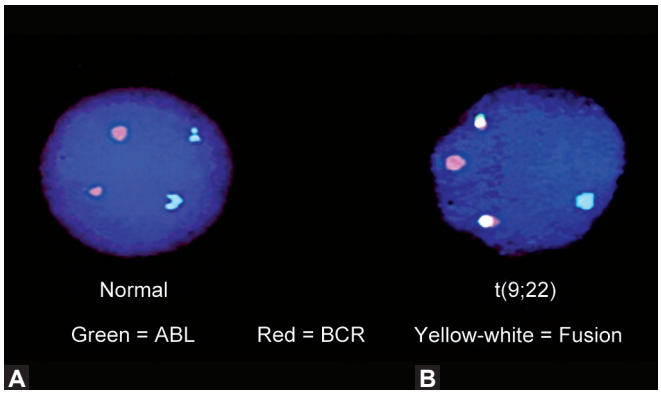


Fig. 3 Bone marrow picture of CML-CP



Figs 4A and B Interphase FISH (A) 2 Red (BCR) and 2 green (ABL) signals of normal nucleus; (B) Two yellow-white fusion signal corresponds to Ph chromosome

- *Cytogenetics:* The "gold standard" for the diagnosis of CML is either the demonstration of the Philadelphia chromosome by conventional cytogenetic techniques, or the demonstration of the products of the underlying t(9;22) translocation, namely the BCR-ABL fusion mRNA or the BCR-ABL protein.
- *Conventional cytogenetics:* Conventional bone marrow cytogenetics should be done for initial workup, as it not only confirms presence of Ph chromosome but also detects other clonal abnormalities that may indicate advanced phase CML.
- *Fluorescence in situ hybridization technique (FISH):* FISH employs large DNA probes linked to fluorophores; it permits direct detection of the chromosomal position of the *BCR* and *ABL1* genes when employed with metaphase chromosome preparations. Its advantage is that it can also be utilized on interphase cells from bone marrow or peripheral blood, in which physical co-localization of BCR and ABL probes is indicative of the presence of the BCR-ABL fusion gene (Figs 4A and B).
- *Reverse transcription polymerase chain reaction (RT-PCR):* Reverse-transcription polymerase chain reaction (RT-PCR) is a highly sensitive technique that employs specific primers to amplify a DNA fragment from BCR-ABL mRNA transcripts. Depending upon the combination of primers used, the method can detect the e1a2, e13a2 (b2a2), e14a2 (b3a2) and e19a2 fusion genes. The use of nested primers and sequential PCR reactions makes the technique extremely sensitive, capable of routine detection of one Ph positive cell in 10^5 to 10^6 normal cells. Quantitative RT-PCR is recommended before starting therapy as well as for monitoring response to therapy.

DIFFERENTIAL DIAGNOSIS

Leukemoid reaction, juvenile myelomonocytic leukemia (JMML) and other myeloproliferative disorders may mimic CML.

- Leukemoid reaction usually has an obvious inflammatory focus, a high LAP score, a less marked splenomegaly and absence of Ph chromosome. JMML is generally characterized by greater involvement of skin, lymphoid tissue and monocytic cell line as compared to CML, with lesser leukocytosis and splenomegaly, and absence of Ph chromosome, though the LAP score may be low.
- CML may be differentiated from other myeloproliferative disorders by more pronounced involvement of the granulocytic cells and the presence of Ph chromosome.
- Blast crisis mostly follows chronic phase and does not pose diagnostic dilemma. However, children who initially present in BC need to be differentiated from *de novo* acute leukemia and *de novo* acute Ph positive leukemia. A combination of massive splenomegaly, basophilia and Ph chromosome helps distinguish BC of CML with *de novo* leukemias. However, BC of CML and *de novo* Ph positive acute leukemias need to be differentiated at molecular and cytogenetic level. Ph-positive acute leukemia will produce a 190 kd protein, while CML-BC produces a 210 kd protein and has specific nonrandom cytogenetic aberrations.

Prognostic Factors

The major predictors of survival are phase of disease and duration of chronic phase. Unlike adults; spleen size, burden of white cells, platelets, basophils or blast percentage in peripheral blood have not been found to be prognostically useful in children and hence prognostic score like Sokal, Hasford or EUTOS are not applicable in children. Early response to imatinib has been found to be prognostic in recent studies.

Treatment (Fig. 5)

The therapy for CML is has evolved significantly over time from earlier (Phase-1) palliative approaches such as arsenic, splenic RT, busulphan and hydroxyurea (late 1860s–1970) to phase-II of aggressive nontargeted curative approaches such as allogenic stem cell transplant, interferon with or without cytarabine (1970s–2000) finally to the current phase of targeted molecular therapies such as imatinib, dasatinib, and other tyrosine kinase inhibitors.

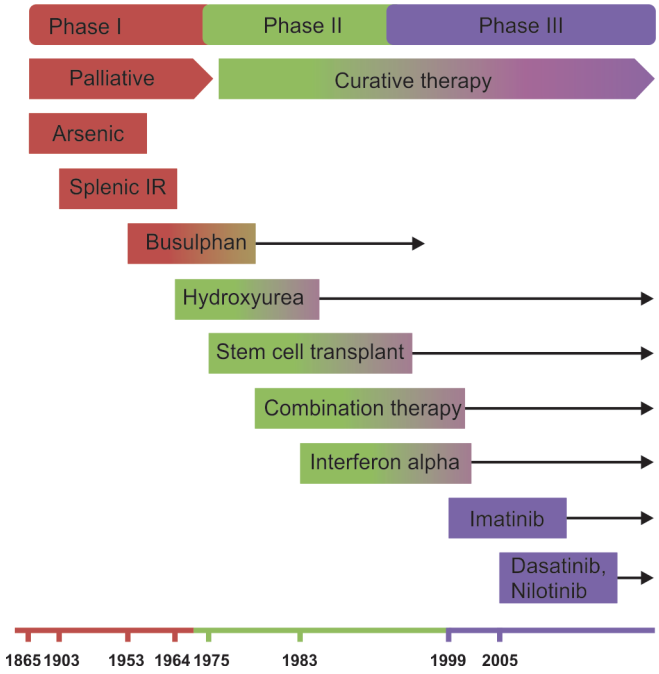


Fig. 5 Evolution of treatment for CML

Medical Management

The initial goal of therapy in CML is to reduce the leukocytosis and organomegaly. Early agents like busulphan and hydroxyurea did achieve this goal but could not achieve cytogenetic remission. Interferon was the first drug to achieve a significant cytogenetic remission. Since its introduction, tyrosine kinase inhibitors have become the frontline therapy and helped achieve rapid and durable complete cytogenetic and molecular remissions.

Hydroxyurea

It is an inhibitor of ribonucleoside diphosphate reductase, preventing conversion of ribonucleotides to deoxyribonucleotides and interferes with DNA synthesis during the S phase. Recommended starting dose is 25 to 50 mg/kg, which is further adjusted according to hematological response.

Interferon (IFN)

In 1980s, interferon with or without cytarabine (AraC) constituted standard management of patient awaiting transplant or not eligible for transplant. IFN exerts antiproliferative effect on myeloid precursors, in particular on those in the late progenitor phase which is preferentially expanded in CML. In addition, it also has an immunomodulatory action.[10,11] In the scant pediatric data available, nearly 58 percent pediatric patients treated with interferon achieved complete hematological response, 50 percent achieved major cytogenetic response and 14 percent showed complete cytogenetic responses, with an overall survival of 60 percent at 8 years.[12,13] In one study combination of IFN and AraC was found to be superior to IFN alone.[14]

Tyrosine Kinase Inhibitors (TKI)

The unique disease biology of CML paved way for the development of first molecular targeted therapy in human cancers in the form of imatinib mesylate which has changed the entire outlook towards management of CML, receiving an accelerated FDA approval for pediatric use in 2003. Imatinib, a small 2-aminopyridine molecule, competitively inhibits the inactive configuration of the BCR-ABL protein tyrosine kinase by blocking the ATP binding site and thereby preventing a conformational change to the active form. It inhibits cellular proliferation without inducing apoptosis, producing a 92 to 98 percent decrease in CML colony growth *in vitro* without inhibiting normal colony growth. Imatinib also inhibits platelet-derived growth factor (PDGFR) and c-kit, but not the SRC family kinases.[15,16] The second generation TKIs inhibit both BCR-ABL and other signaling pathways. Dasatinib and bosutunib inhibit both BCR-ABL and SRC kinases. Nilotinib, like imatinib, is an inhibitor of BCR-ABL, c-kit, and PDGFR. Both nilotinib and dasatinib are >100-fold more potent than imatinib *in vitro*.

Dose of TKI

The recommended standard dose of imatinib in children is 260 to 340 mg/m^2 (maximum absolute dose 400 mg/day) which gives drug exposures similar to the 400 to 600 mg adult dosage levels. The recommended pediatric dose for CML-AP is 400 mg/m^2 daily (maximum absolute dose, 600 mg) and for CML-BC is 500 mg/m^2 daily (maximum absolute dose, 800 mg). The anticipated decrease in the white cell count may be observed not earlier than 2 weeks after the start of treatment and complete hematological response is usually achieved after a median of 4 weeks.

Pediatric experience with second generation TKI is limited though dasatinib is being evaluated in pediatric phase II trial (NCT00777036) with a dose of 60 mg/m^2 daily for CML-CP and 80 mg/m^2 daily for more advanced phases. Dosage recommendations for children concerning

nilotinib cannot yet be made. The dose approved for treating adults is 300 to 400 mg twice daily. Table 4 details the starting dose and administration guidelines for TKI.

Response Rates and Response Assessment to TKI

Effectiveness of therapy with TKI is assessed based on the achievement of landmark responses, i.e. hematologic, cytogenetic and molecular responses as defined in Table 5. The response achieved in relation to time, i.e. milestones is used to grade response as optimal, suboptimal and failure, providing basis for timely alteration of therapy as detailed in Table 6.[17,18]

The pediatric TKI treatment results are comparable to the results achieved in adults. Data from pediatric trials have shown that with use of imatinib in CML-CP, 96 percent achieve CHR and 69 percent achieve CCyR after

Table 4 Starting doses and instruction for administration of TKI[25]

TKI	*Dose (mg/m²)*	*Instructions*
Imatinib	340, OD	To be dispersed in water or apple juice, 50 mL for 100 mg tablet. Take with water or food to avoid esophageal irritation
Dasatinib	60–80, OD	Dissolve in 30 mL lemonade/preservative-free apple or orange juice
Nilotinib*	170–230, BD	Capsules may be dispersed in 5 mL of apple juice/sauce and ingested immediately on an empty stomach, abstain from eating for at least 1 hour

* Pediatric dosing has not been established but is based on 300 mg or 400 mg twice daily, if less than or greater than 40 kg, respectively.

Table 5 Landmark responses to treatment[17,18]

Hematological response (CBC and Clinical)	*Cytogenetic response (Ph+metaphases)*	*Molecular response (BCR-ABL transcripts)*
Complete: 1. WBC <10 ×10³/L 2. Platelets <450×10⁹/L 3. Differential with no immature granulocyte and <5% basophils 4. Spleen not palpable	*Complete:* 0% *Partial:* 1–35% Minor 36–65% Minimal 66–95%	*Complete:* Transcripts not detectable, or >3-log reduction in transcript from diagnosis *Major:* <0.1%

Table 6 Milestones of response in CML-CP[17,18] with therapeutic option

Time (Months)	*Optimal*	*Suboptimal*	*Failure*
Diagnosis	NA	NA	NA
3	CHR and minor CyR	CHR and no CyR	No CHR
6	Partial CyR	Minor/Minimal CyR	No CyR
12	Complete CyR	Partial CyR	Less than partial CyR
18	MMolR	<MMolR	<Complete CyR
Anytime	Stable or improving MMolR	Loss of MMolR, BCR-ABL kinase domain mutations still sensitive to imatinib, >0.05% increase in transcript levels	Loss of CHR, CCyR, new chromosome abnormalities in presence of Ph+, BCR-ABL kinase domain mutations insensitive to TKI
Management Consideration[13-15]	Continue same with monitoring	Increase dose of imatinib Introduce second generation TKI	Introduce second generation TKI Consider HSCT

Table 7 Response rates to imatinib in pediatric CML trials

Study	*Dose*	*CHR (%)*	*CCyR (%) At months (m)*	*MMR (%) At months (m)*	*OS At months (m)*
COG-phase I (N = 20)[19]	260–570	100	83	NA	78.5% (24 m)
European phase II (N = 30)[26]	260–340	80^ 75*	60^ 29*	50 0*	95^ (12 m) 75* (12 m)
French national (N = 44)[20+]	260	98	62 (12 m)	31 (12 m)	98 (36 m)
AAML0123 (N = 50)[23+]	340	78	91 (9 m)		98 (12 m)
CML-PAED II (N = 51)[21+]	300**	95	93 (12 m)	85 (18 m)	–
I-CML-Ped (N = 150)[27+]	–	–	63 (12 m)	awaited	97 (42 m)

^ : In CP, *: advanced phase, ** : 400 for AP, 500 for BC
+ : Patients treated upfront with imatinib

1 year [19-23] as depicted in Table 7. Imatinib is more effective in CML-CP with overall survival of 95 percent at 1 year as compared to 75 percent in advanced phases.[26]

Dasision and ENESTnd Trial in treatment naïve CML adults have demonstrated a significantly faster as well as deeper responses and better transformation free survival with dasatinib and nilotinib as compared to imatinib, however, not showing a difference in the PFS and OS at 3 and 4 years respectively.[28-31] There is very scant data and little experience with second generation TKI in pediatric population, with two phase 1 trials confirming effectiveness of dasatinib in pediatric age group.[24,33] In the COG phase 1 trial in the dose range of 60 to 110 mg/m^2 once daily, dasatinib could achieve CCyR in 3 and partial CyR in 3 of 8 evaluable patients with resistant CML-CP.[24] In another CA180-018 phase I study of 17 children with resistant CML-CP, 14 (82%) achieved complete cytogenetic response (CCyR) and eight (47%) achieved major molecular response. Of 17 patients with advanced-phase CML or Ph-positive ALL, six (35%) achieved CHR and 11 (65%) achieved CCyR.[33]

Intolerance and Resistance to TKI

Up to one-fourth of patients either may not tolerate or may show resistance to TKI.

Intolerance

A patient is considered to be intolerant to therapy when a nonhematologic toxicity of at least grade three recurs despite appropriate dose reductions and optimal symptomatic management. In general <5 percent of patients are intolerant to imatinib therapy.

Resistance

It is divided into two categories:

1. *Primary resistance:* It is defined as failure to achieve a timely response (Table 6). It has been seen in 10 to 20 percent of CML-CP pediatric trials of imatinib. It could be due to inadequate inhibition of tyrosine kinase or BCR-ABL mutations.
2. *Secondary resistance:* Loss of a previously achieved response constitutes secondary resistance. It occurs in 80, 50, and 15 percent of patients in blast crisis, accelerated phase, or chronic phase, respectively after 2 years of imatinib therapy. It is due to reactivation of BCR-ABL signaling (mutation of BCR-ABL, imatinib excretion, overexpression of BCR-ABL) or activation of other signaling pathways including SRC kinases.

Managing Resistance

Once resistance is suspected, the disease status should be re-evaluated. Compliance and tolerance to therapy should be confirmed in patients suspected to have resistance, as none of the patients with compliance ≤80 percent to imatinib could achieve a MMR in past studies. The trough plasma imatinib levels should be obtained since level <1000 ng/mL is associated with higher rate of progression of disease. In addition, mutational analysis of BCR-ABL should be performed. Primary resistance due to inadequate inhibition of tyrosine kinase can be overcome by increasing the dose of imatinib. Resistance due to mutation can be overcome by changing the TKI known to be active for the particular mutation keeping the side effect profile and comorbidities in the child in mind.

Toxicity of TKI

Imatinib is generally well tolerated in children. Table 8 enlists the major acute side effects with TKI as well as their management. Generally, the acute side effects of TKI tend to be manageable and decrease over time.

However, the developing organs and potentially prolonged use of TKI, known to act on off target kinases involved in normal function of organs, in pediatric patients makes them more particularly vulnerable to long-term side effects of TKI, many of which may not be apparent for years (Table 9). Among these, most concerning long-term side effects are growth retardation, impact on bone health, and cardiac dysfunction. Growth delay has been demonstrated in many recent studies due to disturbance in GH:IGF-1 axis. Similarly, altered bone mineralization has been shown in few studies. The cardiac dysfunction is still a topic of debate, however, definite *in-vitro* evidence definitely warrants more evaluation. Thus, TKI administration in children requires close monitoring of side effects.[25] Hence, the risk and benefits of TKI therapy should be weighed against the alternative options available, including allogeneic SCT.

Allogenic Stem Cell Transplant (Allo-SCT)

Prior to imatinib, allogenic stem cell transplant was the treatment of choice for CML and was usually performed during the early chronic phase since outcome of Allo-SCT in BC is dismal with less than 20 percent long-term survival.[32] Allo-SCT is the only known curative treatment available for children with CML. In most studies of pediatric patients, the survival ranges between 60 to 80 percent with better results in matched sibling donors compared to voluntary unrelated donors. However, owing to the complications of high early mortality, growth failure, infertility, graft versus host disease, metabolic syndrome, and secondary malignancies associated with Allo-SCT, it has become a form of rescue treatment for patients in advanced CML (AP/BC), treatment failure after receiving second generation TKI, and patients with T315I tyrosine kinase mutation (till proven role of ponatinib).

However, in developing countries, where one time cost of transplant is significantly lesser as compared to potentially lifelong therapy with TKI and good contemporary outcome and potential cure with matched sibling donor transplant, it remains an attractive option in

Table 8 Major acute side effects of TKI[25]

TKI side effects	*Recommended intervention*
Hematologic toxicity: Neutropenia, anemia, or thrombocytopenia	Hold TKI until count recovery for up to 2 weeks; G-CSF may be administered to treat neutropenia; restart at full dose if cytopenia persists < 2 weeks; reduce dose by 20% if longer than 2 weeks
Rash	Observation; consider topical steroids
Elevated liver function tests	Observation, avoid ibuprofen
Muscle cramps	Check CK and electrolytes, consider electrolyte repletion
Nausea, vomiting	Supportive care, consider ondansetron
Headache	Supportive care

Table 9 Chronic side effects of TKI[25]

TKI side effects	*Recommended intervention*
Cardiac toxicity *Imatinib:* Possible, not proven *Dasatinib:* Possible, not proven *Nilotinib:* QT prolongation and sudden death have been reported	Consider ECG, echocardiogram, troponin, electrolytes, if there is clinical concern Do not use nilotinib with history of cardiac or electrolyte problems
Effusions *Dasatinib:* Pleural effusions	Hold TKI; if multiple sites of edema, give diuretics; and if severe, thoracocentesis and brief course of steroids
Decreased height/growth retardation	Closely monitor height, GH stimulation tests and IGF-1 levels
Poor bone health	Closely monitor calcium and phosphorus; vitamin D repletion as necessary
Teratogen	Avoid TKIs during pregnancy

Table 10 Laboratory monitoring on TKI therapy

Test	*Recommendation*
Bone marrow cytogenetics/FISH	At diagnosis to establish disease phase and additional mutations, if any Every 3 months till complete CyR Rising levels of BCR-ABL(1 log) without MMR
Quantitative RT-PCR (peripheral blood)	At diagnosis to determine baseline transcript type and levels Every 3 month after cytogenetic CR till MMR, then 6–12 monthly Rising levels of BCR-ABL (1 log) with MMR then 1–3 monthly
TKD mutations	Failure to reach (suboptimal response) or maintain CHR, CCyR, or MMR; rise in quantitative PCR after MMR; cytogenetic relapse or increase in Ph chromosomes, if CCyR not obtained

CML-CP in presence of a matched sibling donor. Recent studies have shown promising early results with reduced intensity conditioning transplants in children with CML with significantly lower long-term morbidity. Also, preliminary data using imatinib for cytoreduction pre-transplant has demonstrated a significantly lower risk of death attributable to the lower disease burden at the time of transplant making it an attractive strategy. Also, imatinib has shown to be effective in relapse post-transplant as well as maintenance post-transplant, with most centers using 1 year of imatinib post-transplant to reduce the risk of disease relapse.

Guidelines for Management of CML in Children

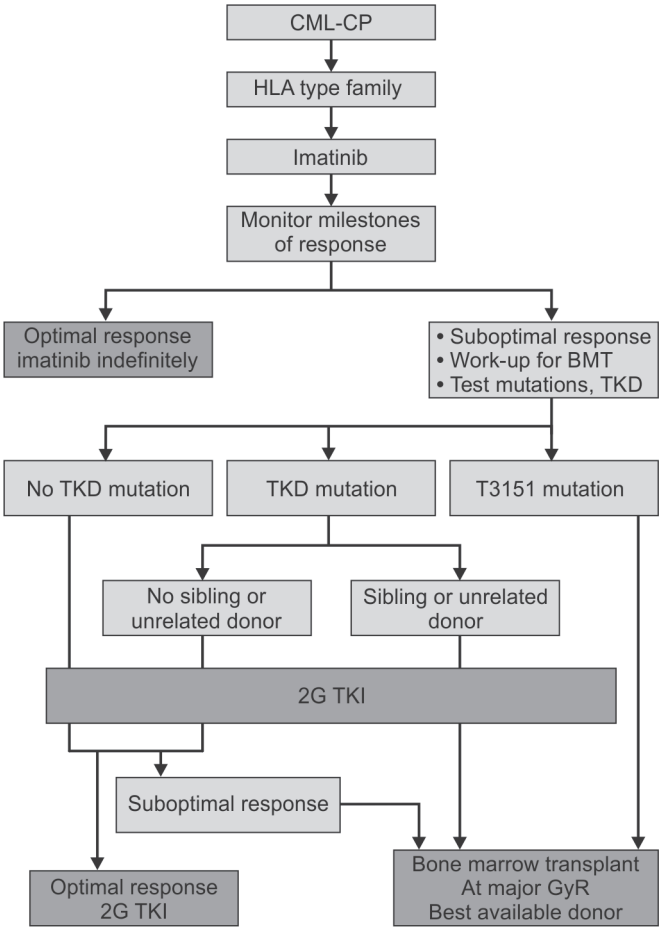
Chronic Phase CML (Flow chart 1)

Initiate treatment with hydration, hydroxyurea, allopurinol and start imatinib at 340 mg/m2 per day after confirmation of diagnosis. Monitor disease status through CBC, differential count and peripheral blood FISH every 3 months till achievement of cytogenetic remission followed by quantitative RQ-PCR every 3 month till MMR, then 6 to 12 monthly (Table 10). If child fails to achieve optimal response (Table 6), check for compliance, imatinib levels, BCR-ABL mutations and switch to dasatinib 60 mg/m^2 per day if none or sensitive mutations are present and initiate screening for related and unrelated allogeneic stem cell donors. If second line-TKI is not affordable/available, or child is noncompliant with low-serum levels consider increasing the dose of imatinib and follow for response. In children with resistant mutations (T315I), or progression or relapse on dasatinib, allo-SCT should be done with the best available donor as soon as possible.

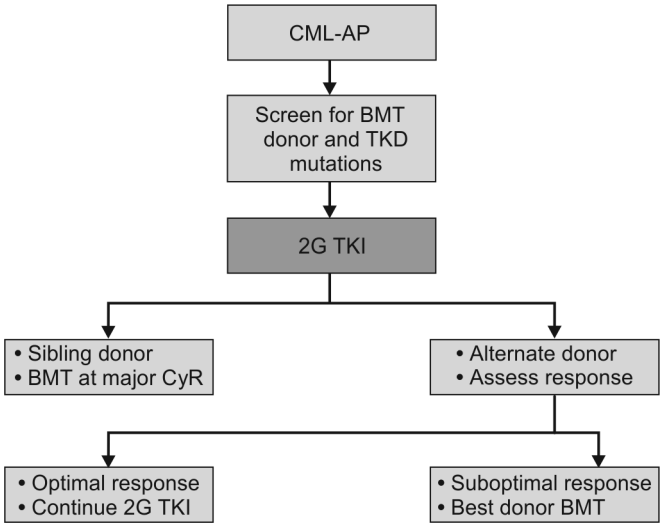
Accelerated Phase CML (Flow chart 2)

Start imatinib 400 mg/m^2 or dasatinib at 80 mg/m^2 per day in 2 divided doses (preferred). Initiate search for HLA-matched sibling or unrelated donors and proceed to myeloablative allogeneic SCT if sibling donor is available after achievement of remission. If patient fails or has a suboptimal response at any time (Table 6), proceed immediately to myeloablative allogeneic SCT with best available donor. These patients may benefit from post-transplant maintenance with TKI in view of high risk of relapse.

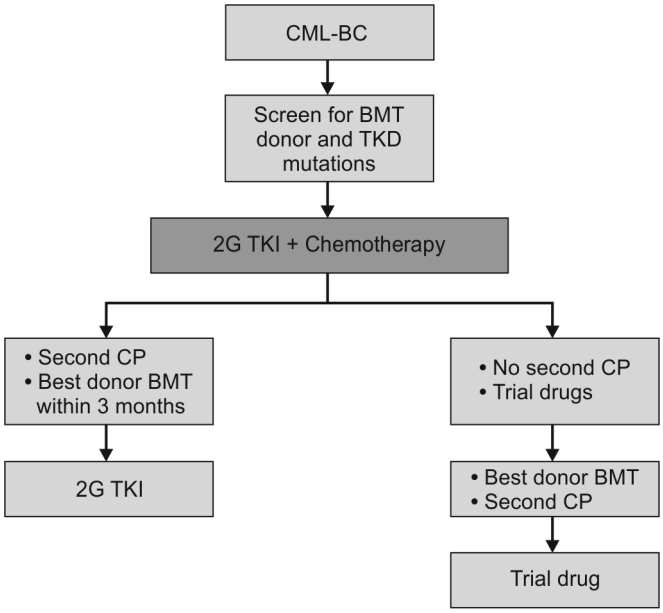
Flow chart 1 Management of CML-CP in children



Flow chart 2 Management of CML-AP in children



Flow chart 3 Management of CML-BC in children



Blast Crisis CML (Flow chart 3)

Start imatinib at 500 mg/m^2 or dasatinib at 80 mg/m^2 per day in 2 divided doses (preferred) along with appropriate anti-leukemic regimen based on type of blast crisis and screen for HLA-matched sibling or unrelated donors. Once in remission or second chronic phase, move to myeloablative allogeneic SCT with best available donor. These patients may benefit from post-transplant maintenance with TKI in view of high-risk of relapse.

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CHAPTER 42

Juvenile Myelomonocytic Leukemia

Gaurav Narula, Nirmalya D Pradhan

Juvenile myelomonocytic leukemia (JMML) is a clonal panmyelopathy and a unique kind of chronic myeloproliferative disorder seen almost exclusively in infants and young children. It is characterized by myeloid proliferation, especially in the monocytic lineage and occurs due to well-characterized molecular defects at the genetic level which directly affect granulocyte macrophage-colony stimulating factor (GM-CSF) invoked downstream signaling pathways involved in cellular proliferation—especially the RAS pathways. This results in a progressive organ infiltration of the spleen, liver and finally lungs in a progressive, relentless march to fatality if untreated. Allogeneic Hematopoietic Stem Cell Transplantation (Allo-HSCT) is the only known cure resulting in long-term remission. The unique leukemogenesis model of JMML has provided in depth understanding of cancer cytogentics and offers a wide array of potential targeted therapies that may have applications across several malignancies.

Juvenile myelomonocytic leukemia (JMML) is a clonal panmyelopathy, which presents with leukocytosis, splenomegaly and organ infiltration due to excessive proliferation of cells of the monocytic and granulocytic lineages. It is one of those enigmatic diseases of childhood that has evaded categorization and generated considerable confusion over its classification over the years until recently. It was first broadly categorized under "myeloproliferative" disorders, a term coined by Dameshek in 1951 when he observed that different chronic proliferative disorders sharing similar clinical and hematologic features often developed a more aggressive form over the course of their illness,[1] and has jumped categories till its molecular basis has been better understood in recent times.

HISTORY AND CLASSIFICATION

Earlier termed a chronic myeloproliferative disorder, it was only after the discovery of the Philadelphia chromosome by Nowell and Hungerford in 1960[2] which characterized adult-type chronic myeloid leukemia (CML), that it was shown that this unique anomaly was absent in children and infants with a similar clinical picture.[3] Hardisty in 1964 had coined the term "juvenile CML" (jCML), which stayed in popular parlance for several decades since,[4,5] leading to much confusion over its classification and its persistent identification as the "ph negative" chronic myeloid disorder. Subsequently, the finding of elevated HbF levels,[6] and the identification of monosomy 7 as a unique chromosomal anomaly in many infants and young children with recalcitrant myeloid proliferations and acute leukemias[7] led to further disorders being broadly clubbed under this entity.

After the French-American-British (FAB) group had successfully classified MDS in adults, there was a move to extend the same for the pediatric entity and called it chronic myelomonocytic leukemia (CMML) instead of jCML. In 1997, Niemeyer reported one of the largest series to date and described in painstaking of CMML in childhood, jCML and the infant monosomy 7 syndrome highlighting their common biological features.[8] Later leading an international group she proposed the term JMML to include all these entities and established its diagnostic criteria.[9] The 2001 classification system of the World Health Organization (WHO) grouped these into a separate category of myelodysplastic/myeloproliferative disorders as it reflects both elements, i.e. dysplastic and proliferative features of the myeloid cells.[10] With the recent identification of certain genetic mutations in JMML, a revised set of diagnostic criteria was proposed by the

International JMML Working Group in 2006.[11] The existing criteria and the proposed ones are given in Tables 1 and 2 respectively.

EPIDEMIOLOGY

From the few population-based studies, it can be derived that JMML represents about 2 to 3 percent of leukemias in children, and has an annual incidence of 1.2/million children per year.[12,13] JMML predominates in infants and toddlers with a median age at diagnosis of 1.8 years, and close to 85 percent of all cases are diagnosed from 4 months to 6 years of age. A male preponderance is seen through all age groups at with a sex ratio of about 2:1.[8] Few reports exist of a familial occurrence in twins pairs and other siblings.[14,15]

JMML is closely associated with neurofibromatosis type 1 (NF1).[16-19] These patients have a 200- to 350-fold higher risk of developing JMML.[8,19] While about 11 percent of patients with a clinical diagnosis of NF1 develop JMML, another 15 percent of JMML patients without a clinical diagnosis of NF1 have mutations in the NF1 gene.[14,20,21] A few cases of JMML have been associated with Noonan syndrome (NS), which is diagnosed in about 2 percent of JMML patients registered. In all such cases a heterozygous PTPN11 mutation, a gene that encodes the nonreceptor protein tyrosine phosphatase SHP-2 was observed.[22]

Table 1 Current JMML diagnostic criteria (2nd International JMML Working Group[11])

All of the following	*At least 2 of the following*
Absence of the t(9;22) BCR/ABL fusion gene	Circulating myeloid precursors
Absolute monocyte count >1000/micL	White blood cell > 10,000/micL
<20% blasts in the bone marrow	Elevated fetal hemoglobin (HbF)
	GM-CSF hypersensitivity

Table 2 Proposed criteria of the 2nd International JMML Working Group[11]

Category 1	*Category 2*	*Category 3*
All of the following	*At least 1 of the following*	*At least 2 of the following*
Splenomegaly	Somatic mutations in RAS or PTPN11	Circulating myeloid precursors
Absolute monocyte count >1000/μL	Clinical diagnosis of NF1 or NF1 gene mutation	WBC >10,000/μL
Blasts in PB/BM <20%	Monosomy 7	Elevated fetal hemoglobin (HbF) for age
Absence of the t(9;22) BCR/ABL fusion gene		Clonal cytogenetic abnormalities excluding monosomy 7
Age less than 13 years		

PATHOPHYSIOLOGY AND GENETICS

As previously noted, JMML came to be defined among the group of disorders primarily identified by the absence of the Philadelphia chromosome. In contrast monosomy 7 was identified in about 25 percent of patients, other abnormalities in 10 percent and a normal karyotype in 65 percent.[8,14,23] When monosomy 7 is present, it is generally the sole abnormality. Among the chromosomal abnormalities other than monosomy 7, loss of material on the long arm of chromosome 7 is the most frequent.[8]

While JMML involves the myeloid, erythroid and megakaryocytic lineages and transforms into myeloid leukemias, clonal involvement of the B-lymphoid lineage is also seen, including lymphoid blast crisis.[24,25] It may be gathered that malignant transformation takes place at a stage of a committed stem cell that has the ability for myeloid, as well as early B-lymphoid differentiation. Involvement of T-lymphoid precursors has only been reported in one child with JMML and T-cell lymphoma.[26]

Molecular Aspects

Molecular pathways in JMML have been extensively studied and still draw interest as they serve as models for studying leukemogenesis. Several molecular events that activate RAS dependent signaling pathways and deregulate growth and survival of leukemic cells, have been described in JMML[27,28] (Fig. 1), primarily including mutations in the genes encoding RAS, NF1 and SHP-2. In addition Casitas B Lymphoma (CBL) is also increasingly recognized as a causative gene.[29] Figure 1 also highlights the targets of JMML therapy covered later in this text.

ONCOGENIC RAS MUTATIONS

A hypersensitivity of JMML cells to GM-CSF has been well-established and was used as a diagnostic test for its confirmation long before the molecular pathways responsible became apparent. A specific defect in the GM-CSF signal transduction pathway was postulated to drive the pathogenesis of this disease. However, no abnormalities in the GM-CSF receptor (GM-CSFR) could be found, but a host of abnormalities in the

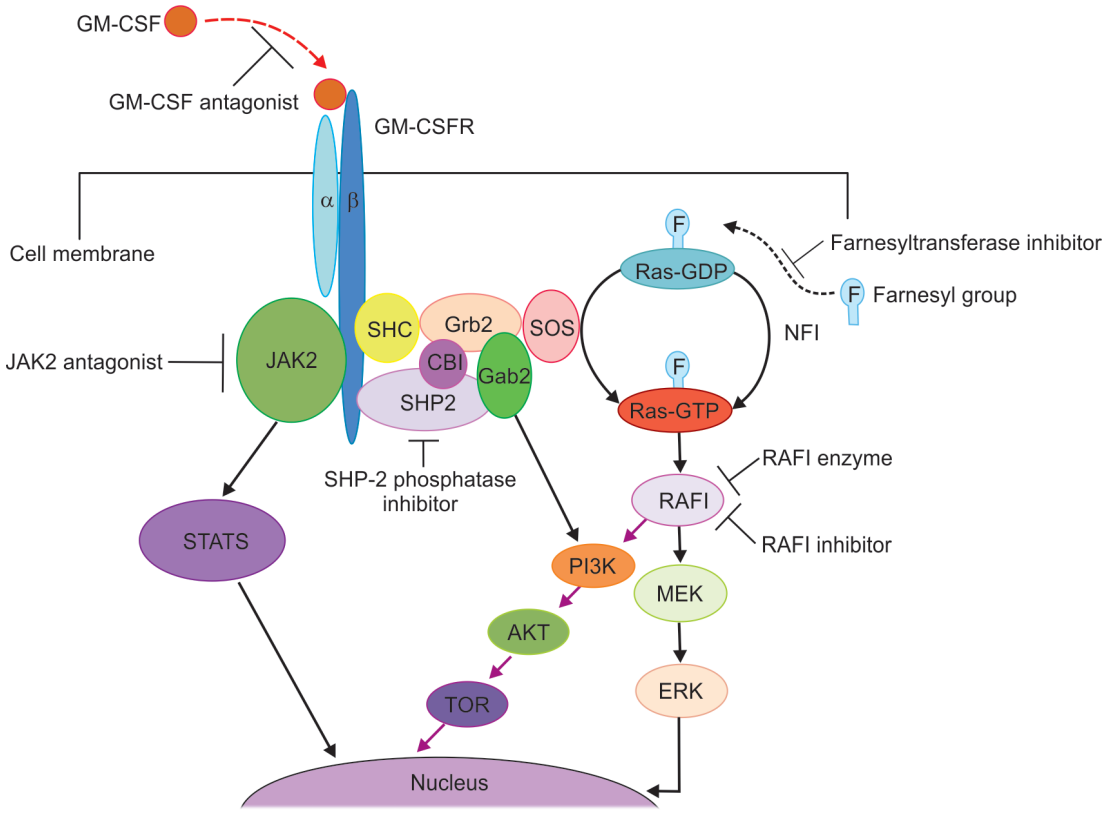

Fig. 1 Molecular activation pathways in JMML and the potential therapeutic targets[28]

downstream pathways were soon identified in the RAS/MAPK pathway.[27] The RAS family of signaling proteins regulate cellular proliferation by cycling between an active guanosine triphosphate (GTP)-bound state (RAS-GTP) and an inactive guanosine diphosphate (RAS-GDP)-bound state. Mutant RAS alleles encode proteins that accumulate in the GTP-bound conformation because of defective GPT hydrolysis. Common sites of these mutations along with other candidate genes are summarized in Table 3.[27,30] Oncogenic point mutations of NRAS and KRAS are seen in 15 to 20 percent of children with JMML.[27,30]

Other genetic pathways include NF1 in neurofibromatosis and PTPN11 in Noonan syndrome. A group of GTPase- activating proteins (GAPs) facilitate the conversion from active RAS-GTP to the inactive RAS GDP state. Neurofibromin, the protein encoded by the gene for NF1, functions as GAP and negatively regulates RAS.[27,28,30] As described above, 25 to 30 percent of JMML cases carry the clinical diagnosis of NF1[14] or are known to harbor NF1 gene mutations.[20,21] Extensive studies in the field by Shannon and co-workers who demonstrated loss of heterozygosity (LOH) by loss of the normal NF1 allele from the bone marrow of children with type 1 neurofibromatosis and malignant myeloid disorders,[31] were instrumental in proving that NF1 works as a myeloid tumor suppressor gene (TSG) that functions by negatively regulating RAS signaling.

Table 3 Summary of gene mutations in JMML[28]

Gene	*Sites of mutation*	*Frequency*
PTPN11	E76K, D61Y, D61V, E69K, A72T, A72V, E76V/G/A	35%
RAS NRAS KRAS HRAS	 Codons 12 and 13 Codon 13 No mutation in codons 12, 13, and 61 was found	25%
NF1	Loss of wild-type NF1 allele	11–15%
CBL	Codons 371, 380, 381, 384, 396, 398, 404, and 408. Splice sites 1227, 1228, and 1096	17%

Noonan syndrome (NS): It is a heterogeneous disorder defined by short stature, facial dysmorphia, cardiac defects (most commonly pulmonic stenosis and hypertrophic cardiomyopathy), skeletal defects, mental retardation

and bleeding diathesis, in which JMML is an occasional association, NS is caused by germline mutations in PTPN11, the gene encoding the nonreceptor protein tyrosine phosphatase (PTP) SHP-2, 130 a member of a small subfamily of cytoplasmic src-homology 2 (SH-2) domain containing PTPases.[32] It is required for hematopoietic cell development and participates in signal transduction of a number of cytokines, including GM-CSF and IL-3.[30,32] Binding of the SH2 domain to phosphorylated tyrosine residues induces a conformational shift that relieves the inhibitory interaction between the SH-2 domain and the catalytic PTP domain. Heterozygous germline mutations of PTPN11 are present in children with NS and JMML.[22] PTPN11 mutations also represent a major molecular event in nonsyndromic JMML. About 35 percent of patients with JMML harbor somatic mutations in PTPN11.[28,30]

The Casitas B-cell lymphoma (CBL, c-CBL) protein, is also now increasingly recognized to have a causative role in JMML.[29] First reported by Loh, et al. c-CBL mutations have been detected in up to 17 percent of cases.[29,30] The germ line mutation represents the first hit, with somatic loss of heterozygosity being the second hit positively selected in JMML cells. Individuals with germ line CBL mutations are at increased risk of developing JMML, which might follow an aggressive clinical course or resolve without treatment.[33,34]

CLINICAL FEATURES

The typical presentation is that of an infant with progressive pallor, fever, infection, petechiae, cough and progressive abdominal distention due to splenomegaly and hepatomegaly.[8] Occasionally, the spleen may be normal at diagnosis, but will rapidly increase thereafter. However, most patients presenting in the author's experience have had large spleens with varying degree of hypersplenism (personal experience, unpublished data). Lymphadenopathy is fairly common too, a feature which distinguishes it from CML.[8,23] The tonsils may be markedly enlarged due to infiltration. Dry cough, tachypnea and interstitial infiltrates on chest X-ray are signs of peribronchial and interstitial pulmonary infiltrates. Patients with advanced disease frequently have cachexia. Skin is usually involved by eczematous eruptions or erythematous maculopapules on the face, trunk, and hands.[23,35] Indurated raised lesions with central clearing,[35] and petechiae may also be seen.

In addition to these often nonspecific lesions, juvenile xanthogranulomas composed of numerous foamy cells may be seen in JMML. They are present by the end of the second year of life and are often multiple.[36] In some but not all children, xanthogranulomas are associated with multiple cafe au lait spots and the clinical diagnosis of NF1.[17] Abdominal distension and discomfort are generally due to hepatosplenomegaly. Gut infiltrates may predispose to diarrhea and gastrointestinal infections. Unlike acute monoblastic leukemia, JMML rarely involves the central nervous system (CNS). A small number of patients with CNS chloroma[8] and with ocular infiltrates[37] have been described. Pituitary infiltration and diabetes insipidus, responsive to antileukemic therapy, has also been described.[38] In addition, clinical features of NF1 with multiple cafe au lait spots, and the characteristic dysmorphisms of NS may be seen in 11 percent and 7 percent of JMML cases respectively.[8]

HEMATOLOGICAL AND LABORATORY FEATURES

- The hematologic profile is characterized by leukocytosis, anemia and thrombocytopenia. Unlike CML, the median white blood cell (WBC) count is 33×10^9/L and rarely exceeds 100×10^9/L.[12,25,47] Rarely the counts may be less than 10×10^9/L, especially in children with monosomy 7.[8,14]
- Both mature and immature myeloid lineage cells are seen along with a characteristic monocytosis, often with dysplastic cell forms. An absolute monocyte count of more than 1×10^9/L has been retained as a diagnostic criteria (Table 2).[9,11]
- Occasionally, eosinophilia and basophilia may be seen.
- The median blast cell percentage in PB smears is less than 2 percent[8] and rarely exceeds 20 percent.
- In about 14 percent of children, the platelet count at diagnosis is below 20×10^9/L.
- Most patients have a hemoglobin level between 7 and 11 g/dL. The reticulocyte count and the number of normoblasts vary over a wide range. Red cells are generally normocytic, while macrocytosis is noted in some patients with monosomy 7. Microcytosis may be due to iron deficiency, but can often be noted even in the absence of laboratory detected deficiency.[8]

  Bone marrow (BM) aspirate shows increased cellularity with predominance of granulocytic cells in all stages of maturation. Monocytosis in BM is usually less than in PB, with a median of 10 percent cells.[8] The BM blast count is moderately elevated, but is more than 10 percent in only 10 percent of patients.[8,10] Dysplasia of granulocytes is usually minimal, with hypogranulation of neutrophil cytoplasm and pseudo-Pelger-Huët forms.[10] Besides macrocytic differentiation in a few cases, erythroid cells mature normally. Megakaryocytes are reduced or absent in about 75 percent of children.[8] Cytochemical and immunophenotypic studies are not specific, but might be helpful in identifying the

monocytic population.[10] Because smears of PB and BM provide sufficient information, BM biopsy may be omitted in most cases. Reticulin fibrosis has been noted in biopsies of some patients.[8,39]

- HbF synthesis is increased especially in those with a normal karyotype resulting from a high number of circulating F cells. In addition, other fetal red cell characteristics, such as increased expression of the I antigen and decreased carbonic anhydrase levels, are present.[40] Despite these changes, maturation of red cells does not seem to be compromised.
- While clinical features of patients with monosomy 7 and those with a normal karyotype are similar,[8] hematological differences are often seen. Monosomy 7 patients have a lower median WBC but similar absolute monocyte count, red blood cells are often macrocytic, and erythropoiesis in BM is more pronounced. In addition, they have a normal or only moderately elevated HbF, which is often elevated in patients with normal karyotype.[8]
- Immunological abnormalities are frequently seen in JMML. Serum IgG, IgM and IgA levels are often increased in a polyclonal fashion. Autoantibodies, such as antinuclear antibodies, antibodies against red cells causing a positive Coomb's test and anti-thyroglobulin antibodies may be present.[8]

DIFFERENTIAL DIAGNOSIS

Several viruses have been implicated in creating a clinical and hematological picture resembling JMML. These include cytomegalovirus (CMV), Epstein-Barr virus (EBV), human herpesvirus 6 (HHV-6) and parvovirus B19.[41-43] Concomitant viral infections must therefore always be carefully excluded, especially in those with a normal karyotype or when a molecular analysis has not been feasible. Other disorders like leukocyte adhesion deficiency (LAD) variant and some metabolic disorders can also mimic JMML. Strict adherence to the diagnostic criteria can usually avoid misdiagnosis.

Hematopoiesis in Cell Culture Studies

When JMML cells are cultured in semisolid media, an increased number of monocyte-macrophage colonies are formed even in the absence of added growth factors.[44] This hypersensitivity of JMML cells to GM-CSF, since its first identification, has become the hallmark of the disease and an essential element of its diagnosis.[9,11,44] The shift to left in colony assays of JMML cells in absence of GM-CSF stimulation, as compared to controls is characteristic. GM-CSF appears to be obligatory for survival of JMML cells. This has led to several novel approaches in blocking colony formation of JMML cells by different strategies. These include fusing of diphtheria toxin to GM-CSF,[45] the use of GM-CSF receptor antagonist E21R,[46] and inhibition of production of GM-CSF, TNF-α and IL-1β by IL-10.[47] "Spontaneous" growth of JMML myeloid progenitors *in vitro* can also be inhibited by 13-cis or all-trans retinoic acid[48-50] possibly due to their antagonistic effect of retinoic acid on the transcription factor AP-1, which is activated by Jun/Fos oncoproteins shown to be upregulated in JMML.[51] Interferon-α (IFN-α)[52] and farnesyltransferase inhibitors (FTIs)[53] have also been shown to inhibit colony formation.

Natural Course and Prognostic Factors

The JMML is a relentlessly aggressive disorder and uniformly fatal in untreated patients. Allogeneic hematopoietic stem cell transplantation (HSCT) offers the only hope of a permanent cure. Thrombocytopenia, age above 2 years and high HbF at diagnosis predict a poor outcome.[8,14] A scoring system was devised in which HbF of 10 percent or higher and a platelet count of 33×10^9/L or less had an adverse impact on outcome.[14] JMML rarely transforms to a blastic stage and infiltration of the lungs leading to respiratory failure is the usual cause of death in progressive disease. Watchful observation may be of use in cases of Noonan's syndrome as some of them are known to spontaneously remit.

MANAGEMENT OF JMML

It has now long been recognized that Allo-HSCT offers the only real chance of cure in JMML. However, historically in the developed countries and in the current reality of developing countries, few have reached the stage of transplant. In the absence of this possibility, either from lack of donor availability or socioeconomic constraints, a host of alternative options have long been practiced. In addition there is now a growing list of molecular directed therapies as the JMML model of leukemogenesis has become increasingly well-defined and newer targets identified.[27,28,30] Comparative evaluation of the efficacy of these different approaches has been hampered to a large extent by the lack of adequate response criteria. This was addressed to an extent by the 2nd International JMML Working Group which evolved the new response criteria reflected in Table 4.[11]

Low-dose Conventional Chemotherapy

Historically 6-mercaptopurine (6-MP) has been used either as a single agent[54] or in combination with low-dose cytarabine or etoposide,[54,55] and was shown to be of benefit. In a later update in 2006 of the original 110 cases reported by Neimeyer, sixty-three patients who did not receive transplant were analyzed and all the above agents

Table 4 Response criteria of the 2nd International JMML Working Group[11]

	Complete clinical response	*Partial clinical response*
White blood cell count	<20000/μL	<50% of initial WBC count but total still greater than 20000/micL
Splenomegaly	Normalization of spleen size	25% decrease from initial size

used either singly or in combination in a maintenance like therapy showed some efficacy in producing partial response or stable disease three months from start of therapy. However, the variability in response evaluation made it unreliable and the setting up of uniform criteria was the main thrust of this paper.[56] This came to fruition with the efforts of the 2nd International JMML Working Group recommendations in 2009.[11] At best, this therapy may be used currently only in the palliative setting.

Intensive Chemotherapy

This has usually involved AML type protocols and has been far more controversial. A few small series have reported benefit,[57,58] with one CCG study reported remission in seven of 12 patients who received intensive chemotherapy.[59] This success has allowed some groups to use a combination of intensive cytoreduction with cis-retinoic acid as a bridge to Allo-HSCT. However, these results have not been widely replicated and many other studies have reported prolonged aplasia which is often fatal.[60,61] In addition, there may be no difference in outcome between those who receive intensive therapy and those who do not, as in the absence of Allo-HSCT, both groups do poorly with overall survival (OS) of 6 percent at 10 years.

Other Measures

In vitro sensitivity of JMML cells to IFN-α led to its initial use in JMML.[52] However, apart from isolated case reports, no benefit has ever been proven. A POG study using an IFN-α dose of 30,000 units/m^2 was stopped for excessive toxicity.[62] None of the evaluable patients had either a partial or complete response. A similar basis was found to justify the use of 13-cis retinoic acid (isotretinoin).[48,49] However, the earlier promise was not borne out a phase II POG study [63] or by other investigators.[54]

The role of splenectomy has remained undecided. It is often offered to reduce respiratory distress or abdominal discomfort due to massive spleen size, and sometimes also to reduce transfusion requirements in the presence of hypersplenism. Its role is largely limited currently to symptomatic relief or to tackle hypersplenism due to massive spleens or prior to Allo-HSCT to accelerate hematologic recovery and reduce the risk of hemorrhagic complications post-transplant. [8,54,56]

Experimental Therapeutic Approaches

The recent focus in JMML has been on designing targeted drug therapy to the many recognized molecular defects. Most of these therapies have been tested and developed on mouse models, while some have undergone early phase human trials. Since RAS hyperactivation remains a crucial element in the pathogenesis, suppression of its activity has been one of the prime targets, and various methods have been used to achieve this end (Fig. 1).

- One such method has been targeting RAF1—a MAP kinase which functions downstream of the RAS subfamily of proteins and plays an important role in the signal transduction. A DNA enzyme designed to specifically cleave mRNA for RAF1, has been found to be very specific for JMML cell lines while sparing normal marrow cells, which indicates a high level of safety.[64] Another means of achieving the same purpose is the use of a RAF1 inhibitor. BAY 43-9006—a low molecular-weight agent binds at the active site of the RAF1 kinase[65] and has undergone further trials.[11]
- Another target has been stopping RAS activation at the cell membrane level where addition of a farnesyl group to the newly translated protein is one of the first steps of RAS activation. Farnesyltransferase inhibitors (FTIs) are able to prevent RAS translocation to the plasma membrane, leading to downregulation of RAS-activated cellular pathways.[53] L-744,832 is one such FTI, which can abolish the *in vitro* growth of myeloid progenitor colonies in response to GM-CSF.[66] Other FTIs too, have reported some *in vitro* activity, however, they have shown only modest to little activity in clinical trials when used as a single agent.
- SHP-2 phosphatase has presented a very tempting target for inhibition due to the direct correlation between the driving mutation of PTPN11 and JMML, much like the tyrosine kinase inhibition of imatinib in CML with a direct driving gene at the bcr-abl locus. However in JMML, this has been plagued with issues related to the highly selective SHP-2 inhibition required due to a shared homology in catalytic pathways with SHP-1 which has a negative role in cytokine signaling[67,68] resulting in neutralized end results of SHP-2 inhibition.

The key role of GM-CSF stimulation and proliferation of JMML cell lines has also led to interest in the role of GM-CSF antagonists for its cure, which has already been covered in this text elsewhere.

Other approaches have focused on the tumor microenvironment. Angiogenesis inhibition with Endostatin and PI-88, have shown promise in mouse xenograft models for JMML.[69] Even newer approaches now are focusing on inhibition of STAT5, which is activated downstream of activated RAS. This has been found to be an effective biomarker[70] and also presents a potential therapeutic target.

Hematopoietic Stem Cell Transplantation

Allogeneic HSCT is the only known curative treatment for JMML, resulting in long-term survival in about a third of the patients.[71-73] The malignant JMML clone is difficult to eradicate even with HSCT,[74] and post-transplant relapse rate is high. For this reason, right from the first successful HSCT in JMML from Seattle, a conditioning regimen including total-body irradiation (TBI) has been used.[75] However, radiation-induced late effects such as severe growth retardation, cataracts, hypothyroidism and neuropsychologic sequelae, have been daunting, and increasingly conditioning regimens without TBI have been used and reported to be equally effective.[72,73]

Younger age at HSCT may predict improved survival.[71,73] Thus, transplant should be recommended soon after diagnosis. This is imperative as long-term survival in children with JMML without HSCT is less than 10 percent.[8] Where feasible, a matched unrelated donor (MUD) must be searched for if a matched sibling donor is not available.[72,74] Though relapse rates in both approaches are comparable, graft rejection[76] and transplant-related mortality[71] are expectedly higher in MUD grafts. Unrelated umbilical cord transplants have also found to have similar rates of success as other sources.[77]

The efficacy of HSCT depends on both the conditioning regimen and the GvL or graft versus leukemia reaction. Children who receive less immunosuppressive therapy for GvHD prophylaxis have a lower relapse rate.[71] Similarly, acute or chronic GvHD is associated with a lower risk of relapse.[71-73] Reducing the intensity and duration of GvHD prophylaxis may significantly contribute to successful leukemia control; however, unlike BCR-ABL positive CML, donor lymphocyte infusion in JMML relapse is largely unsuccessful.[72]

Post-HSCT relapse rates continue to be as high as 50 percent[72,77] and tend to occur within a few months.[71,72] A strategy of pretransplant intensive chemotherapy does not reduce relapse rates.[54,71] The impact of pretransplant splenectomy is also unclear.[54,72] Risk factors for relapse include older age at transplant, increased HbF and abnormal karyotype.[71-73] However, a second transplant often works in children who have relapsed.[54,78]

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CHAPTER 43

Pediatric Hodgkin Lymphoma

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Hodgkin lymphoma (formerly called Hodgkin's disease) is a malignant lymphoma, first described by Hodgkin, in 1832 as *some morbid appearances of the absorbent glands and spleen* that accounts for approximately 5 to 7 percent of childhood cancers and 1 percent of all deaths.[1] Hodgkin lymphoma (HL) is characterized by a progressive painless enlargement of lymph nodes and defined by specific histopathological features. Pediatric HL is one of the few pediatric malignancies that shares aspects of its biology and natural history with its adult counterpart. The odyssey of treatment in HL, which began with radiotherapy and then got revolutionized with multiagent chemotherapy, has continued to grow better in terms of cure rates. With the currently available treatment modalities (multiagent chemotherapy either alone or in conjunction with low-dose involved-field radiation therapy) and the use of risk-adapted therapy, over 90 percent of children diagnosed with HL are long-term survivors. Currently, management designed to achieve maximal cure rates with the fewest late-effects of therapy continues to be the paradigm for pediatric oncologists across the world.

EPIDEMIOLOGY

Variation in the incidence, age, and gender distribution of HL occurs in different populations according to geographic location, socioeconomic status, and immunologic status. In Industrialized countries, HL presents with a bimodal distribution with regard to age, with a rise in incidence in young adults (20–34 years) and in the elderly (55–74 years).[2] In contrast, in low-income countries, there is a trimodal distribution. There is an inverse relationship between the incidence of the HL in children and young adults within countries according to their economic development.[3] Such patterns of occurrence being similar to Epstein-Barr virus (EBV), tuberculosis and poliomyelitis infections; the role of an environmental exposure has been suggested as a possible etiology of HL. As many as 20 to 30 percent of childhood HL cases in developing countries occur before 5 years of age[4,5] against some 5 percent in industrialized countries. Overall, there are three distinct forms of Hodgkin lymphoma:

1. *Childhood form (Ages 14 years and younger):* This type increases in prevalence in association with larger family size and lower socioeconomic status. Early exposure to common infections in early childhood appears to decrease the risk of Hodgkin lymphoma, most likely by maturation of cellular immunity.[2]
2. *Young adult form (Ages 15–34 years):* This is associated with a higher socioeconomic status in industrialized countries, increased number of siblings, and earlier birth order. Delayed EBV infection, particularly when associated with infectious mononucleosis, is a risk factor for the young adult form. It has been proposed that delayed exposure to a common infectious agent may also play a role in EBV-negative young-adult cases, although the identity of the agent has not been established.[6]
3. *Older adult form:* Most commonly presents in individuals aged 55 to 70 years.

In India, lymphomas are the second most common malignancies in children ahead of CNS tumors especially in males unlike west where brain tumors are more common. Importantly, HL exceeds non-Hodgkin lymphoma in India in contrast to west due to significantly higher incidence of HL in males in India. The age standardized incidence of HL in Indian children ranges from 8.2 to 19.6 per million children per year compared to 5.7 in USA and 6.4 in Britain. Furthermore, mixed cellularity is most common

phenotype in India (likely related to early childhood EBV exposure) leading to much younger age peak (median age 8–9 years) compared to 16–30 years in west where nodular sclerosis is most common.[7] Pediatric HL shows a significant male predominance in low-income countries including India (male : female ratios being 2.5:1 to 8:1) compared to ratio of about 1.5:1 in west.[8,9]

ETIOLOGY

The etiology of Hodgkin lymphoma is believed to be multifactorial and may include the following;

- *Infectious agents:* Several studies have documented a link between Hodgkin lymphoma and EBV. EBV DNA can be identified in tumor cells in approximately 50 percent of patients in the United States as well as Western Europe and in more than 90 percent of patients in developing countries.[10] EBV positivity is most commonly observed in tumors with mixed-cellularity histology and is almost never seen in patients with lymphocyte-predominant histology. EBV positivity is more common in children younger than 10 years compared with adolescents and young adults. Patients with a prior history of serologically confirmed infectious mononucleosis have a four-fold increased risk of developing EBV-positive HL but are not at increased risk for EBV-negative HL.[11]
- *Genetic predisposition:* Clustering in families suggests a genetic predisposition, with an increased incidence observed among same-gender siblings, monozygotic twins, and parent-child pairs. Familial Hodgkin lymphoma has been associated with specific human leukocyte antigens. Familial cases account for 4.5 percent of all cases.
- *Immune dysregulation:* The increased susceptibility to HL in patients with T-cell immunodeficiency, human immunodeficiency virus (HIV) infection, or congenital immunodeficiency syndromes suggest a role for immune dysregulation in its development.
- *Environment:* Clustering of cases in families or racial groups supports the idea of a common environmental link. At present, no conclusive association is recognized with common environmental factors other than EBV infection.

BIOLOGY

HL is a B-lineage lymphoma. The malignant cells of HL are clonal Hodgkin/Reed-Sternberg (HRS) cells or lymphocytic and histiocytic (L&H) cells or their morphologic variants, which usually constitute less than 1 percent of the cells in involved lymph nodes. Characteristic RS cells are binucleate or multinucleate giant cells with prominent nucleoli and abundant cytoplasm. The rest of the lymph node contains a variable cellular infiltrate consisting of lymphocytes, eosinophils, macrophages, plasma cells, and fibroblasts. These infiltrating cells secrete an array of cytokines and chemokines, which are important for HRS cell survival and maintenance of the characteristic cellular infiltrate. These malignant cells have three distinct origins; in nodular lymphocyte predominant HL, the tumors cells (L&H) derive from germinal centre (GC) or postgerminal centre B-cells and retain expression of all B-cell specific antigens. In classical HL, HRS cells are GC B-cells but have crippling mutations that destroy the coding capacity of their functional IgV gene rearrangements. In a minority (2%) of classical HL cases, HRS cells display a cytotoxic T-lymphoid phenotype.

In EBV-positive Hodgkin lymphoma, EBV-encoding genes play a role in preventing apoptosis. Latent membrane protein-1 (LMP-1) expressed in EBV-positive HRS cells mimics an activated CD40 receptor, activating the anti-apoptotic nuclear factor–kappa-B (NF-κB) pathway. A paracrine activation of NF-κB in Hodgkin lymphoma is observed; both HRS cells and the surrounding supporting cells produce cytokines that upregulate several members of the TNF receptor superfamily, including CD30, CD40, or EBV latent membrane protein-1 (LMP-1). The production of the ligand for these receptors is responsible for the phosphorylation and translocation to the nucleus of NF-κB. The constitutive translocation of NF-κB to the nucleus of HRS cells is essential for the malignant transformation of HRS cells. It leads to inhibition of apoptosis, proliferation, and secretion of proinflammatory cytokines.[12]

PATHOLOGIC CLASSIFICATION

The current World Health Organization classification classifies HL in two broad types based on both morphologic appearance as well as immunophenotypic characterization including type of neoplastic cells, inflammatory mileu and overall growth pattern as detailed here.[13]

Classical Hodgkin Lymphoma

The hallmark of classic HL is the Reed-Sternberg cells (R-S) cells and their mononuclear (Hodgkin cells) and multinucleate variants which lack the immunophenotypic evidence of B-cell differentiation. R-S cells almost always express CD30, and approximately 70 percent of patients express CD15. CD20 is expressed in approximately 6 to 10 percent of cases, and generally RS cells do not express B-cell antigens such as CD45, CD19, and CD79A.

The classical HL is subclassified into four subtypes according to the number of R-S cells, characteristics of the inflammatory milieu, and the presence or absence of

Table 1 Histopathological classification of classical Hodgkin lymphoma

REAL subgroups	*Distinctive features*	*Relative frequency (%)*
Lymphocyte rich (LR)	Benign appearing lymphocytes with or without histiocytes. Few Reed-Sternberg (RS) cells. No fibrosis	10–15
Nodular sclerosis (NS)	Thickened capsule with proliferation of orderly collagenous bands that divide lymphoid tissue in nodules: Lacunar variant of RS cells	20–50
Mixed cellularity (MC)	5–15 RS cells per high power field. Fine fibrosis in interstitium. Focal necrosis may be present	20–40
Lymphocyte depletion (LD)	Abnormal cells with relative paucity of lymphocytes. Fibrosis and necrosis common but diffuse	5–16

fibrosis. The histologic features and clinical symptoms of HL have been attributed to the numerous cytokines secreted by the R-S cells, which include interleukin-1, interleukin-6, and tumor necrosis factor. Classical HL subtypes are detailed in the Table 1.

Nodular Lymphocyte—Predominant Hodgkin Lymphoma

This pathologic class of Hodgkin lymphoma is characterized by large cells with multilobed nuclei, referred to as popcorn cells. These cells express B-cell antigens, such as *CD19, CD20, CD22*, and *CD79A*, and are negative for *CD15* and may or may not express *CD30*. The *OCT-2* and *BOB.1* oncogenes are both expressed unlike classical HL. Nodular lymphocyte-predominant Hodgkin lymphoma (NLPHL) is most common in males younger than 10 years and constitutes 5 to 10 percent of all HL cases. Patients with NLPHL generally present with slow growing, localized, non-bulky disease usually in axilla and inguinal regions without any B symptoms.

PRESENTING SYMPTOMS AND SIGNS

Presenting symptoms and signs of HL in children include lymphadenopathy, systemic symptoms, and mediastinal mass. HL almost always presents at a site above the diaphragm, with only 3 percent of cases presenting in a purely subdiaphragmatic location.[14]

Lymphadenopathy

Most common presenting sign is painless lymphadenopathy. Approximately, 80 percent of young children present with cervical lymphadenopathy. The affected lymph nodes typically feel rubbery and more firm than inflammatory adenopathy; they may be sensitive to palpation, if they have grown rapidly.

Hepatosplenomegaly

Hepatic and/or splenic enlargement may be present in patients with advanced stage HL. Overall, children are more likely than adults to present with stage I/II disease and less likely to present with stage IV disease.

Mediastinal Mass

Unlike adolescents and young adults, only few young children with HL have mediastinal disease at presentation (approximately 75 *vs* 33 percent, respectively), in part reflecting the tendency of these patients to have mixed cellularity histology. Mediastinal masses are almost always present in association with low cervical or supraclavicular adenopathy. Such bulky mediastinal disease may cause dysphagia, dyspnea, cough, stridor and the superior vena cava syndrome.

Systemic Symptoms

Patients with HL may present with nonspecific systemic symptoms including fatigue, anorexia, and weight loss. Fewer than 20 percent of children with HL have B symptoms which include unexplained persistent fever (above 38°C or 100.4°F), drenching night sweats and weight loss (more than 10 percent of body weight) in the previous six months. These symptoms have important implications for staging and prognosis. As in adults, pruritus, which typically resolves with treatment, has been described. Rarely, patients present with autoimmune disorders such as autoimmune hemolytic anemia, thrombocytopenia, or neutropenia.[15–18]

DIFFERENTIAL DIAGNOSIS

The presenting symptoms and signs of HL in children and adolescents may be caused by a variety of diseases and the differential diagnosis includes other malignant, infectious,

and inflammatory diseases. In India, mycobacterial infections are the most common differential diagnosis. Others include EBV infection, Non-Hodgkin lymphoma, metastatic adenopathy from other primary tumors (e.g. nasopharyngeal carcinoma, soft tissue sarcoma), toxoplasmosis, systemic lupus erythematosus, and other disorders causing reactive hyperplasia of lymph nodes.[19]

DIAGNOSTIC EVALUATION

A complete evaluation of patients with suspected HL is mandatory before beginning treatment. The goal is to confirm the diagnosis, stage the disease, document other prognostic factors, and to evaluate organ function that may influence the selection of therapy.

ESTABLISHING THE DIAGNOSIS

After a careful physiological and radiographical evaluation of the patient, the least invasive procedure should be used to establish the diagnosis of lymphoma by biopsy of one or more peripheral lymph nodes. Fine-needle aspiration cytology alone is not recommended because of the lack of stromal tissue, the small number of cells present in the specimen, and the difficulty of classifying Hodgkin lymphoma into one of the subtypes. An image-guided biopsy may be used to obtain diagnostic tissue from intrathoracic or intra-abdominal lymph nodes. Patients with large mediastinal masses are at risk of cardiac or respiratory arrest during general anesthesia or heavy sedation. In these patients, peripheral lymph node biopsy or image-guided core-needle biopsy of mediastinal lymph nodes may be feasible using light sedation and local anesthesia before proceeding to more invasive procedures. Supine position should be avoided and procedures should be done with the patient on his or her side or prone. If airway compromise precludes biopsy, immediate treatment with steroids or localized radiation therapy should be considered and biopsy performed as soon as feasible preferably within 48 hours.

LABORATORY STUDIES

Hematological and chemical blood parameters show nonspecific changes that may correlate with disease extent. Abnormalities of peripheral blood counts may include neutrophilic leukocytosis, lymphopenia, eosinophilia, and monocytosis. Acute-phase reactants such as the erythrocyte sedimentation rate and C-reactive protein, if abnormal at diagnosis, may be useful in follow-up evaluation. Patients with history of recurrent infections, autoimmune and inflammatory disorders, or a family history of immune deficiency should undergo a detailed immunologic evaluation.

IMAGING STUDIES

The goal of imaging is to define the accurate extent and stage of the disease. The following studies should be obtained:

Anatomical Imaging

CT of neck, thorax, abdomen, and pelvis (with and without intravenous contrast) should be obtained. Establishment of lymphomatous involvement on CT-scan is complicated by great variability of normal nodal size with body region and age as well as the frequent occurrence of reactive hyperplasia. However, contiguous nodal clustering or matting, focal mass lesion in a visceral organ, size on long axis of 2 cm or greater or between 1 cm and 2 cm with other suggestive clinical features should be considered significant. Currently, definitions of bulky disease are not uniform and often depend on the clinical protocol with bulky peripheral (non-mediastinal) lymphadenopathy varying from aggregate nodal masses exceeding 4 to 6 cm and bulky mediastinal mass as a transverse mediastinal diameter over one-third of the maximum intrathoracic diameter on an upright posterior-anterior (PA) chest radiograph. However, the Cotswolds modification of the Ann-Arbor classification has defined lymph nodes more than 10 cm in greatest dimension on CT imaging as bulky.

Combined Anatomical and Metabolic Imaging

PET-CT, which integrates functional and anatomic tumor characteristics, is being increasingly used for staging and monitoring of pediatric patients with HL. In PET scan, uptake of the radioactive glucose analog, 18-fluoro-2-deoxyglucose (FDG) correlates with proliferative activity in tumors undergoing anaerobic glycolysis and adds to the anatomical information from CT scan. In recent studies, PET findings resulted in a change in staging in more than 50 percent of patients and subsequent adjustments in involved-field radiation therapy treatment volumes in 70 percent of patients. Concordance between PET and CT is generally high for nodal regions, but lower for extranodal sites such as spleen, lung nodules, bone/bone marrow, and pleural and pericardial effusions. Generally, a suspected anatomic lesion which is PET-negative should not be considered involved unless proven by biopsy and areas of PET positivity that do not correspond to an anatomic lesion should be disregarded in staging. PET scan is more accurate in detecting viable HL in post-therapy residual masses. Residual or persistent FDG avidity has

Table 2 Cotswold's revision of Ann Arbor staging classification

Stage	*Definitions*
I	Involvement of a single lymph node (LN) region (I) or of a single extranodal organ or site (IE)
II	Involvement of two or more LN regions, on the same side of the diaphragm (II) or localized involvement of an extralymphatic organ or site and one or more LN region on the same side of the diaphragm (IIE)
III	Involvement of LN regions on both sides of the diaphragm (III), which may be accompanied by involvement of the spleen (III S) or by localized involvement of an extralymphatic organ (III E) or both (IIISE)
IV	Noncontiguous involvement of one or more extralymphatic site with or without LN involvement
Annotations	*Definitions*
A	No B symptoms
B	At least one of the following within the last 6 months: a. Weight loss >10% b. Unexplained persistent or recurrent fever c. Drenching night sweats
X	Bulky disease (>6 cm in diameter or mass >1/3 of mediastinal) diameter
E	Extension to a single extralymphatic organ adjacent to a known involved site

been correlated with prognosis. Rapid early response documented by significant reduction in disease volume and PET negativity at an early stage (after one or two cycles of chemotherapy) is associated with a favorable outcome. PET scanning should be performed at baseline and a not earlier than 3 weeks postchemotherapy completion and 8 to 12 weeks post-radiation.[20–22]

Staging and Prognostic Factors

Current Hodgkin's lymphoma staging is based on Cotswold's modification of Ann Arbor staging proposed in 1998 (Table 2). The assessment to determine stage of disease involves scrupulous history for B symptoms and laboratory studies including a blood count, LDH, ESR, and liver function tests apart from imaging studies detailed here. Bone marrow biopsy is indicated in those with advanced disease (stage III/IV), B symptoms, elevated alkaline phosphatase or hematologic abnormalities. Recently, with the advent of PET-CT scan, which is highly sensitive for bone marrow involvement, bone marrow biopsy is considered mandatory only in ambiguous cases. Sites of bulk disease, disease infiltration into extranodal tissues, presence of B-symptoms, ESR, hemoglobin, WBC count, and gender should be documented.

These prognostic factors may be able to identify a group of patients with an extremely low risk of relapse for whom therapy may be minimized. Because the treatment of HL has improved and fewer patients relapse after initial therapy, many previously reported clinical factors have lost their prognostic significance. Also, these factors may be interrelated in the sense that disease stage, bulk, and biologic aggressiveness are frequently codependent. Consequently, no uniform system of prognostic stratification exists in pediatric HL. Different clinical trial groups have established various risk categorizations as outlined in Table 3. In general, these incorporate stage of disease, disease bulk, and systemic (B) symptoms.[23-26]

TREATMENT

The treatment of Hodgkin lymphoma (HL) in children requires a careful balance between providing enough therapy to eradicate the tumor and avoiding unnecessary treatment that could result in excessive long-term treatment-related side effects. Hence, focus is on maximizing treatment efficacy and minimizing risks for late toxicity associated with both RT and chemotherapy.

The treatment paradigm for childhood HL is risk, gender and response adapted use of non-crossresistant combination chemotherapy with or without low-dose involved-field radiation therapy. This approach is supported by information obtained from clinical trials and meta-analyses of randomized trials evaluating the influence of radiation field size, dose and the role of chemotherapy in children with HL.

Combined Modality Therapy in Children with Hodgkin Lymphoma

A number of randomized clinical trials have investigated the benefit of adding chemotherapy to radiation therapy in the treatment of HL. While the radiation therapy acts to control known sites of tumor, the chemotherapy is aimed at occult disease outside the radiation field. The judicious combination of the two modalities allows for a decrease in the dose and size of the radiation field used and a reduction in the intensity or duration of chemotherapy

Table 3 Prognostic factors and risk stratification in pediatric Hodgkin lymphoma

Study group/Trial	*Low risk*	*Intermediate risk*	*High risk*
Children's Oncology Group	• IA/IIA no bulk or extranodal extension	• IA bulk or "E" extension • IB • IIA bulk or "E" extension • IIB • IIIA • IVA	• IIIB, IVB
German studies/Euronet PHL	• IA/B • IIA	• IIB • IIEA • IIB	• IIEB • IIIEA/B • IIIB • IV
St Jude/Stanford/Dana Farber†	• IA/IIA no bulk	• IA bulk • IB • IIA bulk • IIB • III • IV	
Children's Cancer Group 5942	• IA/B patients no adverse features* • IIA patients no adverse features*	• IA/B patients with adverse features* • IIA patients with adverse features* • IIB • IIIA/B	• IV

*Adverse factors include hilar lymphadenopathy, >4 sites of nodal disease, or bulky disease.
†Patients categorized as favorable or unfavorable risk.

below toxicity thresholds that would not be possible if single modality chemotherapy or RT were used, thus decreasing overall acute and late toxicities. The use of RT in pediatric HL permits reduction in dose-related toxicity of anthracyclines, alkylating agents, and bleomycin that may preserve cardiopulmonary as well as gonadal function and reduce the risk of secondary leukemia. The results of prospective and controlled randomized trials indicate that combined modality therapy, compared with chemotherapy alone, produces a superior event-free survival (EFS). Thus, combined modality treatment approach has become the preferred initial therapy for children with HL.[27]

Chemotherapy Regimens in Children with Hodgkin Lymphoma

Contemporary chemotherapy regimens in HL combine non-crossresistant agents wherein each agent is individually active against tumor but targets different cellular events to prevent drug-resistance and have non-overlapping toxicities which allow delivery of each agent at full dose which are detailed in Table 4. All of the agents in original MOPP and ABVD regimens continue to be used in contemporary pediatric treatment regimens. However, COPP with less leukemogenic and gonadotoxic potential (substituting cyclophosphamide for mechlorethamine) has replaced MOPP as the preferred alkylator regimen. In contrast to adult HL, pediatric chemotherapy approaches have focused on 2 unique strategies.

Avoidance of Late Toxicity

Non-crossresistant regimen like ABVD has demonstrated superior efficacy (i.e. freedom from progression) and less toxicity when compared with MOPP in adults with Hodgkin lymphoma.[28] However, due to cardiac and pulmonary toxicity of ABVD in children, many investigators have evaluated regimens either devoid of anthracyclines, alkylators and/or bleomycin such as VAMP (St Jude), VBVP (French MDH-90 study) or hybrid regimens that utilize lower total cumulative doses of alkylators, doxorubicin, and bleomycin such as COPP/ABV (CCG) or ABVE-PC (POG). VAMP like less-toxic regimens can be safely used in favorable risk HL but not in intermediate or high-risk HL.[29]

Gender-Adapted Chemotherapy

In an effort to decrease risk for male infertility, etoposide has been substituted for procarbazine in the initial courses of therapy in studies of the German pediatric HL group (OPEA) and Pediatric Oncology Group (DBVE and DBVE-PC)[30] and dacarbazine (COPDAC) has been used to replace procarbazine (COPP) with preservation of efficacy and minimization of infertility.[31]

Table 4 Contemporary chemotherapy regimens for children with Hodgkin lymphoma

Name	*Drugs*
COPP	Cyclophosphamide, vincristine (oncovin), procarbazine, prednisone
COPDAC	Dacarbazine substituted for procarbazine in COPP
OPPA	Vincristine (Oncovin), procarbazine, prednisone, doxorubicin (Adriamycin)
OEPA	Vincristine (Oncovin), etoposide, prednisone, doxorubicin (Adriamycin)
ABVD	Doxorubicin (Adriamycin), bleomycin, vinblastine, dacarbazine
COPP/ABV	Cyclophosphamide, vincristine (Oncovin), procarbazine, prednisone, doxorubicin (Adriamycin), bleomycin, vinblastine
VAMP	Vinblastine, doxorubicin (Adriamycin), methotrexate, prednisone
DBVE	Doxorubicin, bleomycin, vincristine (Oncovin), etoposide
ABVE-PC	Doxorubicin (Adriamycin), bleomycin, vincristine (Oncovin), etoposide, prednisone cyclophosphamide
BEACOPP	Bleomycin, etoposide, doxorubicin (Adriamycin), cyclophosphamide, vincristine (Oncovin), prednisone, procarbazine

Radiotherapy

Consolidative radiation therapy (RT) after risk-adapted chemotherapy is an integral part of treatment of children with HL.[27] Radiation has been used as an adjunct to multiagent chemotherapy in intermediate/high-risk pediatric HL with the goal of reducing risk of relapse in initially involved sites and preventing toxicity associated with second-line therapy. Compared with chemotherapy alone, adjuvant radiation produces superior EFS for children with intermediate/high-risk HL who achieve a complete remission (CR) to multiagent chemotherapy, but it does not affect overall survival (OS) because of the success of second-line therapy.[29,30] Since, adjuvant radiation therapy may be associated with excess late effects or mortality; there has been a movement to decrease the field of radiation as well as dose of radiation therapy in order to limit toxicities while maintaining survival rates.

Radiation Dose

The dose of radiation is variously defined and often protocol specific. However, doses of 15 to 25 Gy are typically used with modifications based on patient age, the presence of bulky or residual (postchemotherapy) disease, and normal tissue concerns.[37] Some protocols prescribe a boost of 5 Gy in regions with suboptimal response to chemotherapy.

Radiation Therapy Volume

Involved field radiation therapy (IFRT) is the current standard of care in children in place of total nodal RT. The IFRT treats the clinically involved region(s), with coverage of the whole nodal region but accounts for tumor regression with chemotherapy and avoids extensive inclusion of uninvolved regions. With contemporary low-dose RT, treatment of contralateral uninvolved sites is not necessary in most children. Currently, targeted RT, which entails restricting RT to areas of initial bulky disease (generally defined as ≥5 cm at the time of disease presentation) or post-chemotherapy residual disease (generally defined as ≥2.5 cm or residual PET-avidity), and involved-nodal RT, which treats only the initially involved nodes with a margin (typically 2 cm), are under investigation.

Radiation Therapy Technique

While CT-based two-dimensional RT remains the standard technique for radiation delivery, three-dimensional conformal RT (3D CRT) or intensity-modulated radiation therapy (IMRT) are often used in situations where the more conformal techniques would reduce dose to surrounding normal critical structures. Proton therapy is currently being investigated and may further decrease the mean dose to the surrounding normal tissue compared with IMRT or 3D CRT.

Response-Adapted Therapy

Response to therapy is one of the most robust prognostic factors in HL as in many other pediatric tumors. The concept of tailoring the extent as well as dose of radiotherapy and duration of chemotherapy based on response to therapy in HL is the focus of many recent and future trials.

With regards to avoiding radiotherapy in patients with early stage favorable risk HL, finding from three recent studies[32,33] (*COG*-9542, *GPOH HD*-95 and Euronet *PHL*

C-1) have shown that RT can be safely avoided in patients who achieve CR after initial chemotherapy. However, omission of RT was found to be detrimental in intermediate and high-risk subgroups except in a recent North American study (*COG AHOD* 0031) which showed that RT can be avoided in rapid early responders in intermediate risk HL.[34]

In advanced HL, a recent POG study (P9425) has shown that in rapid early responders to a dose dense chemotherapy, further chemotherapy can be safely curtailed. In this study, 216 children with intermediate or high-risk HL received ABVE-PC every 21 days. Rapid early responders (RER, 63% of patients) to 3 cycles received 21 Gy RT to involved regions. Slow early responders received two additional cycles before 21 Gy radiation.[35] Five-year EFS was 86 percent for the RER and 83 percent for the slow early responders (P = 0.85). Five-year OS was 95 percent. Cumulative doses of alkylators, anthracyclines, and epipodophyllotoxins were below thresholds usually associated with significant long-term toxicity.

Similarly, Children's Cancer Group (CCG) (CCG-59704) evaluated response-adapted therapy featuring four cycles of the dose-intensive BEACOPP regimen followed by a gender-tailored consolidation for pediatric patients with high-risk HL. For rapid early responding girls, an additional four courses of COPP/ABV (without IFRT) was given in an effort to reduce breast cancer risk. Rapid early responding boys received two cycles of ABVD followed by IFRT. Slow early responders received four additional courses of BEACOPP and IFRT. RER was achieved by 74 percent of patients after four BEACOPP cycles and 5-year EFS among the cohort was 94 percent.[36]

RECOMMENDATIONS FOR TREATMENT OF PEDIATRIC HODGKIN LYMPHOMA

Treatment of Low-risk Classical Hodgkin Lymphoma

The preferred treatment option for early stage, favorable prognosis HL (stages I–IIA; no bulky disease; no B symptoms, less than four sites of disease) is combined modality treatment including hybrid chemotherapy with less or no alkylators and anthracyclines (VAMP/VBVP/OPPA/OPEA or equivalent) for two to four cycles with or without low-dose IFRT of 15 to 25 Gy. IFRT can be safely avoided in children who achieve CR after initial chemotherapy in some regimens (Table 5). Ongoing trials for patients with low-risk HL are evaluating the effectiveness of treatment with fewer cycles of combination chemotherapy alone that limit doses of anthracyclines and alkylating agents.[29-31]

Table 5 Risk-adapted treatment of newly diagnosed Hodgkin lymphoma in children

Risk group	*5 yr EFS*	*5 yr OS*
Low risk disease		
• Four cycles of VAMP with LD-IFRT (if not in CR after 2 cycles) or without IFRT (if in CR post 2 cycles)	92%	98%
• Four cycles of COP/ABV plus LD-IFRT		
• ABVE, administered for two to four courses depending on response, followed by LD-IFRT		
• Two cycles of OEPA or OPPA with LD-IFRT (if not in CR after two cycles) or without IFRT (if in CR post two cycles)		
Intermediate risk disease		
• Six cycles of COPP/ABV plus LD-IFRT	85%	95%
• ABVE-PC, administered for three to five courses depending upon response, with or without LD-IFRT		
• Two cycles of OPPA (for males) or OEPA (for females), followed by two cycles of COPP (for females) or COPDAC (for males) plus LD-IFRT		
High-risk disease		
ABVE-PC, administered for three to five courses depending upon response, followed by LD-IFRT	83%	94%
Two cycles of OPPA (for males) or OEPA (for females), followed by four cycles of COPP (for females) or COPDAC (for males) plus LD-IFRT		
Two cycles of cytarabine/etoposide, COPP/ABV, and CHOP plus LD-IFRT		
Four cycles of BEACOPP with subsequent dependent upon response; rapid responders: four cycles of COPP/ABV without IFRT (for females) or two cycles ABVD with IFRT (for males); slow responders: four additional cycles of BEACOPP plus IFRT		

Treatment of Intermediate Risk Classical Hodgkin Lymphoma

Patients with intermediate risk HL (all stage I and stage II patients not classified as early stage; stage IIIA) generally qualify for combined modality treatment. However, the ideal chemotherapy and radiation combinations are not yet clearly defined, and there is an ongoing desire to optimize treatment in this risk group. These children require 3 to 6 cycles of dose-intense alkylator based chemotherapy followed by low-dose IFRT. In some studies, chemotherapy is given to maximal tumor response, as judged by CT scan and PET, after which two additional cycles of consolidation chemotherapy are given followed by limited RT (Table 5). The patients with rapid early response may be treated with less number of chemotherapy cycles as shown in the recent studies (*POG* 9425)[35] or may not require RT (*COG AHOD* 0031).[34]

Treatment of High-risk Classical Hodgkin Lymphoma

Children with high-risk (Stages IIIB, IV) HL require 6 to 8 cycles of dose-intense alkylator based chemotherapy regimens followed by low-dose IFRT (Table 5).[37] Current trials for patients with intermediate/high-risk HL are testing, if chemotherapy and radiation therapy can be limited in patients who achieve a rapid early response to dose-intensive chemotherapy regimens.

Treatment of Nodular Lymphocyte-Predominant Hodgkin Lymphoma

NLPHL, an uncommon subtype, represents a more indolent disease than classical HL, and is therefore managed uniquely. Most information concerning its therapy has come from reports of single institutions or pooled, multi-institutional retrospective analyses in children and adults. Generally, patients with stage I/II NLPHL without B symptoms are treated with less intensive therapy than patients with classical HL. In contrast, patients with stage III/IV are treated in a similar fashion to patients with classical HL. The current strategies for treating NLPHL in children are modest intensity chemotherapy regimens, some without anthracyclines, with or without IFRT.

Given the indolent nature of NLPHL and because deaths observed among individuals with this histological subtype are more frequently related to complications from cytotoxic therapy, several pediatric study groups have evaluated treatment de-escalation in an attempt to avoid toxicities associated with treatment.[38,41,42] Some have evaluated the use of chemotherapy alone or observation without treatment following excision in children with stage I NLPHL. The overall survival (OS) in most series is 100 percent, with lower progression-free survival (PFS) and EFS in series with surgery alone (67 to 82 percent)[38,39] or with CVP (cyclophosphamide, vincristine, prednisone) chemotherapy (74 percent).[40]

Relapsed or Refractory Disease

Most relapses in children with HL occur in first 3 years. Treatment and prognosis after relapse depends upon the timing of relapse, the initial stage of disease, and the initial treatment given. Also, presence of B symptoms, extranodal disease and inadequate response to second-line therapy portend poor prognosis. Relapsed patients may be classified in 2 groups for prognostication and treatment planning.

Low-risk (Favorable) Group

Children with localized late (≥12 months after completing therapy) recurrences after limited risk-adapted therapy or with chemotherapy alone and/or IFRT have a high likelihood of achieving long-term survival following treatment with more intensive conventional chemotherapy alone. Intensive non-crossresistant regimens using agents not part of initial treatment such as cytarabine at moderate or high doses, carboplatin and cisplatin, ifosfamide, etoposide, vinorelbine, gemcitabine, and vinblastine are used. Approximately, two-third of these patients may be salvaged with second-line chemotherapy.

High-risk Group

Children who develop refractory disease during therapy or relapsed disease within 1 year after completing therapy require aggressive salvage chemotherapy and consolidation with high dose chemotherapy and subsequent autologous hematopoietic cell transplantation (HCT).[43,44] Autologous source of stem cells is preferable to allogeneic because of the high transplant-related mortality (TRM) associated with allogeneic transplantation. Following autologous HCT, the projected overall survival rate is 45 to 70 percent and progression-free survival (PFS) is 30 to 89 percent. Patients who fail autologous HCT or for patients with chemoresistant disease, allogeneic HCT has been used with encouraging results. Salvage rates for patients with primary refractory HL are poor even with autologous HCT and range from 20 to 40 percent. Brentuximab vedotin (Anti-CD30 monoclonal antibody) has been evaluated in adults with relapsed/refractory HL and has shown promising response rates of 50 to 70 percent in phase-I/II studies.

Table 6 Late effects in Hodgkin lymphoma survivors

Adverse effects	*Predisposing therapy*	*Clinical features*
Thyroid	Radiation to thyroid	Hypothyroidism, hyperthyroidism, thyroid nodules
Cardiovascular	Radiation to heart	Left ventricular dysfunction, cardiomyopathy pericarditis, heart valve dysfunction, conduction disorders, vascular disease, myocardial infarction, stroke
	Anthracyclines	Left ventricular dysfunction, cardiomyopathy, congestive heart failure
Pulmonary	Radiation to lungs	Subclinical pulmonary dysfunction
	Bleomycin	Pulmonary fibrosis
Musculoskeletal	Radiation to musculoskeletal tissues	Growth impairment
	Glucocorticosteroids	Bone mineral density deficit
Reproductive	Alkylating agents	Hypogonadism
	Gonadal irradiation	Infertility
Subsequent neoplasm or disease	Alkylating agents	Myelodysplasia/acute myeloid leukemia
	Epipodophyllotoxins	Myelodysplasia/acute myeloid leukemia
	Radiation	Solid benign and malignant neoplasms

FOLLOW-UP AND LATE EFFECTS

Current 5-year relative survival for HL is approximately 90 percent with higher rates reported in younger populations.[45] However, emergence of late toxicity among survivors can limit long-term survival and affect quality of life. Mortality in the first 15 years after diagnosis relates to the primary disease and following that to second cancers (SMNs) and cardiovascular disease (CVD).[46] Hence, children treated with HL should be closely followed for relapse as well as late effects. Imaging is not recommended for routine follow-up, as a recent Children's Oncology Group Study, which evaluated surveillance CT for detection of relapse, found that most relapses were detected based on symptoms, laboratory, or physical findings without any incremental value of imaging. The method of detection of late relapse, whether by imaging or clinical change, did not affect overall survival.[47]

HL survivors continue to be at risk of treatment related mortality for decades beyond their initial disease. In a recent Childhood Cancer Survivor Study of morbidity and mortality risks among 2742 survivors of HL in the USA, substantial excess absolute risk of mortality per 10000 person-years was identified: overall 95.5; death due to HL-38.3, SMNs-23.9, and CVD-13.1.[48] Commonly, adverse treatment effects may impact musculoskeletal development, endocrine, reproductive, cardiovascular and pulmonary function and risk of secondary carcinogenesis. Adverse effects have reduced overtime with use of risk-adapted less toxic chemotherapy and low-dose radiotherapy. Table 6 summarizes the common late effects and their etiology in children with HL.

SUMMARY

Pediatric Hodgkin lymphoma is currently one of the most curable childhood malignancies with more than 90 percent cure rates in recent studies. Risk and response-adapted combined modality therapy is the current standard of care and is stratified based on disease stage and the presence of adverse prognostic factors. Long-term follow-up of pediatric HL patients should take place in a comprehensive pediatric-oncology center, where late complications can be anticipated, monitored, and treated. Recent trials are exploring to reduce toxicities of treatment with the selective elimination of radiotherapy, devising less toxic chemotherapy regimens and assessing the role of functional imaging with PET in assessing response and predicting outcome.

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CHAPTER 44

Non-Hodgkin Lymphoma in Children and Adolescents

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Non-Hodgkin lymphoma (NHL) is diverse collection of neoplasms of lymphoid system derived from numerous cell types comprising the immune system including B-cell progenitors, T-cell progenitors, mature B-cells, or mature T-cells. Lymphomas are systemic diseases and have patterns of spread that mimic the migration patterns of their normal cellular counterparts. Progress in therapy of childhood NHL is one of the greatest success stories of the pediatric oncology in past two decades. More than 75 percent of children with NHL can now be cured with modern therapy. These extraordinary advances in treatment have resulted from enhanced understanding of the biology, immunology, and molecular biology of the NHL; improvements in imaging and staging systems; advances in supportive care; and more rational application of risk adapted chemotherapy by cooperative group trials. Consequent to such high cure rates, the current focus is on optimization of therapy to reduce the acute and long-term consequences of treatment.

Childhood NHL is a heterogeneous collection of diseases derived from both mature and immature cells of B- and T-lineage. Early morphology based classification systems have given way to a practical approach utilizing available immunologic and molecular genetic techniques in addition to the standard morphologic criteria in the current World Health Organization (WHO) classification of hematopoietic and lymphoid tumors. Although nearly all pathologic subtypes of NHL can be seen in children, majority of NHL that occur in children appear in four major categories in the WHO classification systems which are detailed in Table 1. Unlike adults where more than 60 percent lymphomas are indolent, childhood NHL are diffuse, intermediate-high grade, clinically aggressive and are predominantly multifocal and disseminated at diagnosis.[1]

Table 1 Major biologic subgroups in childhood NHL

Histology	*Immunology*	*Clinical features*	*Cytogenetics*
Burkitt and Burkitt-like	B-cell	Abdominal masses, GIT tumors, Waldeyer's ring	t(8;14)(q24;q32) t(2;8)(p11;q24) t(8;22)(q24;q11)
Diffuse large B-cell lymphoma (DLBCL) Primary mediastinal DLBCL	B-cells (germinal center or post-germinal center) B-cells (medullary thymus)	Abdominal masses, GIT tumors, Waldeyer's ring Mediastinum	t(8;14)(q24;q32) t(2;17)(p23;q23)
Anaplastic large cell	T-cell, null cell or NK cell ($CD30^+$)	Skin, nodes, bone, lung	t (2;5) (p23;q35) t (1;2) (q21;p23) t (2;3) (p23;q21)
Precursor T lymphoblastic	T-cell	Anterior mediastinal mass	t (1;14) (p32;q11) t (11;14) (p13;q11) t (10;14) (q24;q11) t (7;19) (q35;p13)
Precursor B lymphoblastic	B-cell precursors	Skin, lymph node	

EPIDEMIOLOGY AND ETIOLOGY OF CHILDHOOD NHL

Lymphoma (Hodgkin and non-Hodgkin) is the third most common childhood malignancy, and NHL accounts for approximately 7 to 10 percent of cancers in children younger than 20 years. NHL occurs most commonly in the second decade of life, and occurs less frequently in children younger than 3 years.

Immunodeficiency, both congenital and acquired (HIV infection or post-transplant), increases the risk of NHL more than 100-fold compared with general population. Epstein-Barr virus (EBV) has been shown to transform human B-cells and has been associated with lymphomas in immunocompromised hosts. However, its role in pathogenesis of NHL in immunocompetent individuals is unproven. EBV DNA has been found in more than 95 percent of tumor cells in endemic Burkitt lymphoma (BL) in Africa in contrast to only 15 to 20 percent in sporadic BL in Europe and North America.[1,2]

The incidence and relative frequency of various subtypes of lymphoma in children varies considerably in different world regions. In India, the estimated incidence is between 6 to 10/million/year with an almost equal distribution of B- and T-cell tumors.[1,2] In India, there is no population-based study with sufficient immunohistochemical backup to allow assignment according to the WHO classification. However, data from lymphoma registry at Tata Memorial Hospital (TMH) suggests that B-cell lymphomas form 48.1 percent of NHLs whereas T-cell lymphomas form 44.3 percent of all the lymphomas. Of B-cell, diffuse large B-cell lymphoma (DLBCL) is the most common (22.9%) followed by BL (15.3%) and in T-cell, lymphoblastic lymphoma (LL) is the most common (31.5%) followed by anaplastic large cell lymphoma (ALCL) seen in 11.1 percent cases. Overall, there seems to be a higher prevalence of DLBCL and LL and lower frequency of BL compared to western countries.[3]

CLINICAL FEATURES

Burkitt Lymphoma

Burkitt lymphoma (BL) is the most common subtype and accounts for about 30 to 50 percent of childhood NHL. It exhibits consistent, aggressive clinical behavior. The malignant cells display a mature B-cell phenotype with expression of surface immunoglobulin M with either kappa or lambda light chains, CD20, CD22, CD10 and are negative for the enzyme terminal deoxynucleotidyl transferase (TdT). BL expresses a characteristic chromosomal translocation, t(8;14) in 80 percent cases and t(8;22) or t(2;8) in rest 20 percent of children; which is considered the gold standard for diagnosis of BL. Each of these translocations results in the inappropriate expression of *cMYC*, the gene involved in cellular proliferation due to juxtaposition of the *cMYC* oncogene on chromosome 8 and immunoglobulin locus regulatory elements on chromosome 14, 2 or 22. Endemic BL possesses breakpoints upstream of *cMYC* while sporadic BL have breakpoints within *cMYC*. The two most common primary sites of disease are the abdomen and head-neck region. Other sites of involvement include testes, bone, peripheral lymph nodes, skin, bone marrow (BM), and central nervous system (CNS).[1,4]

Diffuse Large B-cell Lymphoma

Diffuse large B-cell lymphoma (DLBCL) represents 10 to 20 percent of pediatric NHL. DLBCL occurs more frequently during the second decade of life. Pediatric DLBCL is clinically similar to BL, though it is more often localized and less often involves the BM or CNS. These cells have a mature B-cell immunophenotype with expression of CD19, CD20, CD22, CD79a, and PAX5. Most cells express monoclonal surface immunoglobulin light chain. They have a high mitotic rate but MIB-1 index is less than 90 percent. In contrast to adults, pediatric DLBCL have high expression of *cMYC* and low expression of BCL-2. Majority of pediatric DLBCL have a germinal center B-cell phenotype with expression of normal germinal center markers such as the *BCL6* gene product and CD10. Unlike adult DLBCL, pediatric diffuse DLBCL rarely demonstrates the t(14;18) translocation but 30 percent of will have a gene signature similar to BL.

About 20 percent of pediatric DLBCL present as primary mediastinal disease (primary mediastinal B-cell lymphoma [PMBCL]). This presentation is more common in older children and adolescents. These tumors arise from thymic B-cells and show diffuse large cell proliferation with classic compartmentalizing sclerosis. Cells have surface markers similar to DLBCL but lack surface immunoglobulins and commonly express CD30. It is associated with distinctive chromosomal aberrations (gains in chromosome 9p and 2p in regions that involve *JAK2* and *c-REL*, respectively) and has an inferior outcome compared with other pediatric DLBCL.[1,4]

Lymphoblastic Lymphoma

Lymphoblastic lymphoma (LL) makes up approximately 20 percent of childhood NHL. More than 75 percent of LL usually have a T-cell immunophenotype (T-LL) and the remainders have a precursor B-cell phenotype (B-LL). These are part of a spectrum of precursor blast cell neoplasms seen in children. By definition, patients with more than 25 percent marrow blasts are considered to have leukemia, and those with fewer than 25 percent marrow

blasts are considered to have lymphoma. However, this is arbitrary and current WHO classification labels them in the category of lymphoblastic lymphoma/leukemia. Notably, despite the clinicopathologic overlap between ALL and LL, there is suggestion of different gene expression profile and loss of heterozygosity at 6q indicating biologic differences between them. Cytologically, cells have a high mitotic rate and express TdT. T-LL display cortical thymocyte origin and express CD1a, CD2, CD5 and CD7 along with co-expression of CD4 and/or CD8. B-LL displays early pre-B or pre-B immunophenotype with expression of CD19, CD10 and TdT. Majority of patients with T-LL present with an anterior mediastinal mass and may have symptoms of dyspnea, wheezing, stridor, dysphagia, or swelling of the head and neck due to compression of mediastinal structures which is called superior vena cava syndrome (SVCS). Pleural effusions and supradiaphragmatic lymph nodes may also be present. There may also be involvement of bone, skin, bone marrow, CNS, abdominal organs (but rarely bowel), and occasionally other sites such as lymphoid tissue of Waldeyer ring and testes.[1,4]

Anaplastic Large Cell Lymphoma

Anaplastic large cell lymphoma (ALCL) is a peripheral T-cell lymphoma (PTCL) as per WHO classification and accounts for approximately 10 percent of childhood NHL. Majority of ALCL are mature T-cell, but 20 percent may have null-cell disease (i.e. no T-cell, B-cell, or NK-cell surface antigen expression). However, ALK positive null ALCL shows *TCR* gene rearrangements. Morphologically, classic variant shows large anaplastic cells and horseshoe like multinucleate "hallmark" cells. Ten percent would have lymphohistiocytic variant with lots of benign histiocytes and a small percentage would show the small cell variant. The latter two variants have relatively poor prognosis. Immunophenotypically, tumors cells variably express CD30, CD45, epithelial membrane antigen and ALK protein. More than 90 percent of ALCL cases have the translocation t(2;5)(p23;q35) leading to the expression of the fusion protein NPM/ALK. Clinically, ALCL has protein presentations, including involvement of lymph nodes and a variety of extranodal sites, particularly skin and bone and, less often, gastrointestinal tract, lung, pleura, and muscle. Involvement of the CNS and bone marrow is uncommon. ALCL is often associated with systemic symptoms (e.g. fever, weight loss) and a prolonged waxing and waning course, making diagnosis difficult and often delayed.[1,4]

Rare Lymphomas in Children

Indolent mature B-cell lymphomas, are rare in children but follicular lymphoma (FL) and nodal marginal zone lymphoma (MALT) have been described and accepted by WHO as unique entities. Pediatric follicular lymphoma predominantly occurs as cervical adenopathy and tonsillar enlargement generally in males, and is more likely to be localized disease. It can rarely involve extranodal sites such as the testis, kidney, gastrointestinal tract, and parotid. The outcome of pediatric follicular lymphoma is excellent. MALT lymphomas present as low-stage (stage I or II) disease both in nodal and extranodal sites such as stomach (associated with *H. pylori* infection) and conjunctiva (associated with *chlamydial psittaci* infections).[4-6]

Diagnostic and Staging Evaluation

The diagnosis of NHL is based upon the pathologic evaluation of involved tissue, usually an abdominal mass, extranodal site, or lymph node, interpreted within the clinical context. Subtypes of NHL are identified using histology, immunophenotype, and genetic studies. Most children present with advanced-stage disease including BM invasion or/and malignant effusions. In such cases, correct diagnosis can be made by cytology and immunophenotyping by flow cytometry. If this is not possible, diagnosis is based on biopsy, and most cases are correctly classified by cytology of tumor touch imprints, histomorphology, and immunohistochemistry. In most cases, these enable correct classification and allocation of patients to appropriate treatment subgroups. In certain cases, cytogenetics is also required for diagnosis, such as variant BL/BL-like lymphomas. Fluorescence *in situ* hybridization (FISH), which can be performed on tumor touch preparations, or paraffin sections, is a standard method for confirming most of the chromosomal translocations.[1,4]

- Routine staging of pediatric NHL should include contrast enhanced computerized tomographic (CT) imaging of the neck, chest, abdomen, and pelvis. Baseline CT serves to help determine disease stage at diagnosis and to provide a baseline for comparison to determine response to treatment. Examination of cerebrospinal fluid and BM is crucial for staging evaluation. Laboratory tests also may be abnormal in patients with newly diagnosed pediatric NHL such as unexplained anemia, thrombocytopenia, or leukopenia due to extensive bone marrow infiltration, hyperuricemia as well as other features of tumor lysis syndrome and elevated level of serum lactate dehydrogenase (LDH) due to high tumor burden.[1,4]
- *Emerging role of PET-scan:* PET-CT scan of whole body for staging and response evaluation in children is currently investigational and being evaluated in many current studies. Although PET-CT is recognized to be advantageous in the primary staging of adult NHL, this has not been demonstrated in childhood NHL.

This may be, because majority of children present with advanced disease (stages III or IV) which is easily detectable by CT-scan. However, PET-CT appears to have a higher level of sensitivity than bone marrow biopsy in the detection of bone marrow infiltration and hence may be useful as a noninvasive modality for detecting bone marrow involvement in pediatric NHL.[1,4,7]

Similarly, early response assessment to chemotherapy with an interim PET is now routine done in the management of adults with NHL; this is not regarded as standard practice in children due to limited data. However, PET may be potentially useful for assessing the speed of response and confirmation of post-therapy remission (CR).[7]

- *Staging and risk stratification (Tables 2 and 3):* The Ann Arbor staging classification used for HL does not adequately reflect prognosis in childhood NHL because of the unique biology, clinical behavior and outcome of the four major subtypes of NHL seen in children.

Currently used St. Jude children research hospital (Murphy's) staging classification takes into consideration increased extranodal involvement, metastatic spread to the BM or CNS and noncontiguous spread of disease in this group (Table 2).[1,4] However, with the evolution of more intensive therapy, it is becoming redundant. For example, in BL, the cure rates for stages 2, 3 and 4 (CNS-negative) have become almost equal. Hence, the French Society of Pediatric Oncology (SFOP) and BFM group have modified the St. Jude's system with incorporation of other clinical and biological parameters for better risk-assignment (Table 3). This classification is being applied in the ongoing B-NHL international study (FAB-LMB).[8-13]

St. Jude's system is also not ideal for LL and ALCL. In LL, patients presenting with stage 1 or 2 disease are rare and majority present in stage 3. Moreover, there is no significant difference in outcome in those with stage 3 and stage 4 disease.[1,4] Similarly, ALCL frequently involves sites atypical of childhood lymphoma (such as skin, bone and lung). Le Deley evaluated prognostic factors for ALCL in

Table 2 St. Jude's staging system for childhood NHL

Stage	*Definition*
I	Single tumor (extranodal) Single anatomic area (nodal) excluding mediastinum or abdomen
II	Single tumor (extranodal) with regional node involvement Primary gastrointestinal tumor with or without involvement of mesenteric node only On same side of diaphragm: (a) Two or more nodal areas (b) Two single extranodal tumors with or without regional node involvement
III	All primary intrathoracic tumors All extensive primary intra-abdominal disease Two or more nodal or extranodal areas on both sides of diaphragm
IV	Any of the above with CNS or bone marrow involvement

Table 3 Pediatric B-NHL—current risk grouping

Protocol	*Group*	*Definition*	*5 years EFS*
B-NHL (LMB89)	A	Completely resected stage-1 and abdominal stage 2	98%
	B	Unresected stage-1, nonabdominal stage 2 All stages 3 and 4 B-ALL <25% blasts, CNS –ve	92%
	C	B-ALL >25% blast or CNS +ve	84%
B-NHL (BFM)	R1	Stage I, II initial complete resection	94%
	R2	Stage I, II unresected, stage III with LDH <500 U/L	94%
	R3	Stage III with LDH <500–999 U/L BM+ve and LDH <1000 U/L	85%
	R4	LDH >1000 U/L and/or CNS +ve	81%

culled data from BFM, SFOP and UKCCSG studies and found that, mediastinal involvement (p=0.004), lung, spleen and/or hepatic disease (p=0.006) and skin lesions (p=0.02) were associated with a significantly poorer outcome. Based on this, two risk groups were delineated: standard (EFS 87%), and high risk (skin, mediastinal and/or visceral disease; EFS 61%).[14]

Furthermore, in B-NHL, adolescent age, primary mediastinal DLBCL subtype, involvement of CNS with bone marrow, high LDH (more than 2.5 times upper limit of normal), and poor response to COP prophase (<20% reduction in tumor burden) are associated with poor prognosis.[15] Also, secondary cytogenetic abnormalities, other than *cMYC* rearrangement including gain of 7q or deletion of 13q have been shown to be strong adverse factors in two recent studies in BL. Similarly, deletion of 6q has been demonstrated to be a poor prognostic factor in LL.[16]

- *Upcoming role of minimal residual disease (MRD) evaluation:* Monitoring residual clonal lymphoma cells in the blood and/or BM by means of aberrant immunophenotype or PCR-based identification of specific fusion gene products is an emerging tool for evaluating the kinetics of treatment response in childhood NHL. The cumulative incidence of relapse was 71 percent in children with ALCL having > 10 copies of NPM-ALK/10,000 copies ABL in BM or blood. Quantitative PCR for NPM-ALK in BM or blood allowed identification of 20 percent of patients experiencing 60 percent of all relapses with an event-free survival of only 20 percent in one study. Similarly, in BL, a assay that can detect the t(8;14) has been used at diagnosis or during therapy and has been found superior to BM aspirate and BM biopsy in the assessment of MRD.[17,18]

TREATMENT

Principles of Management

Childhood NHL are extremely chemosensitive tumors. Surgery plays a very limited role, mainly for arriving at a diagnosis. Radiation of primary sites is used very rarely in emergency situations. Hence, multiagent chemotherapy directed to the histologic subtype and stage of the disease remains the cornerstone of therapy.

There are two potentially life-threatening clinical situations that are often seen in children with NHL at presentation:

- Superior vena cava syndrome (or mediastinal tumor with airway obstruction), most often seen in LL
- Tumor lysis syndrome, most often seen in lymphoblastic and BL. These emergent situations should be anticipated and addressed immediately.

Patients with large mediastinal masses are at risk of cardiac or respiratory arrest during general anesthesia or heavy sedation. If peripheral blood counts are normal, the least invasive procedure should be used to establish the diagnosis of lymphoma such as pleural tap, bone marrow examination, a lymph node biopsy under local anesthesia or a computed tomography–guided core needle biopsy should be contemplated. These children should be closely monitored in intensive care units in propped-up lateral position and may be started on steroids if it is unsafe to perform a diagnostic biopsy because of the risk of anesthesia or sedation. Biopsy should be obtained as soon as patient is able to undergo the procedure safely.[1,4]

Tumor lysis syndrome (TLS) results from rapid breakdown of malignant cells resulting in a number of metabolic abnormalities, most notably hyperuricemia, hyperkalemia, and hyperphosphatemia. Hyperhydration and allopurinol or rasburicase (urate oxidase) are essential components of therapy. Rasburicase, a recombinant urate oxidase rapidly lowers serum uric acid levels and prevents the metabolic problems associated with TLS. Use of rasburicase (0.05 to 0.1 mg/kg IV [max 1.5 mg]) preserves renal function and allows early administration of planned therapy. The use of rasburicase has dramatically reduced the requirement for dialysis in this population. Gastrointestinal bleeding, obstruction, and (rarely) perforation may also occur during the initial phase of therapy in B-NHL with gut involvement.[19]

Principles of Chemotherapy in NHL

Many studies including the seminal Children's Oncology Group (COG) trial that randomized all children with NHL to be treated with short duration pulse intensive COMP regimen (cyclophosphamide, vincristine, methotrexate, and prednisone) or to a long duration modified LSA_2L_2 regimen (used for acute lymphoblastic leukemia) have shown that LL fare better when treated with long duration LSA_2L_2 leukemia regimen and short duration COMP was better for patients with B-cell NHL.[20,21]

DLBCL has a similar pattern of initial disease distribution and the same rapid response to chemotherapy as BL. Results from the large studies such as LMB and BFM suggest that with similar therapy there is no difference in outcome between BL and DLBCL and these should be treated with the same approach. In B-NHL, in view of high growth fraction and short doubling time; short, pulse-intensive, multi-agent chemotherapy is given in courses of 3 to 5 days with a schedule characterized by fractionation or continuous infusion of drugs. To prevent rapid re-growth, courses are administrated at shortest intervals. Treatment intensity is adapted to tumor burden (stage, LDH level, BM involvement, CNS involvement) and response to COP pre-phase. In addition, intensive CNS directed therapy using high dose methotrexate or cytosine-arabinoside and intrathecal therapy (single agent methotrexate +/- ara-c or triple intrathecal) is usually necessary. Cranial radiotherapy is not necessary.[8-13]

T-cell Lymphomas (Table 4)

Table 4 Treatment and outcome of T-NHL

ALCL (T-cell type approach)

Protocol	*Number*	*Stage*	*EFS (%)*	*Duration /number of cycles*
LSA2L2	19	III/IV	56	14–36 months
CCG-5941	86	III/IV	78	12 months
POG 9315	86	III/IV	72	12 months
AIEOP (LNH-92)	34	II/III/IV	65	24 months
ALCL (B-Cell type approach)				
BFM 90	8 20 55 6	I II III IV	100 79 74 50	*Stage I/II (completely resected):* 3 cycles *Stage II (unresected)/stage III:* 6 cycles *Stage IV/bone disease:* 6 intensified cycles
SFOP-HM 89/91	82	I/II III/IV	94 55	7–8 months
UKCCSG	72	III/IV	59	*Stage III/IV (CNS–neg):* 5 cycles *CNS positive:* Intensified 5 cycles
MCP-842	27	I/II III/IV	67 40	6–8 (alternating A and B) cycles
Lymphoblastic lymphoma				
LSA2L2/ADCOMP with LSA2L2	281	I/II III/IV	84 64	18 months
POG8704	218	III / IV	67	24 months
LMT 81	76 8	I / II III / IV	76 73	12 months
UKCCSG	59	III / IV	65	24 months
BFM- 90	82 19	III IV	90 95	24 months
BFM- 95	22 169	I / II III / IV	95 78	24 months

Precursor T Lymphoblastic Lymphoma

- *Localized LL:* For localized LL patients (stage I/II disease), induction therapy with short, pulsed chemotherapy (CHOP) results in a 95 percent CR rate. However, only 60 percent can achieve long-term DFS due to late relapses in bone marrow. Majority of these patients can be salvaged, giving an overall survival (OS) of >90 percent at 5 years. There is no survival benefit of involved field irradiation. Using a leukemia like approach with induction, consolidation, and maintenance therapy for a total of 24 months, most studies have shown more than 90 percent survival for localized LL. No reinduction therapy and local or cranial radiation is given for stage I and II patients.[21,22]
- *Advanced stage LL:* Advanced LL have event free survival (EFS) rates higher than 80 percent with protocols designed for high-risk ALL consisting of a four-drug induction, consolidation incorporating high-dose methotrexate, cyclophosphamide, cytarabine, CNS directed therapy including intrathecal therapy and long maintenance with total 24 months of therapy.[21-26] Some recent studies have shown that patients receiving high-dose asparaginase regimen had a superior outcome.[23] Several studies also suggest that high dose methotrexate (MTX) results in survival advantage. No benefit on outcome has been observed with use of high dose cytarabine. The results of the various chemotherapeutic regimens are shown in Table 4.[21-26]

Until recently, CNS directed therapy included the combined use of cranial irradiation and intrathecal chemotherapy. However, recent studies have demonstrated that cranial irradiation can be safely omitted if systemic high-dose methotrexate combined with intrathecal chemotherapy is administered. However, cranial irradiation may be necessary for children who present with CNS disease (fewer than 5% of children).[26] Irradiation of primary sites such as mediastinum has not been shown to improve outcome when added to chemotherapy. Even in patients with testicular disease at diagnosis, testicular radiation is only indicated for residual disease after systemic therapy incorporating high-dose MTX.[21-26]

In NHL-BFM-95, prophylactic cranial radiation was omitted, and the intensity of induction therapy was modified (reduction of asparaginase and/or doxorubicin). There was no significant increase in CNS relapses, suggesting cranial radiation may be reserved for patients with CNS disease at diagnosis. However, survival was worse in BFM-95 than in BFM-90 (90% vs. 82%), possibly due to reduced intensity induction and increased number of secondary malignancies in BFM-95.[26] Currently, ongoing BFM-based trials are trying to determine whether dexamethasone instead of prednisone during induction can further improve outcome of patients with LL, as was observed in children with ALL.

B-Precursor LL

The correct treatment for B-lineage LL constituting around 20 percent of LL has not been clearly defined because of rarity of this disease. The results of the largest review of 98 patients (64% <18 years old) showed that majority had skin (with or without adjacent nodal disease), lymph node, bone, head and neck and retroperitoneal disease. Mediastinal disease was uncommon. The disease free survival was 74 percent at a median follow-up of 28 months. In BFM-NHL trials, 27 children with precursor B-cell LL were treated; 21 on ALL-type therapy (<10% relapses) and 6 on Burkitt type therapy (50% relapses). All relapses on the latter regimen were salvaged with ALL-type therapy leading to 73 percent EFS and 92 percent OS for the group at 10 years. This suggests that patients with B-lineage LL should be treated with ALL like therapy duration of 18 to 24 months.[27]

- *Anaplastic large cell lymphoma (Table 4):* There is no consensus on management of ALCL due to small number of patients treated in various studies, heterogeneity in inclusion criteria, different staging systems and diverse treatment approaches used in past trials. However, following broad principles can be derived.
- *Localized ALCL:* For localized ALCL (grossly resected, i.e. >90% stage I/II disease), the best results have come from using pulsed chemotherapy similar to B-NHL therapy. BFM group has shown results similar to those obtained with the BFM-90 regimen for B-NHL with use of 3 cycles after cytoreduction. In POG studies, Stage I and II disease was very effectively treated with CHOP for three cycles without RT. The recent ALCL-99 trial used three cycles of chemotherapy following prophase for patients with stage I completely resected disease with good results. Primary cutaneous ALCL may be treated successfully with surgical resection and/or local radiotherapy without systemic chemotherapy. Thus, children with standard risk disease (Stage I/II completely resected with no high risk features such as involvement of skin, mediastinum, viscera, CNS or BM) can be managed with 3 cycles (10–12 weeks) of B-cell type regimen and intrathecal therapy.[28-32]
- *Disseminated ALCL:* Children with disseminated ALCL have EFS of approximately 60 to 75 percent. Majority of European studies (BFM, SFOP, UKCCSG, ALCL-99) have used short duration (5–8 months) pulse intensive B-cell type approach with good results while American (CCG, POG) and Italian groups (AIEOP) have used leukemia type long duration (12–24 months) less intensive approach with almost equivalent survival but increased hematological toxicity. The recent POG studies demonstrated no benefit of adding methotrexate and high-dose cytarabine to 52 weeks of cyclic chemotherapy. Cranial prophylaxis using high or intermediate dose methotrexate with (BFM) or without intrathecal (SFOP) or only intrathecal therapy (COG, MCP-842) have shown equivalent results with less than 1 percent incidence of CNS relapses. Cranial radiotherapy (RT) is not recommended.[28-32]

  Recently conducted international ALCL 99 study has shown that HD MTX (3 g/m^2 intravenously over 3 hours) is sufficient to protect the CNS in CNS-negative patients in the absence of additional intrathecal chemotherapy and addition of vinblastine during induction and as maintenance for a total treatment duration of 1 year did not reduce the risk of failure.[31,32] Thus, disseminated ALCL may be managed with 6 cycles (6 months) of B-cell type regimen using short infusion HD MTX.

B-Cell Lymphoma (Table 5)

- *Treatment of limited stage B-NHL:* Children with limited stage B-cell NHL (stage I or stage II, BFM R1 or FAB group A) have a good prognosis with an estimated five-year EFS of 90 to 95 percent with minimal

Table 5 Outcome of B-NHL in International Studies

Protocol	*Number*	*Stage*	*EFS (%)*	*Duration/number of cycles*
COMP	57 135	I/II III/IV	84 53	6 months
LMB 89	52 386 123	Gp A Gp B Gp C	98 92 84	*Group A:* 2 cycles, No IT *Group B:* 5 cycles *Group C:* 8 cycles
FAB-LMB96	136 760 238	Gp A Gp B Gp C	98 90 79	*Group A and C:* As LMB 89 *Group B:* 4 cycles with reduced dose cyclophosphamide *Group C:* 8 cycles
BFM-95	98 233 82 142	R1 R2 R3 R4	94 94 85 81	*R1:* 2 cycles *R2:* 4 cycles *R3:* 5 intensified cycles *R4:* 6 intensified cycles
CHOP	266	I/II	90	6 cycles
Orange (CCG)	34	III/IV	77	5–7 months

chemotherapy (range 6 weeks to 6 months). There are several multiagent chemotherapy regimens that have resulted in this excellent outcome, including 6 weeks of COPAD (FAB), 3 to 6 months of COMP (CCG and POG), or two cycles of multiagent chemotherapy (BFM). In the most recent BFM study (BFM-95), it was shown that reducing the dose or duration of methotrexate infusion did not affect the results for localized disease.[8-13,21]

- *Treatment of advanced B-NHL:* The prognosis of advanced B-NHL has improved significantly over the past decade with the use of short intensive chemotherapy regimens such as FAB/LMB 96 (FAB), Orange (CCG) or BFM NHL 95 with more than 90 percent 5-year disease-free survival except in patients with CNS disease.[4-9] Recent studies have demonstrated that cranial irradiation can be eliminated in patients with CNS-positive disease with the substitution of more aggressive high-dose methotrexate and additional intrathecal chemotherapy.[8-13,21]

  Recently conducted FAB/LMB 96 study showed that intermediate-risk patients (group B) with and good response to prophase COP can be treated with reduced intensity therapy with 4 courses but high-risk patients should receive standard FAB/LMB therapy (8 courses).[12,13] Children with BM and/or CNS involvement have an inferior outcome if they have a poor response to reduction chemotherapy with COP prophase or have combined BM and CNS disease.[15] Rituximab, a mouse/human chimeric monoclonal antibody targeting the CD20 antigen, has shown good responses in relapsed B-NHL and a promising response rate of 41.4 percent in a phase II window study in newly diagnosed B-cell NHL and Burkitt leukemia. Based on results of a COG pilot study (ANHL01P1) that it is feasible and safe to include rituximab in the current chemotherapy backbone, current studies are evaluating rituximab in high-risk B-NHL patients.[33]
- *Primary mediastinal DLBCL (PMBL):* The response to chemotherapy is slow and outcome is poor in PMBL. In one CCG series of 20 children with PMBCL, where almost half received local irradiation, the 5-year EFS was only 75 percent. In a BFM report of 30 children, the 5-year EFS was 70 percent using chemotherapy alone. In FAB/LMB-96 study of stage III primary mediastinal large B-cell lymphoma, the 5-year event-free survival (EFS) was 66 percent, versus 85 percent for adolescents with nonmediastinal DLBCL. Recently, a single-arm study in adults showed excellent event-free survival utilizing the DA-EPOCH-R regimen (dose-adjusted etoposide, doxorubicin, cyclophosphamide, vincristine, prednisone, and rituximab; usually six cycles) with filgrastim and no radiation therapy. The 5-year EFS was 93 percent and overall survival (OS) was 97 percent. This is currently being tested in pediatric clinical trials. Early mediastinal irradiation in incomplete initial responders may be considered.[34]
- *Outcome of pediatric NHL in India (Table 6):* Before 1986, survival of pediatric NHL patients in India was less than 30 percent. To improve survival rates and to overcome the barriers of limited resources, sub-optimal supportive care, high prevalence of infectious diseases, impaired nutritional status and delayed

Table 6 Outcome with MCP-842 (1986–2006) at Tata Memorial Hospital

Histology	*Number*	*Stage*	*Modified MCP-842 EFS (%) (10 years)*	*OS (%) (10 years)*
Burkitt-lymphoma	107	I/II III/IV	100% 78%	83%
DLBL	53	I/II III/IV	96% 82%	82%
ALCL	27	All stages	75%	71%

diagnosis; a moderately intensive, short duration protocol (MCP842) was designed in 1984. Protocol consisted of 6 to 8 alternating cycles of two different drug combinations designated as regimens A and B with intrathecal therapy during the first four cycles. The drugs used in regimen A were cyclophosphamide, vincristine, doxorubicin and cytarabine, while regimen B included ifosfamide, etoposide and MTX. Intrathecal MTX and cytarabine were used for central nervous system (CNS) prophylaxis. Neither cranial nor local radiotherapy were included in the treatment protocol.

Over last 2 decades, MCP 842 has been found to be an effective protocol for the management of patients with B-cell NHL and may be an ideal protocol for patients in centers with limited resources since it involves no high dose chemotherapy, there is no need for methotrexate level monitoring, no central line/hyperalimentation requirement and there is minimum blood component usage. This protocol has been recently modified with addition of vinblastine as well as maintenance (6–12 months) for ALCL, addition of COP prephase (for patients with bulky disease or poor GC), use of urate oxidase in tumor lysis. The survival with modified MCP-842 is significantly better compared to standard MCP-842.The results of this protocol are summarized in Table 5.[35-37]

Management of Relapse

Relapse is a significant obstacle to long-term survival for children with advanced-stage NHL. In LL, most relapses occur within 2 years of diagnosis, but occasional late relapse is observed. In contrast to relapse for early-stage disease, the outcome after salvage chemotherapy is poor for children with advanced-stage disease at initial presentation. However, survival rates of 30 to 50 percent have been reported after allogenic stem cell transplantation (SCT).[38,39]

The outcome of relapsed patients with BL is dismal because most relapses tend to occur early during active chemotherapy, and drug resistance is a major obstacle to successful salvage. Rituximab have been reported to be active in the relapse setting. Current investigational protocols combine chemotherapy with rituximab followed by allogeneic or autologous SCT. The outcome is more favorable for patients who achieve a second remission before proceeding to SCT.[38]

Children with relapsed DLBCL are often treated with salvage chemoimmunotherapy regimens such as ICE, GDP (Gemcitabine, dexamethasone and cisplatinum) and DECAL (dexamethasone, etoposide, cisplatin, HD cytarabine, and L-Asp) with rituximab followed by autologous SCT based on the adult experience. The outcome is quite favorable for those children who have chemosensitive disease at the time of relapse.[38]

The outcome for survival after relapse of ALCL is relatively favorable in contrast to the less optimistic outcome for children with relapsed LL and BL. A French study demonstrated excellent responses to single-agent vinblastine followed by some very durable second remissions. A survival rate of 69 percent at 3 years with courses of CCNU, vinblastine, bleomycin or cytarabine followed by autologous HSCT in some of the patients has been reported.[40] Even in high-risk patients with on-therapy relapse or relapse after autologous HSCT, long-term remissions have been observed after allogeneic HSCT.[41] The potential benefit of vinblastine and anti-CD30 antibody combined with either an APO or BFM-like regimen is currently under investigation. Phase 1 study of ALK oncogenic tyrosine kinase inhibitor (Crizotinib) in pediatric patients with anaplastic large-cell lymphoma has shown response rate of 88 percent in ALK positive patients.[42] Brentuximab vedotin is a novel antibody-drug conjugate that targets CD30, a cell surface antigen expressed by HL and ALCL. A phase II trial in adults with relapsed anaplastic large cell lymphoma has shown CR rates of approximately 55 to 60 percent and PR rates of 29 percent.[43]

CONCLUSION

Refinements in systemic chemotherapy fuelled by better understanding of NHL biology in children have led to cure in approximately 80 to 85 percent of all patients. This improved outlook for childhood NHL, however, has come

with a certain price. The use of intense chemotherapy has resulted in long hospitalizations, severe hematopoietic as well as non-hematopoietic toxicity and late effects, such as sterility, cardiomyopathy, and secondary malignancies. Consequently, the emphasis for the near future is to decrease the therapy in good risk patients as well as better identification and development of new therapeutic approaches for high-risk cases. In future, as the molecular pathogenesis of the malignant lymphomas is better elucidated using molecular diagnostic tools, new targets for therapy will emerge. Also, it is likely that targeted therapy will substitute for some of the toxic chemotherapy and thereby minimize the chemotherapy related morbidity. This novel molecular biologic information will also be valuable for developing more sensitive diagnostic tools, measurement of early response to therapy as well as submicroscopic disease and for identifying new prognostic subgroups. Superior risk-adapted therapy based on these advances would maximize the chance for cure while avoiding both acute and chronic toxicities of treatment.

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CHAPTER 45

Langerhans Cell Histiocytosis

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Langerhans cell histiocytosis (LCH) is an enigmatic disorder occurring due to a reactive clonal proliferation of dendritic cells, which are immunophenotypically and functionally immature and comprises a group of idiopathic disorders characterized by the presence of these cells in a background of hematopoietic cells, including T-cells, macrophages, eosinophils and occasional multinucleated giant cells.[1] Accumulation of these cells at various sites in the body are responsible for its clinical manifestations. More recent work has shown that the pathologic LCH cells have a gene expression profile of a myeloid dendritic cells rather than the skin Langerhans cells (LC) that are closer to the sites where the disease usually occurs. Controversy too exists whether the clonal proliferation of LCH cells results from a malignant transformation or due to immune dysregulation of LCs.[2]

Earlier, LCH was subcategorized based upon clinical presentation (i.e. Letterer-Siwe disease, Hand-Schüller-Christian, eosinophilic granuloma, etc.). Lichtenstein was the first to suggest integration of these disorders under the common term histiocytosis X, the first word recognizing their common origin, while the "X" underlined his uncertainty as to what the origin was![3] However, over the past few decades since then, there has been unprecedented progress in the understanding of this enigmatic disease by means of collaborative trials notably the LCH trials by the Histiocyte Study Groups, and large single institutional studies.[4-6] The common etiology between Lichtenstein's groups is now well understood. Minimum diagnostic criteria have been evolved to ensure clarity and uniformity allowing comparison between various studies—a prerequisite all the more essential for rare disorders like LCH. For treatment purposes too, the disorders are now clubbed and categorized by the number and nature of organs involved at diagnosis. Involvement of the liver, spleen, lung, and bone marrow, and their degree of dysfunction has been shown to be the key determinant to outcome irrespective of the therapies offered. This was even corroborated when the new risk classifications were applied retrospectively to past cases, to see how they fared, in a large single institution study from India, showing the robustness of these risk stratifications.[7]

EPIDEMIOLOGY

The reported incidence of LCH from various studies is two to ten cases per million children aged 15 years or younger.[8,9] The male/female (M/F) ratio is close to one and the median age of presentation is 30 months.[10] Solvent exposures in parents, family history of cancer, and perinatal infections have been weakly associated with LCH.[11,12]

CLINICAL FEATURES

LCH is a rare childhood malignancy. In a large Indian cancer center, only 52 cases were registered over a 17-years-period.[7] In addition to its rarity is the fact that it has myriad presentations and its clinical course can range from low grade chronic and persistent to the rapidly progressive and fatal, making its behavior unpredictable to most individual clinicians. Collaborative trials and large single institutional studies therefore, have been the major means of shedding light on the understanding of this disease and deciding its management. The most important concept that has evolved has been that multiorgan involvement has worse outcome than single-organ disease and needs some form of cytotoxic therapy. Also, among those with multiorgan disease, some patients are more "at risk"

for disease progression, sequelae, and death. High-risk organs include liver, spleen, and bone marrow. Low-risk organs include skin, bone, lymph nodes, gastrointestinal tract, pituitary gland, and central nervous system (CNS).[4-7,9,10,13,14]

LCH in children usually presents with a skin rash or painful bone lesions. Systemic symptoms of fever, weight loss, diarrhea, edema, dyspnea, polydipsia, and polyuria may relate to specific organ involvement by disease.[7, 9, 10]

Patients may present with a single organ involvement (single-system LCH), which may be a single site (unifocal) or involve multiple sites (multifocal). Involvement of more than one organ is categorized as multisystem LCH. In this group, involvement may be in a limited number of organs or it may be disseminated. Patients may present with LCH of the skin, bone, lymph nodes, and pituitary in any combination and are still considered at low-risk of death. Multisystem LCH patients' have relatively high-risk for long-term consequences of the disease.[7, 9, 10, 13, 14]

Single-System Disease

In single-system LCH, the patient presents with involvement of a single site or organ, including skin and nails, oral cavity, bone, lymph nodes and thymus, pituitary gland, and thyroid.

- **Skin and nails:** Dermal involvement occurs in 35 to 50 perent cases in most series.[10,15] However, in Indian literature, the reported incidence was lower at 25 percent. This may have been due to under-reporting of minor lesions such as seborrheic dermatitis, the data being from a large referral center.[7] In infants, seborrheic involvement of the scalp may be mistaken for prolonged cradle cap. Infants may also present with brown to purplish papules over any part of their body (Hashimoto-Pritzker disease). These lesions in infants may be self-limited as the lesions often disappear without treatment during the first year of life; however, they need to be followed up closely for systemic disease manifestations which may present later on after the initial skin lesions.[15-17] Children may present with a red papular rash in the groin, abdomen, back, or chest that resembles a diffuse candidal rash. Seborrheic involvement of the scalp may be mistaken for a severe case of dandruff in older children. Ulcerative lesions behind the ears, involving the scalp, under the breasts, or genitalia or perianal region may be misdiagnosed as bacterial or fungal infections on presentation. Involvement of nails is an unusual presentation. They may present as a single site or in conjunction with other sites, and often show longitudinal, discolored grooves with loss of nail tissue.[15-17]
- **Oral cavity:** The oral cavity lesions may precede evidence of LCH in other organs. Presenting symptoms may be gingival hypertrophy, and ulcers of the soft or hard palate, buccal mucosa, or on the tongue and lips. Hypermobile teeth (floating teeth) and tooth loss may occur.[18]
- **Bone:** Bones are the most common site of involvement in LCH. Skeletal involvement with or without other sites occurs in 80 to 100 percent of patients in most large series.[7,10,13] Hands and feet are often spared, but it can involve almost any other bone, the most frequent site being the skull. It presents as a lytic bony lesion with associated soft tissue swelling, which may be asymptomatic or painful. In the skull, the soft tissue mass may impinge inwards on the dura.[19] Other frequent sites involved are the femur, ribs, humerus, and vertebra.[19,20] Amongst the vertebra, the cervical are the most common to be involved. Vertebral lesions may result in collapse of the vertebral body (vertebra plana). Vertebral lesions with soft tissue extension may present with pain and neurologic deficits.[20] Disease of the facial bones or anterior or middle cranial fossae (e.g. temporal, sphenoid, ethmoid, zygomatic) with intracranial tumor extension comprise part of a CNS-risk group. These children have a threefold increased risk of developing diabetes insipidus and an increased risk of other CNS disease.[21, 22]
- **Lymph nodes and thymus:** Lymphadenopathy has been reported more commonly in a large Indian series than in western literature, where it is reported to be less than 10 percent.[7,22] Cervical nodes are most frequently involved. Nodes may be soft- or hard-matted with accompanying lymphedema. An enlarged thymus or mediastinal node due to LCH can mimic lymphoma or an infectious process, or asthma.
- **Pituitary gland and thyroid:** Posterior pituitary gland involvement presents with central diabetes insipidus (DI). Involvement of anterior pituitary results in growth failure and delayed or precocious puberty. DI more commonly manifests in multisystem LCH where it can afflict nearly 1/3rd of all such patients and can develop at any time during the course of the illness.[4,7,21,22]

Multisystem Disease

In this presentation, multiple organs are involved at the outset. The involvement of certain organs like the liver, spleen and hematological system put the patients into a higher risk category. Other organs that can be involved are the same as in single system disease but in various combinations.

- **Bone and other organ systems:** LCH patients may present with multiple bone lesions (single-system multifocal bone) or bone lesions with other organ involvement (multisystem including bone). As already discussed above, the later group has a higher incidence of diabetes insipidus, probably due to the higher frequency of lesions in the facial bones (temporal bone, mastoid/petrous bone, orbit, and zygomatic bone).
- **Abdominal/gastrointestinal system:** Liver and spleen are considered high-risk organs. Enlargement of these organs may be due to direct infiltration of LCH cells or as a secondary phenomenon of excess cytokines leading to macrophage activation or infiltration of lymphocytes around bile ducts.

  Hepatomegaly is often present in systemic disease and pathological changes might be present in the liver on histology, even in the absence of liver dysfunction.[23] LCH in liver has a portal (bile duct) tropism and can cause biliary damage and ductal sclerosis. Hepatomegaly may be accompanied by hypoalbuminemia with ascites, hyperbilirubinemia, clotting factor deficiencies, elevated alkaline phosphatase, liver transaminases, and gamma glutamyl transpeptidase levels. Cholestasis and sclerosing cholangitis is one of the most serious complications of liver involvement in LCH.[23, 24] Sonography, computed tomography (CT), or MRI of the liver will show hypoechoic or low-signal intensity along the portal veins or biliary tracts when the liver is involved with LCH.[25] This usually occurs months after initial presentation, but occasionally may present at diagnosis. Children with sclerosing cholangitis will not respond to chemotherapy as the disease is not active and the fibrosis and sclerosis remain. Liver transplantation is the only treatment when hepatic function worsens.[24] Rare cases of LCH infiltration of the pancreas and kidneys has been reported.[26]
- Splenic involvement has been noted to be much higher (25%), at presentation in a large Indian center,[7] while the French in a series of nearly 350 patients found it in only 5 percent at presentation.[10] This may represent natural evolution in a country like ours where many patients tend to present late. Massive splenomegaly may lead to cytopenias due to hypersplenism and may cause respiratory compromise. Splenectomy may provide transient relief of cytopenias, but should be done only as a life-saving measure.
- Other gastrointestinal manifestations in LCH are diarrhea, hematochezia, perianal fistulas, or malabsorption.[27,28] Diagnosis of gastrointestinal involvement in LCH is difficult because of patchy involvement. Endoscopic examination with multiple biopsies is usually needed.
- **Lung:** Lung is less frequently involved in children than in adults, in whom smoking is an etiologic factor.[29] It was seen in 15 percent cases in an Indian series. Criteria used to diagnose pulmonary lesions play a major role in frequencies reported in various series and these have been the most diverse criteria used among all organ involvements ranging from plain radiographs to more sophisticated anatomical and functional imaging and even pulmonary function tests. The incidence was as high as 50 percent in a large series that also included patients with isolated abnormal pulmonary function tests.[30] The lung is usually involved in a symmetrical manner and predominantly involves the upper and middle lung fields, while sparing the costophrenic angle.[31] Confluence of cysts may cause bullous formation, which can sometimes rupture spontaneously leading to a pneumothorax. Occasionally this may be the first sign of LCH involvement of the lung. As the disease progresses, widespread fibrosis and destruction of lung tissue leads to severe pulmonary insufficiency, and patients can present with progressive tachypnea or dyspnea. Eventually declining diffusion capacity may lead to pulmonary hypertension.[32, 33]
- **Bone marrow:** Patients with bone marrow involvement are usually younger, with multisystem disease and often have diffuse disease in the liver, spleen, lymph nodes, and skin. They present with variable thrombocytopenia and anemia with or without neutropenia.[34] Patients with LCH may sometimes present with hemophagocytosis involving the bone marrow.[35]
- **Endocrine system:** The most frequent endocrine manifestation in LCH is diabetes insipidus. This is caused by damage to the antidiuretic hormone (ADH)—secreting cells of the posterior pituitary. MRI scans show nodularity and/or thickening of the pituitary stalk with loss of the pituitary bright spot on T2-weighted images. Pituitary biopsies are rarely done for diagnosis, and are only indicated when the stalk is greater than 6.5 mm or there is a hypothalamic mass. Most often the diagnosis is established by biopsying the other sites of involvement in patients who also have pituitary abnormalities. LCH patients with diabetes insipidus have a 50 to 80 percent chance of developing other organ involvement diagnostic of the disease within one year of onset of diabetes insipidus.[7, 21, 23, 36-38]
- **Ocular:** Ocular involvement in LCH is very rare. Sometimes it may lead to blindness. Patients may have other organ systems involved, and this form of LCH rarely responds well to conventional chemotherapy.[39]

- **Central nervous system:** Apart from mass lesions in the hypothalamic-pituitary region, LCH may also involve the choroid plexus, the gray matter, or white matter. CD1a-positive LCH cells and CD8-positive lymphocytes are present in these lesions.

  Chronic neurodegenerative syndrome manifested by dysarthria, ataxia, dysmetria, and sometimes behavioral changes may develop in one to four percent of LCH patients, and sometimes, these neuropsychologic dysfunctions may be severe.[37] MRI scan may show hyperintensity of the dentate nucleus and white matter of the cerebellum on T2-weighted images or hyperintense lesions of the basal ganglia on T1-weighted images and/or atrophy of the cerebellum.[38] These radiologic findings may precede the onset of symptoms by many years or found coincidently. The neurodegenerative form of the disease has been compared to a paraneoplastic inflammatory response.[37, 38]

DIAGNOSTIC EVALUATION OF LANGERHANS CELL HISTIOCYTOSIS

Diagnostic evaluation of LCH must proceed along logical and established lines. This helps to correctly identify risk groups as well established by the LCH trials. Incorrectly assigning risk groups due to faulty or incorrect assessment test or overdiagnosis due to a very sensitive test makes comparisons difficult in what still remains a rare disorder. The strength of the diagnostic criteria established by the LCH trials has been validated even when applied in a retrospective analysis, proving there robustness.[7] The current recommendations have been recently summarized in an exhaustive review.[14]

Tests and Procedures

- **Blood tests:** These include complete blood count and biochemical evaluations that include liver and renal function tests and serum electrolytes. A coagulation work-up with prothrombin time/partial thromboplastin time in patients with hepatomegaly and jaundice should also be done.
- **Urine tests:** Apart from routine urinalysis, a water-deprivation test must be done if diabetes insipidus is suspected.
- **Bone marrow aspirate and biopsy:** This is indicated in all patients with multisystem disease who have unexplained anemia or thrombocytopenia. The bone marrow biopsy sample should be stained with anti-CD1a and/or anti-CD207 (langerin) and anti-CD163 immunostains for the detection of LCH cells.
- **Radiologic and imaging evaluation:** This is the most important aspect of work-up for a case of LCH. Mandatory in all cases as a first screening, is a complete skeletal survey with skull series, bone scans, and a chest X-ray. Radionuclide scanning has been shown to provide no additional benefit as against its suggested complimentary role in earlier studies.[40-42]

Fludeoxyglucose F18 (18F-FDG)

The PET scans have proved to be the most sensitive technique in detection of involvement and for assessing response to treatment and in follow-up of patients with LCH,[43] but is as yet not recommended to replace the standard tests[14] as its exact role is still being evaluated. Depending on the clinical scenario in a given case,

Table 1 Recommended additional diagnostic testing in a case of LCH[40]

Clinical scenario and recommended additional testing
History of polyuria or polydipsia
• Early morning urine specific gravity and osmolality • Blood electrolytes • Water deprivation test if possible • MRI of the head
Bicytopenia, pancytopenia, or persistent unexplained single cytopenia
• Other causes of anemia or thrombocytopenia has to be ruled out according to standard medical practice. If no other causes are found, the cytopenia is considered LCH-related • Bone marrow aspirate and trephine biopsy to exclude causes other than LCH • Evaluation for features of macrophage activation and hemophagocytic syndrome (triglycerides and ferritin in addition to coagulation studies)
Liver dysfunction
• If frank liver dysfunction (liver enzymes >5-fold upper limit of normal/bilirubin >5-fold upper limit of normal): consult a hepatologist and consider liver MRI which is preferable to retrograde cholangiography • Liver biopsy is only recommended if there is clinically significant liver involvement and the result will alter treatment (i.e. to differentiate between active LCH and sclerosing cholangitis)

Contd...

Contd...

Lung involvement
• Further testing is only needed in case of abnormal chest X-ray or symptoms/signs suggestive of lung involvement, or pulmonary findings not characteristic of LCH or suspicion of an atypical infection
• High resolution-computed tomography (HR-CT) is preferred mode
• Only cysts and nodules are typical of LCH; all other lesions are not diagnostic
• In children already diagnosed with MS-LCH, low dose CT is sufficient to assess extent of pulmonary involvement, and reduce radiation exposure
• Lung function tests (if age appropriate)
• *Bronchoalveolar lavage (BAL):* >5% CD1a + cells in BAL fluid may be diagnostic in a nonsmoker
• Lung biopsy (if BAL is not diagnostic)
Suspected craniofacial bone lesions including maxilla and mandible
• MRI of head including the brain, hypothalamus-pituitary axis, and all craniofacial bones. If MRI not available, CT of the involved bone and the skull base is recommended
Aural discharge or suspected hearing impairment/mastoid involvement
• Formal hearing assessment
• MRI of head or HR-CT of temporal bone
Vertebral lesions (even if only suspected)
• MRI of spine to assess for soft tissue masses and to exclude spinal cord compression
Visual or neurological abnormalities
• MRI of head
• Neurological assessment
• Neuropsychometric assessment
Suspected other endocrine abnormality (i.e. short stature, growth failure, hypothalamic syndromes, or delayed puberty)
• Endocrine assessment (including dynamic tests of the anterior pituitary and thyroid)
• MRI of head
Unexplained chronic diarrhea, failure to thrive, or evidence of malabsorption
• Endoscopy
• Biopsy
Adapted from Haupt, et al[40]

additional specific testing is required. These are summarized in Table 1.

- **Biopsy:** This remains the gold standard for establishing LCH, and is indeed mandatory for a confirmed diagnosis, except in the cases of isolated vertebra plana without a soft tissue mass or isolated pituitary stalk disease when the risk outweighs the benefits.[14]

Lytic bone lesions, skin, and lymph nodes are the most frequent sites biopsied.[7,14] Biopsies from other sites are indicated only in specific situations already summarized in Table 1.

The LCH cells are large cells with abundant pink cytoplasm on hematoxylin and eosin staining with a bean-shaped folded nucleus. LCH cells stain with anti-CD1a or anti-langerin (CD207) and any one of these is essential to confirm the diagnosis of LCH. Other types of histiocytes and macrophages may stain with S-100, which is not considered sufficient to establish the diagnosis of LCH.[14]

TREATMENT OF LANGERHANS CELL HISTIOCYTOSIS

Treatment of langerhans cell histiocytosis (LCH) depends on the site(s) and extent of disease. Treatment historically included surgery, radiation therapy, or oral and topical medications. Later intravenous chemotherapy was also used. The earliest chemotherapy trials were from the German-Austrian-Dutch (Deutsche Arbeitsgemeinschaft für Leukämieforschung und-therapie im Kindesalter [DAL]) Group trials.[44] In updated results from these trials guidelines for formulations for single-system and multi-system disease were made.[4,22] However, since the mid nineties, most of the European study groups merged under the umbrella of the LCH trials, and were increasingly joined by the North American groups. By the time of the launch of the LCH IV trial in 2013–14, will become a truly global study group, with representation from all continents. Most of the current recommendations on treatment are based on the LCH I, II and III trials.

Low-Risk Disease (Single-System or Multisystem)

- Isolated skin involvement has been historically treated with topical steroids, oral methotrexate, oral thalidomide, topical application of nitrogen mustard, and later with psoralen and UV light. However, if no other site is involved, most lesions resolve spontaneously and

only observation would be required. Single-site, single lesion disease similarly requires observation only.[14, 22]

- Skull lesions in the mastoid, temporal, or orbital bones form a risk group for later CNS involvement and DI (CNS-risk lesions) and need to be treated with 6 to 12 months of vinblastine and prednisone to decrease the risk of developing DI.[5]

  For instability of the cervical vertebrae and in patients with neurologic symptoms bracing or spinal fusion may be needed. Chemotherapy is often successful in patients with soft tissue extension from the vertebral lesions.[20, 22]
- Multiple bone lesions; or combinations of skin, lymph node, or pituitary gland with or without bone lesions—should be treated with 12 months of vinblastine and prednisone. A short (≤ 6 months) treatment course with only a single agent (e.g. prednisone) results in a higher number of relapses compared to combination chemotherapy.[22,45] Pamidronate is also effective in LCH with bone lesions.[46]

High-Risk Multisystem Disease

- The standard therapy length recommended for LCH involving the spleen, liver, or bone marrow (high-risk organs) is based upon LCH-I, LCH-II, and the DAL-HX-83 studies and varies from 6 months (LCH-I and LCH-II) to 1 year (DAL-HX-83).[4-6,44] The LCH-II and LCH-III studies used a standard arm consisting of vinblastine and prednisone but 6-mercaptopurine was added to the continuation phase of the protocol. These two studies also conclusively proved that treatment intensification,[47] and prolongation,[6] works better for multisystem LCH. The LCH-II study was a randomized trial which compared treatment of patients with vinblastine, prednisone, and mercaptopurine or vinblastine, prednisone, mercaptopurine, and etoposide.[47] There was no statistical significance in outcomes (response at 6 weeks, 5-year probability of survival, relapses, and permanent consequences) between the two treatment groups. Hence, etoposide has not been used in subsequent Histiocyte Society trials. The LCH-III study randomized risk organ-affected patients to either velban/prednisone/6-mercaptopurine or velban/ prednisone/6-mercaptopurine plus methotrexate (intravenous during the induction phase and oral in the continuation phase).[6] The response rates at 6 and 12 weeks and overall survival were not improved. Significantly increased grade 3 and grade 4 toxicities were seen in patients who received methotrexate.
- **Treatment of CNS disease:** CNS LCH arises initially at areas where the blood brain barrier is deficient. So drugs that cross the blood-brain barrier, such as cladribine (2-CdA), or other nucleoside analogs, such as cytarabine, seem to be the best option for active CNS LCH lesions.[48-50] For treatment of symptoms of LCH CNS neurodegenerative syndrome, dexamethasone, retinoic acid, intravenous immunoglobulin (IVIg), infliximab, with or without vincristine have been used. [51, 52]

Treatment of Recurrent, Refractory, or Progressive Childhood Langerhans Cell Histiocytosis

Various strategies have been evolved to manage LCH patients with recurrent, refractory, or progressive LCH. Optimal therapy for these patients has not been determined. Low-risk recurrence occurring after completion of planned treatment can be treated with a reinduction of vinblastine and prednisone for 6 weeks. Cladribine (2-CdA) has also been used effectively for recurrent low-risk LCH (multifocal bone and low-risk multisystem LCH).[53]

For patients having refractory high-risk organ involvement therapy needs to be changed early. Evaluation points at 6 and 12 weeks post initiation of induction are predictive of outcome. For example, those with progressive disease after 6 weeks of standard treatment, or partial response by 12 weeks require new treatment plan, as they have only a 10 to 50 percent chance of surviving.[5,6,47] Patients with refractory high-risk organ (liver, spleen, or bone marrow) involvement and resistant multisystem low-risk organ involvement have been treated with an intensive acute myeloid leukemia–like protocol. Prompt change of therapy to cladribine (2-CdA) and/or cytosine arabinoside may provide an improvement in overall survival (OS).[54, 55]

Hematopoietic stem cell transplantation (HSCT) has been used for multisystem high-risk organ disease refractory to chemotherapy.[56, 57]

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CHAPTER 46

Hemophagocytic Lymphohistiocytosis: Revisited

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INTRODUCTION

Hemophagocytosis lymphohistiocytosis (HLH) is a disorder of immune dysregulation and is not an uncommon disorder encountered at a tertiary care. It is a potentially fatal hyperinflammatory syndrome with high-grade fever, organomegaly and characteristic laboratory abnormalities like pancytopenia, coagulopathy, hyperferritinemia, hypertriglyceridemia and hemophagocytosis.[1-4]

It was first described in 1939 by Scott and Robb Smith and was initially considered a malignant histiocytic disorder called Malignant Histiocytic Reticulosis.[5] Subsequently Farquhar and Claireaux provided its correct description in 1952.[6] Risdall et al. in 1979 was the 1st to recognize this as a reactive hemophagocytosis secondary to viral infection in a cohort of 19 highly immunocompromised postrenal transplant patients and he coined the term virus-associated hemophagocytic syndrome (VAHS). He observed that though it was a benign disorder the mortality was extremely high and most had infections with herpes group viruses like EBV and CMV.[7,8]

Acquired HLH is commonly seen with infections (IAHS–Infection associated Hemophagocytic Syndrome). The most common infections, which trigger HLH are EBV, CMV, HSV, HHV, Koch's, Salmonella, Malaria, Kala azar in the Indian set up but virtually any infection can trigger HLH.[9,10]

PATHOGENESIS

The hallmark of HLH is defective NK cell and cytotoxic T cell activity. NK cells and cytotoxic T cells can be recognized morphologically as large lymphocytes with azurophilic granules in wright preparation. These granules contain apoptosis inducing machinery the Perforin protein and Granzyme B.

To understand HLH we must understand the function of NK cell and cytotoxic T cells. Both are designed to kill virally infected cells.

Natural killer cells are our innate immune system cell that comprise 10 to 15 percent of peripheral blood lymphocytes and are like Rambo's of the immune system with ready to kill receptors but have to be inhibited by receptors for MHC class I molecule, which override the kill signal. Thus, MHC class I molecule protect us from NK cell toxicity. Cytotoxic T cells are produced as an adaptive response to any infection. They recognize cytosolic protein antigen (e.g. viral proteins) which are presented on the major histocompatibility complex (MHC) class I molecule. Cytotoxic T cells are specific for that infection and are produced later after 4 to 5 days of infection. They are the atypical lymphocytes seen in infectious mononucleosis and express Cd^{3+} and Cd^{8+} on their surface.[11]

Familial Hemophagocytic Lymphohistiocytosis

Inheritance of familial HLH is autosomal recessive. Up till now there are 5 genes discovered in familial HLH. Of which most common is perforin gene (10q21) i.e. PRF 1 mutations seen in 20–40 percent of cases.[12] NK cell and cytotoxic T cells eliminate a virally infected target cell via the Perforin Granzyme pathway. Perforin is a protein like Complement C5-9, it perforates the target cell membrane forming a channel thus allowing Granzyme B to enter the target cell and induce apoptosis by activating the apoptotic mechanism.[13-15] Recent studies suggest that granzyme B can enter into target cells, independent of perforin,[16-18] but granzyme alone is not sufficient to induce toxicity. Once NK cell or Cytotoxic T cell binds the virally infected

cell they form an immunologic synapse at the contact site. All the subsequent events occur at the immunologic synapse. The azurophilic granule undergoes steps of vesicle maturation (LYST protein), polarization to the immunologic synapse (AP3B1 and SH2D1A), docking (RAB 27α), and priming (Unc13)[19], vesicle fusion (Syntaxin 11), vesicle docking, priming and fusion (MUNC 18-2) before it fuses with the surface membrane and releases its content with in the immunological synapse (Fig. 1). XLP (X-linked lymphoproliferative disorder) is of two types XLP1 due to defect in SAP protein and XLP2 due to defect in XIAP (X-linked Inhibitor of Apoptosis) gene. 60% of XLP1 patients develop EBV induced HLH while 90% of XLP2 develop HLH. Currently it XLP2 is being reclassified as HLH causing disease rather than causing lymphoproliferation.[20-25]

Genes Associated with Familial Hemophagocytic Lymphohistiocytosis (Table 1, Fig. 2)

Inability to kill target cells by NK cell and cytotoxic T cells following events can occur. There is excessive stimulation of the immune system, increase in antigen presentation and increase in T cell proliferation with infiltration of various organs like CNS, liver, spleen, and lymph nodes. Ultimately this results in hyper cytokine storm producing mainly TNF alpha, INFG (most important), IL1, Gm-CSF. The Antigen Presenting Cell needs to be culled by NK cells to achieve immune homeostasis. This does not happen and results in further activation of immune system by antigen presenting cell. The cytokine IFNG activate the macrophages that result in hemophagocytosis, thus giving the name to this syndrome[30] (Fig. 2).

Not only NK cells and cytotoxic CD8+ve T cells but also other cells with cytotoxic activity like CD4+ cytotoxic T cells and iNKT cells participate in pathogenesis of HLH. There is some correlation between hyper cytokinemia and the diverse clinical manifestations in HLH.

Secondary Hemophagocytic Lymphohistiocytosis

In most cases of secondary HLH cytotoxicity and cytotoxic lymphocyte degranulation are not impaired[31] (Figs 3 and 4). There is increases APC activation that disrupt the balance between APC activation and CTL-mediated control. APC can directly activated by intracellular pathogens for example via toll-like receptor (TLR) activation.

Based on *in vitro* analyses, four distinct pathways of macrophage activation have been described. Classical activation via interferon-γ or lipopolysaccharide induces microbicidal activities and upregulation of expression of class II MHC. Alternative activation by interleukin 4 or 13 induces the expression of genes that are involved in tissue repair or suppression of inflammation. Innate activation via toll-like receptor ligands also, not surprisingly, induces microbicidal activities. Deactivation by stimulation of interleukin-10 or transforming growth factor-β reduces class II expression and increases secretion of anti-inflammatory cytokines.[32-34]

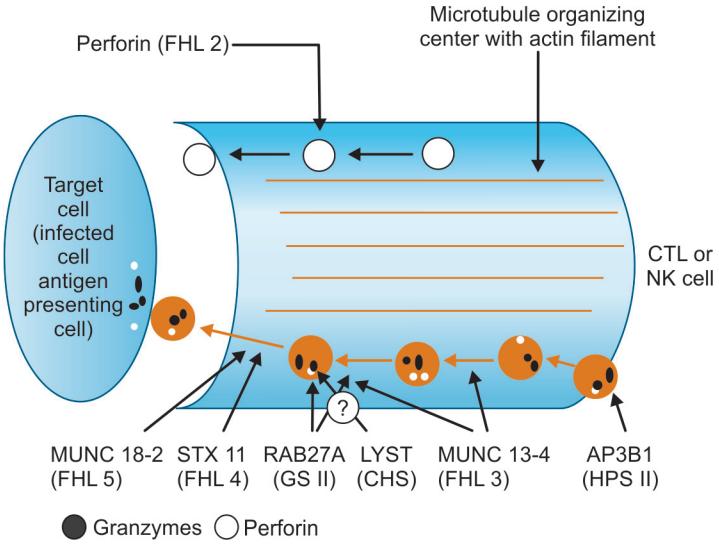


Fig. 1 Pathogenesis of hemophagocytic lymphohistiocytosis. Depicts the events occurring at the immunologic synapse and highlights molecules important in process of granule exocytosis. *Abbreviations:* CHS: Chédiak-Higashi syndrome; FHL: Familial hemophagocytic lymphohistiocytosis; GS: Griscelli syndrome; HP: Hermansky–Pudlak; NK: Natural killer cell; CTL: Cytotoxic T lymphocyte[1, 29]

Table 1 Genes associated with familial HLH

Disease	*Locus*	*Gene*	*Gene symbol*	*Function*
FHLH 1	9q22.1-23	Unknown	Unknown	
FHLH 2	10q22	Perforin	PRF 1	Pore forming protein
FHLH 3	17q25	*C. elegans* Unc13	MUNC 13-4	Vesicle priming
FHLH 4	6q24	Syntaxin 11	STX11	Vesicle fusion
FHLH 5	c. 1697G > A p. G566D	MUNC 18-2 (STXBP2 gene)	MUNC 18-2	Vesicle Bocking, priming and fusion
Griscelli syndrome type 2	5q21	Ras ass protein	RAB27A	Vesicle docking
HSP (Hermansky-Pudlak type II)	Chr. 10	AP3B1	AP3B1	Granule polarization
CHS (Chédiak-Higashi syndrome)	1q42.1–42.2	Lysosomal trafficking regulator	LYST	Vesicle maturation
XLP 1	Xq25	SLAM ass protein (SAP)	SHD2D1A	Granule polarization
XLP 2	Xq24-25	XIAP	BIRC4	Inhibition of apoptosis

Abbreviations: EBV: Epstein-Barr virus; FHLH: Familial hemophagocytic lymphohistiocytosis. Adopted from[1,26-28]

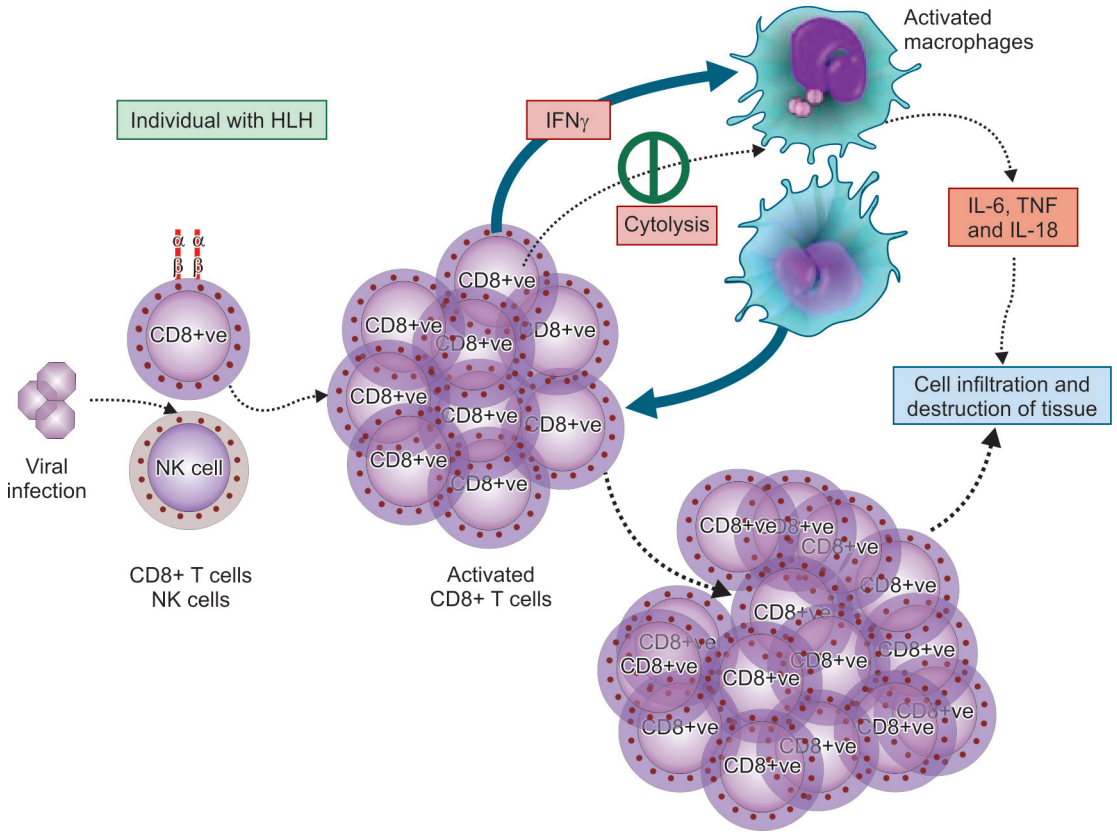


Fig. 2 Patients of HLH due to genetic defect; there is inability to kill virally infected cell as well as antigen presenting cells resulting in massive clonal expansion of CTLs, secretion of their cytokines like interferon gamma (IFNγ), tumor necrosis factor (TNF) alpha, IL6, IL18 and granulocyte macrophage colony stimulating factor (GM-CSF). IFNγ activate macrophages, which then phagocytose blood cells resulting in hemophagocytosis. Since the cytokines are produced in a massive amount by macrophages, T cells and NK cells, hyperstimulation continues and the patient has a cytokine storm with signs and symptoms of HLH. The normal contraction of the immune system also does not take place resulting in persistent cytokine secretion, infiltration of various organs by T cells, massive tissue necrosis and organ failure

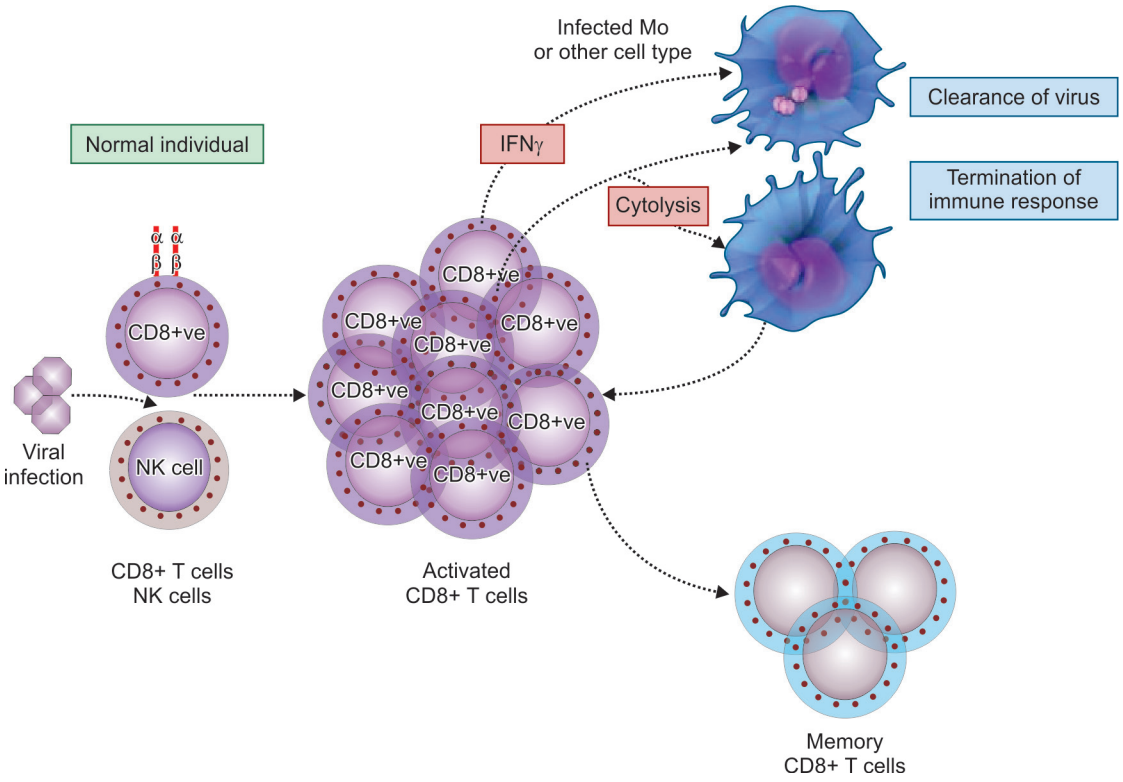
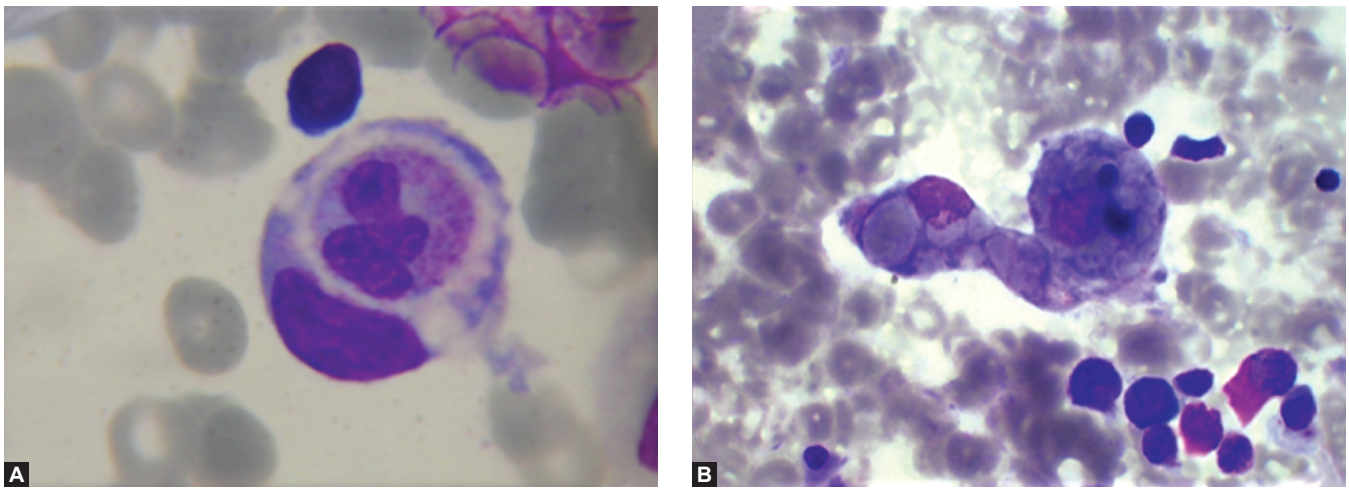


Fig. 3 Natural killer cell and cytotoxic T lymphocyte (CTL) response to virally infected cells. In normal patients there is clonal expansion, secretion of interferon gamma and killing of virally infected cell resulting in control of viral infection. Once infection is controlled the CTL are culled and immune homeostasis achieved. Some of these cells become memory cells



Figs 4A and B Neutrophil phagocytosis and erythrophagocytosis respectively

Types and Causes of HLH[35]

- Genetic causes of HLH
  - Familial HLH
  - Pigmentary dilution disorders
    - Chédiak-Higashi syndrome
    - Griscelli syndrome type 2
    - Hermansky-Pudlak syndrome type II
  - X-linked lymphoproliferative (XLP) disease type 1 and 2
- Infection associated HLH
  - Viruses-EBV, CMV, HHV-6, HHV-8, HIV, adeno, hepatitis, parvo virus.
  - Bacteria numerous including Koch's, Salmonella.
  - Parasites-Malaria, Kala azar in the Indian set-up
  - Spirochetal, and fungal-associated infections.
- Malignancy associated HLH: Leukemia lymphoma, GCT.
- Macrophage activation syndrome (MAS) associated with autoimmune disease like rheumatoid arthritis, SLE.

Clinical Features

Presentation of HLH in initial period is nonspecific and easily confused with common infection, autoimmune disorders and malignancy.[1] HLH typically presents with prolonged fever; unresponsive to antibiotics, hepatosplenomegaly, rash (6–65%), lymphadenopathy, cytopenias, liver dysfunction, hypofibrinogenemia, hypertriglyceridemia, hypoalbuminemia, hyponatremia. In initial course,CNS manifestations in form of irritability, hypo- or hypertonia, cranial nerve palsies, meningismus, signs of increased intracranial pressure and altered sensorium seen in 30% of cases.[36,37] In early course of the disease, hemophagocytosis may not be obvious on bone marrow examination study and may need repeat bone marrow if clinical suspicion is strong[38] (Figs 4A and B).

Incidence of FHLH is 1 in 5000 live births.[39] Ratio of male and female affection is equal. Almost 70% of FHLH are diagnosed in first year of life, with peak age between 1 and 6 month. With availiblity of genetic testing, it is possible to point out the first significant episode of FHLH throughout life,[40] including *in utero*.

In 1987, the Histiocyte Society adopted the unifying term hemophagocytic lymphohistiocytosis (HLH) and defined a set of diagnostic criteria to assist clinicians and researchers (Table 2). The criteria have subsequently been refined to account for advances in our understanding of the syndrome, and to simplify it for practical usage. In 2009, Filipovich et al has revised the HLH diagnostic criteria, which are practical usage and very much applicable in our set-up as shown in Table 3.

The HLH must be suspected in setting of rapidly evolving cytopenias, LFT dysfunction, organomegaly, coagulopathy. It is prudent to ask for serum ferritin and triglycerides with 'D' dimer. If ferritin is >500 ng/mL and specially >3000 ng/mL a BMA done to rule out HLH.

Macrophage Activation Syndrome Associated with Autoimmune Disease

Macrophage activation syndrome (MAS) is observed in number of autoimmune disorders, infections and neoplasms. It is caused by an excessive proliferation and activation of macrophages. Incidence of MAS in systemic onset juvenile inflammatory arthritis is 7-30% and usual triggers

Table 2 Diagnostic guidelines for hemophagocytic lymphohistiocytosis (2004)[35,38]

The diagnosis of HLH can be established if one of either 1 or 2 below is fulfilled

1. A molecular diagnosis consistent with HLH
2. Diagnostic criteria for HLH fulfilled (five out of the eight criteria below)

Clinical criteria

i. Fever
ii. Splenomegaly

Laboratory criteria

i. Cytopenias (affecting >2 of 3 lineages in the peripheral blood): HB<9 g/dL (in infants <4 weeks: HB<10.0 g/dL), platelets <100,000/mm^3, neutrophils <1,000/mm^3.
ii. Hypertriglyceridemia and/or hypofibrinogenemia: Fasting triglycerides > 3 SD, fibrinogen < 3 SD
iii. Hemophagocytosis in bone marrow or spleen or lymph nodes. No evidence of malignancy.

New diagnostic criteria

i. Low or absent NK-cell activity (according to local laboratory reference)
ii. Ferritin >500 µg/L
iii. Soluble CD25 (i.e. soluble IL-2 receptor) >2,400 U/mL.

Adapted from Treatment Protocol of the 2nd International HLH Study, 2004[35,38]

Table 3 Filipovich HLH diagnostic criteria 2009[41]

Molecular diagnosis of HLH or XLP OR
At least 3 of 4
• Fever
• Splenomegaly
• Hepatitis
• Cytopenias
And at least 1 of 4
• Hemophagocytosis
• Hyperferritinemia
• Increased soluble IL2R alpha
• Absent or very decreased NK cell function
Supportive of HLH
• Hypertriglyceridemia
• Hypofibrinogenemia
• Hyponatremia
Adapted from hematology ash education book. 2009;1:127-31.

are gold therapy, aspirin, viral infection and some reports with anti TNF α antibodies.[41] Other diseases associated with MAS are SLE, Kawasaki disease and other rheumatic diseases.[42,43] Specific parameters taken into consideration are falling WBC and platelet counts, hyperferritinemia, hypofibrinogenemia, hemophagocytosis in bone marrow, elevated liver enzymes, elevated erythrocyte sedimentation rate, and hypertriglyceridemia.[44,45] It can be presenting manifestation of autoimmune disorder and features of such diseases (such as arthritis or rash) should therefore be carefully looked in patient with HLH.

Work-up for Patient of Hemophagocytic Lymphohistiocytosis[38]

Laboratory evaluation of HLH is directed with following considerations:

- Establish diagnosis of HLH
  - CBC platelet
  - ESR
  - PS (look for peripheral blood HLH)
  - LFT
  - Creatinine
  - LDH
  - Serum electrolytes
  - Serum ferritin
  - Serum triglycerides
  - Coagulation profile (PT, PTT, plasma fibrinogen and 'D' dimer)
  - CSF for pleocytosis and elevated proteins (50% of cases).
- Supportive evidence for HLH
  - PB or bone marrow aspiration
  - Liver biopsy
  - Lymph node biopsy
- Sophisticated lab investigations
  - sCD25 >2400 IU/L
  - NK cell activity (may be normal in 30% of cases)
  - Serum Beta 2 microglobulin
- Etiological work-up
  - Anti-EBV VCA IgM, PCR
  - Anti-CMV Abs, antigen, PCR
  - Appropriate microbiological cultures
  - Hair mount studies for pigmentary dilution disorders.
- Work-up for familial HLH (Flow chart 1)
  - Perforin by flow cytometry
  - Granule release assay (GRA)
  - Gene sequencing to identify mutations.

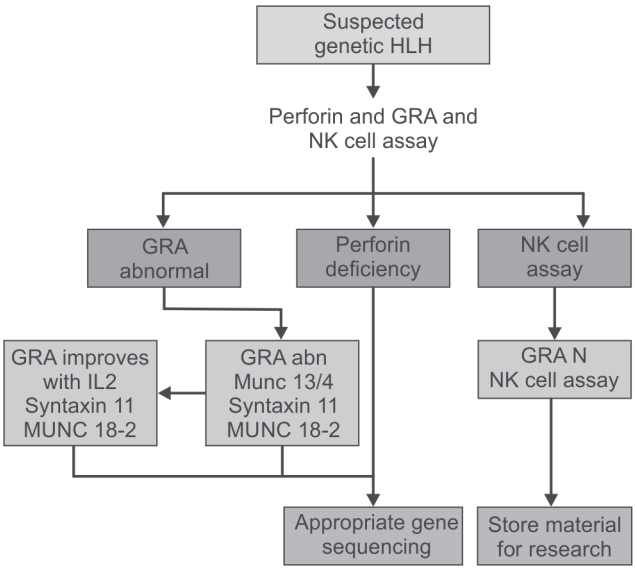
MANAGEMENT OF HEMOPHAGOCYTIC LYMPHOHISTIOCYTOSIS[31]

Principle of Treatment

The immediate aim of treatment is to suppress the severe hyperinflammation. The secondary aim is to eliminate pathogen activated antigen presenting cells (APCs) so as to remove the stimulus for ineffective activation of T cells. The treatment recommended is HLH 2004 protocol that is devised by Histiocytic Society. It essentially consists of 3 drugs:

- Dexamethasone is lympholytic, inhibit expression of cytokines, and suppresses maturation of APCs, better CNS penetration hence preferred over prednisolone.
- Cyclosporine A prevents T cell activation and proliferation.

Flow chart 1 Algorithm for investigation of HLH



- Etoposide (VP-16) has activity against monocytes and macrophages, inhibits EBNA synthesis and EBV infected cells.
- IV gammaglobulins provide cytokine and pathogen specific antibodies and immunomodulation.

HEMOPHAGOCYTIC LYMPHOHISTIOCYTOSIS 2004 PROTOCOL

Initial Therapy (8 Weeks)

- Dexamethasone, 10 mg/m^2/day for 2 weeks followed by a decrease every 2 weeks to 5 mg/m^2, 2.5 mg/m^2 and 1.25 mg/m^2 for a total of 8 weeks.
- Etoposide IV, etoposide 150 mg/m^2 IV, twice weekly for the first two weeks, then weekly during the initial therapy. Paradoxically even if ANC $<0.5 \times 10^9$/L and the bone marrow is hypocellular, at least the first two doses should be given.
- Cyclosporine A, aiming at levels around 200 microg/L (monoclonal, trough value). Start with 6 mg/kg daily orally (divide in 2 daily doses), if normal kidney function.
- Intrathecal methotrexate (IT MTX), age-adjusted doses of intrathecal methotrexate weekly for 3 to 6 weeks as follows if there are progressive neurological symptoms or if abnormal cells persist in the CSF.
- *Supportive therapy:* Cotrimoxazole eq 5 mg/kg of trimethoprim 2 to 3 times weekly (week 1 and onwards), an oral antimycotic (from week 1 to 9), IV immunoglobulin (0.5 g/kg) every 4 weeks.

Continuation Therapy (9–40 Weeks)

The continuation therapy is a continuation of the initial therapy with the major aim to keep the disease nonactive week 9 to 40. Increasing disease activity may make it necessary to intensify the treatment in some children. Patients with nonfamilial disease and no genetic evidence of HLH, are suggested to start continuation therapy only if the disease is active after the initial therapy. Etoposide 150 mg/m^2 IV, every second week. Dexamethasone pulses every second week, 10 mg/m^2 for 3 days. Cyclosporin A aims for blood levels around 200 microgram/L, as above. Monitor GFR.

Indications for BMT

- Familial hemophagocytic lymphohistiocytosis (FHLH)
- Relapsing secondary HLH.

Results of BMT are:[46]

- Matched related donor: 71 percent ± 16 percent
- Matched unrelated donor 71 percent
- HLA mismatched unrelated donor: 54 percent ± 27 percent
- Haploidentical related donor: 50 percent ± 24 percent.

Disease Directed Therapy

Antileishmania therapy, antilymphoma therapy. Please note in secondary HLH pathogen directed therapy is not sufficient to control the hyperinflammation. Leishmaniasis treated with liposomal amphotericin B is the only exception. In patients with EBV-induced HLH or XLP with fulminant EBV induced HLH use injection rituximab (monoclonal Ab to CD 20) at doses of 375 mg/m^2 weekly for 4 weeks, to knock out the B cells harboring EBV in addition to HLH directed therapy. This strategy works very well to control EBV induced HLH and in fulminant infectious mononucleosis. In desperate situations, anti-TNF alpha-receptor blocking agents have been tried.[47-50]

Macrophage activation syndrome: In addition to corticosteroids, CSA has been found effective in patients with corticosteroid-resistant MAS.[51]

The French group does not use etoposide and they use anti-thymocyte globulin (ATG) to control HLH.[52]

Once inflammation is controlled a search for a potential bone marrow donor is done and if a match is available the child should be transplanted to achieve a cure. In HLH, 1994 protocol median survival was 64 percent with overall survival (OS) of 55 percent.[46]

If there is poor or no response to 4 weeks of HLH 2004 protocol treatment, HLH is probably refractory and continuing HLH 2004 protocol will be of no further benefit. Salvage treatment options that can be tried in such a situation are ATG, fludarabine, alemtuzumab,[53,54] Daclizumab, anti TNF-alpha receptor blocking agents, anti-interferon gamma antibodies, chemotherapeutic agents. There is no established salvage regime. ATG is rarely effective, if etoposide-based regimen has been ineffective. Search for BM donor should be done and one should attempt to transplant these patients.

In secondary HLH treatment is given for 8 weeks. HLH re-evaluation is done and if normal treatment is stopped and close follow-up including signs of reactivation are warranted (such as fever, hepatosplenomegaly, neurological abnormalities; hemoglobin, platelets, WBC, ANC, ferritin, transaminases). Once hyperinflammation is controlled there can be reactivation of HLH or development of CNS events. Intrathecal MTX would benefit for CNS activation. The HLH therapy is reinforced in case of systemic reactivation. If the patient develops a reactivation, it is recommended to intensify therapy, such as to restart from week 2, but the initial therapy may be less than 8 weeks, and then continue with modified continuation therapy. Add intrathecal therapy in case of

CNS reactivation. Consider dexamethasone daily, also between the dexa-pulses, in continuation therapy, but be aware that it may lead to severe side-effects, so an early SCT is then suggested.

Poor prognostic factors: Degree CSF pleocytosis, severity of thrombocytopenia, hyperbilirubinemia and hyperferritinemia are important risk factors for outcome in HLH. HLH associated with EBV infection with high viral load is associated with poor outcome.[56] Persistent fever and thrombocytopenia also associated with poor outcome.[55]

Increasing awareness among pediatricians regarding HLH; with early diagnosis and initiation of early treatment decreases morbidity and mortality. Bone marrow transplantation cures disease with good success rate.

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CHAPTER 47

Bone Marrow Transplantation

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Hematopoietic Stem Cells

Hematopoietic stem cells reside primarily in the bone marrow but do circulate in the peripheral blood. These cells may replenish damaged or missing components of the hematopoietic and immunologic system.[1]

Hematopoietic stem cell transplantation was originally conceived more than 50 years ago. Initial studies done in animals showed that transplantation of genetically identical material or the animal's own marrow averted death. Studies in animals later translated to work in humans and a team led by Dr E Donnall Thomas pioneered this. In 1959, he reported that a patient with end stage leukemia sustained a remission for more than 3 months following total body irradiation and infusion of bone marrow from her identical twin. Later in 1970s the same group performed identical sibling transplant in leukemic patients. This work laid the foundation for further advances in hematopoietic stem cell transplantation and this was recognized by the 1990 Nobel Prize awarded to Dr E Donnall Thomas.[2]

GRAFT TYPES IN HEMOTOPOIETIC STEM CELLS

There are three different graft types that can be used for bone marrow transplant.[3]

1. Autologous
2. Allogeneic
3. Syngeneic

Autologous Transplants

Autologous transplants use stem cells derived from the patient's own marrow or peripheral blood. Initially, this was developed in order to rescue the bone marrow of patients undergoing chemotherapy.[1]

Autologous transplants are being increasingly incorporated in protocols for solid tumors like neuroblastoma. This now is the most important form of stem cell transplantation performed worldwide. Stem cells can be stored without loss of viability and mortality is low with this procedure. In addition, there is no risk graft versus host disease.

Allogeneic transplants are hemopoietic stem cells from the bone marrow, peripheral blood, or umbilical cord blood of a healthy donor matched for HLA type, who may be a family member or an unrelated volunteer. Initially, allogeneic was developed for treatment of hematological malignancies. Now it is being utilized for a variety of hematological disorders and for non-hematological disorders like inborn errors of metabolism and autoimmune diseases.[1]

Syngeneic transplant: Syngeneic transplant involves transplantation from a person sharing identical genetic material, i.e. an identical twin.

SOURCES OF STEM CELLS

Bone marrow: Bone marrow obtained by repeated aspiration of posterior iliac crests while donor is under general or local anesthesia is the traditional source of stem cells (Fig. 1). This does not cause any side effects to the donor except for slight discomfort at the site of aspiration and the requirement of packed cell transfusion in some cases of pediatric donors.

Fig. 1 Bone marrow harvesting

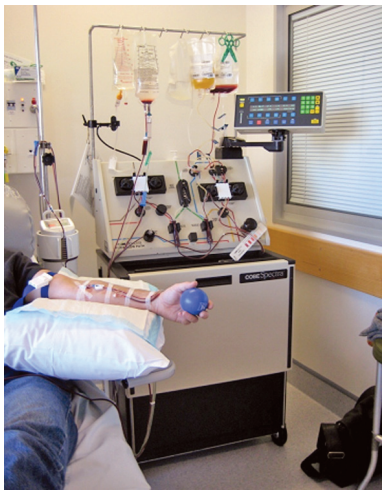


Fig. 2 Peripheral blood stem cell apheresis

Peripheral blood stem cells (PBSC): It was noted in early 1980s that marrow stem cells circulated in the peripheral blood. Stem cell yield from peripheral blood can be increased by giving bone marrow growth factors like granulocyte colony stimulating factor. Stem cells are then harvested by leukapheresis (Fig. 2). CD 34 cell surface molecule is used as a surrogate marker of stem cells.[4] G-CSF increases the proliferation of neutrophils and causes release of proteases. PBSC causes the most rapid hematopoietic reconstitution. But, it contains more T cells than bone marrow. Peripheral blood stem cells are associated with increased risk of chronic GVHD.

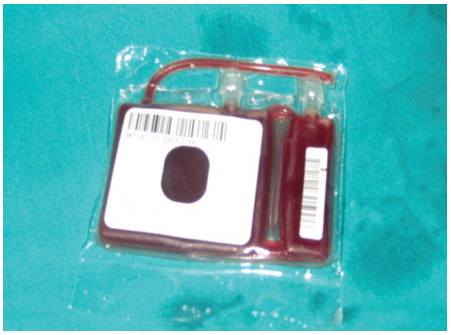


Fig. 3 Umbilical cord blood stem cells

Peripheral Blood Stem Cell Apheresis

Umbilical cord blood stem cells (Fig. 3): Cord blood of neonates contains substantial number of hematopoietic stem cells that can be harvested at delivery, frozen and then transplanted into a patient (Fig. 3). They can be matched with potential donors without much delay. Cord blood requires less stringent HLA matching than marrow/peripheral blood stem cells. The first umbilical cord transplantation was done in 1988 for a child with Fanconi anemia from cryopreserved cord stem cells from an HLA matched sibling.[5] With the emergence of cord blood banks with public cord blood banking facilities, they form an important source of stem cells especially for ethnic minorities and countries where marrow donor registries do not exist.

Cord blood is also associated with:

- Minimal GVHD due to naïve T cells in it.
- The disadvantage with cord blood is delayed engraftment due to the small number of stem cells
- Hence infections are more common during the prolonged neutropenic period.
- The cell dose in one cord blood unit may not be sufficient for an older child or an adult.

The use of double cords has overcome this problem.[6]

A comparative analysis of the various sources of stem cells is given in Table 1.

Table 1 Comparison of various sources of stem cells

	Bone marrow	*Peripheral blood*	*Umbilical cord*
Stem cell content	Usually adequate	Good can be increased, if needed	Fixed source
T cell content	Low	High	Low, naïve
HLA matching	Close matching required	Close matching required	Close matching not very important
Engraftment	Fast	Fastest	Slowest

HUMAN LEUKOCYTE ANTIGEN MATCHING

Allogeneic transplantations became feasible with the identification and typing of human leukocyte antigen (HLA) located on major histocompatibility locus on chromosome 6. There are two sets of genes on both alleles and hence they are inherited as haplotypes. Thus two siblings have one chance in four of being HLA identical. The HLA loci that are important in the transplant setting include Class I antigens (HLA A, B and C) and Class II antigens (HLA DR). For a successful transplant, it is necessary to have matching at all these loci.[1] The immune reaction against HLA molecules can cause problems. Mismatching at Class I antigenic loci, increase the chance of rejection of the donor, where as that at Class II locus increases graft vs host disease.

DONOR REGISTRIES

Only 20 to 25 percent of patients eligible for allogeneic transplantation will have suitable sibling donors. To make transplants available to a greater number of eligible patients, bone marrow donor registries have been established in several countries. This will identify unrelated but matched donors for prospective patients. With the establishment of international marrow donor registries there are good chances of finding a matched unrelated donor depending on the ethnic group. For patients from Asia and Indian subcontinent, the probability of finding a donor of Asian origin is low due to the poor representation in these registries and due to the absence of local registries. The strongest transplant reactions occur when the major histocompatibility antigens of the donor and of the recipient are incompatible.

The HSCT has resulted in sustained remission in patients with autoimmune diseases. SCT results in re-education of the immune system and hence is now used for refractory rheumatoid arthritis and other autoimmune diseases. HSCT cures many genetic diseases like thalassemia and sickle cell disease in developing countries such 'one shot' treatments are highly desirable because chronic treatments often are difficult to sustain.

Among the above mentioned indications, immunodeficiencies, certain genetic disorders and severe aplastic anemia deserves urgent referral to a transplant center for consideration for an early transplant (Table 2).

Table 2 Common indications for hematopoietic stem cell transplantation

Diseases commonly treated with hematopoietic stem cell transplantation	
Malignant conditions	*Nonmalignant conditions*
Autologous	
Acute myeloid leukemia Hodgkin's disease Non-Hodgkin's lymphoma Neuroblastoma, Ewing's sarcoma	Autoimmune diseases
Allogenic	
Acute myeloid leukemia Acute lymphoblastic leukemia Chronic myeloid leukemia Myelodysplastic syndromes Myeloproliferative syndromes Non-Hodgkin's lymphoma	Aplastic anemia Fanconi's anemia Paroxysmal nocturnal Hemoglobinuria Diamond blackfan anemia Dyskeratosis congenita Thalassemia Sickle cell disease Glanzmann thrombasthenia Severe combined immunodeficiency Wiskott-Aldrich syndrome Chronic granulomatous disease Congenital neutropenia Congenital megakaryocytosis Inborn errors of metabolism

COLLECTION OF HEMATOPOIETIC STEM CELLS

Bone marrow is harvested from posterior iliac crest and is generally well tolerated. The donor needs to be admitted and the procedure is done under general anesthesia. The harvested marrow is collected in a special harvest bag with adequate anticoagulation. The harvest bag should be gently shaken during the procedure to avoid the formation of clots. The puncture site is changed after 10 to 15 mL is aspirated from the site. After collection of the desired volume, bony spicules and clumps are filtered out and the final product is made ready for infusion.[7]

Peripheral blood stem cells can be collected after being mobilized from the bone marrow by G-CSF given at a dose of 10 μg/kg/day for 4 to 5 days. PB stem cells are then collected by leukapheresis. Neither anesthesia nor hospitalization is required for the donor.

Children may experience minor side effects related to G-CSF use like body aches and influenza like illness. Adequate vascular access and extracorporeal volume in the circuit of leukapheresis are the main limiting factors for peripheral blood stem cell collection in small children. Central venous catheter is usually inserted in subclavian or femoral veins and should be sufficiently stiff to avoid collapse under the negative pressure while drawing blood into the apheresis machine. In children adequate priming of the extracorporeal circuit may be required in order to avoid hypotension.[8] As the anticoagulation most commonly used during is citrate dextrose (ACD A),

hypocalcemia should be anticipated and managed with calcium boluses given under proper monitoring. Heparin may also be used for anticoagulation, but has been observed to have higher frequency of bleeding during catheter removal.

Umbilical cord blood is collected at the time of delivery by clamping the cord and cutting the umbilical cord. The median volume collected is around 60 mL. Once collected, it is then processed and stored in liquid nitrogen till further use.

Umbilical cord blood is collected after clamping the cord. The collected blood is tested, processed and cryopreserved. At the time of use, the cord blood cassette is transported in liquid nitrogen.

After collection of the marrow/peripheral blood stem cells, the product can be infused immediately, or may be cryopreserved and stored till need arises. Graft manipulation like T cell depletion may be done when required. In case of major or minor ABO incompatibility, it is necessary to either deplete the red cells or plasma in the product as required.

PREPARATIVE REGIMENS/CONDITIONING REGIMENS

The chemotherapy or irradiation given prior to stem cell infusion is called conditioning regimen. The regimen is intended to be myeloablative as well as immunosuppressive. The objective of myeloablation of the recipient prior to transplant is to eradicate the recipient's own bone marrow stem cells. In case of malignancies, such high doses of chemotherapy help in eradicating the cancer cells.[1] The preparative regimen also augments the antitumor immune response by causing a breakdown of tumor cells, which results in flood of tumor antigens into the antigen presenting cells. This results in proliferation of T cells that attack the surviving malignant cells.

Total body irradiation: It is both myeloablative and immunosuppressive. The effects are independent of blood supply, and the effects reach sites that are not accessible by chemotherapy. It is also not associated with cross-resistance to chemotherapy. Local shielding of organs and fractionation of the total dose can reduce toxicity. The toxicity and scarcity of facilities for TBI have led to development of radiation free regimens.

Busulfan-Cyclophosphamide (Bu-Cy): In 1983, a regimen of Bu with high doses of Cy proved effective in treatment of acute myeloid leukemia. Acute adverse effects are associated with high plasma levels of busulfan and metabolites of cyclophosphamide. The dose of cyclophosphamide was later lowered to reduce toxicity. Toxicity can also be reduced by adjusting the dose of busulfan according to the plasma levels or by using intravenous instead of oral busulfan.

Non-myeloablative regimens are those that usechemotherapy agents/radiation in lower doses than mentioned above. These regimens are immunosuppressive, but do not destroy the entire recipient's marrow. The advantage of this regimen is that the toxicity associated with the conditioning regimen is significantly less. It is immunosuppressive as well and can be used in patients with comorbidities. So also in case of malignancies the residual cells of the recipient can exert a graft versus tumor effect. However, the risk of rejection of the graft increased with non-myeloablative regimens.[1]

STEM CELL INFUSION

After the preparative regimen, the processed stem cells are infused intravenously. The patients are kept in HEPA filtered rooms and are on prophylactic antifungals, antibiotics and antiviral agents (Fig. 4).

Engraftment is defined as absolute neutrophil count more than 500/cumm for more that 3 days consecutively.

COMPLICATIONS (FIG. 6)

Early Effects

Mucositis: It is the most common complication of myeloablative preparative regimes especially with the use of total body irradiation and melphalan. Other factors that contribute to mucositis include GVHD, use of methotrexate for GVHD prophylaxis and co-existent infections. Oropharyngeal mucositis results in painful ulcers in the mouth and throat. It can also lead to mucoid diarrhea and pain abdomen. In addition to the considerable pain and need

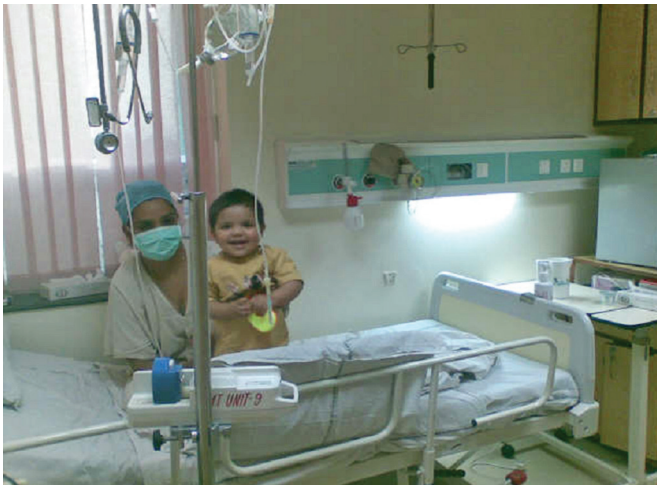


Fig. 4 Bone marrow transplantation room (HEPA filtered)

for narcotics, mucositis leads to compromised enteral nutrition and predisposition to infections due to breach in mucous membranes. Management includes prophylaxis against herpes infections with acyclovir as well as against candidal infections. There are several ongoing studies with agents like glutamine, palifermin, medicated pastes, topical lidocaine which have not yet been of proven benefit.[9]

Sinusoidal obstruction syndrome/veno-occlusive disease (SOS/VOD): Hepatic veno-occlusive disease is an organ injury syndrome that occurs after high dose chemotherapy employed in HSCT. After myeloablative conditioning in allogeneic transplantation VOD is seen in up to 10 percent of patients. It is potentially fatal syndrome of painful hepatomegaly, jaundice and fluid retention. Total body irradiation, busulfan, cyclophosphamide and many other preparative regimens cause SOS. The metabolites of these drugs and irradiation result in sloughing of the sinusoidal endothelium, which results in obstruction of hepatic circulation and injury to the centrilobular hepatocytes.[9] Recognized risk factors for VOD includes older transplant age, HLA disparity between donor and recipient, preexisting liver disease, etc. The type and intensity of transplant conditioning regimen are probably the greatest determining factor for development of severe VOD. High plasma levels of Busulfan or metabolites of cyclophosphamide are associated with increased risk of VOD.

Because there is no effective treatment of this complication, prevention is critical. Use of reduced intensity conditioning regimens and the substitution of fludarabine for cyclophosphamide appears to reduce the risk. Defibrotide is a mixture of single-stranded oligonucleotides that have local antithrombotic, anti-ischemic and anti-inflammatory properties. It protects the sinusoidal endothelium without compromising the cytotoxic therapy. Defibrotide modulates endothelial cell injury and protects the sinusoidal endothelium. It also modulates platelet activity and enhances fibrinolytic activity. Hence, it is used now for prophylaxis as well as treatment of SOS. Other agents used with variable results include tissue plasminogen activator, antithrombin III and prostaglandin E1, low molecular weight heparin, Ursodeoxycholic acid, etc. Despite emerging therapies, VOD remains a much feared transplant complication and severe cases are associated with dismal prognosis.[10]

ACUTE GRAFT VERSUS HOST DISEASE

The graft versus host disease (GVHD) is the most important complication of allogeneic transplantation. The development of GVHD is a complex process that is dependent on many factors including the conditioning regimen used. The pathogenesis of acute GVHD is described as a three-step process that includes

- Conditioning induced tissue damage phase:
- Donor lymphocyte activation phase
- Cellular and inflammatory effector phase.

The first phase consists of tissue damage induced by conditioning regimen and infection. As a result of the inflammatory cytokines released, there is maturation/activation of host dendritic cells with subsequent recognition of host major and minor histocompatibility antigens by mature donor T cells. Despite matching of major HLA antigens, there may be minor HLA mismatches that may be recognized as foreign. This results in activation of the donor T cells against the recipient antigens. The IL 2 and IFN gamma produced by T helper 1 cells result in activation of NK cells and cytotoxic T lymphocytes. Thus various cytokines and T cell subtypes are involved in the pathogenesis of aGVHD and they are potential targets for intervention.[11]

Acute GVHD is characterized by manifestations in the skin, liver, and GI tract. Grading of the severity of aGVHD is based on evaluation of the degree of involvement in each of these organs (Table 3).

- Skin involvement in GVHD starts as redness and maculopapular rash, initially of face, ears and palms and soles. Later the rash may progress to other parts of the trunk and may progress to bullae formation and desquamation (Figs 5A to C).
- Hyperbilirubinemia is the primary hepatic manifestation of liver involvement in GVHD. The other causes of jaundice in transplant patients include veno-occlusive disease, drug toxicity and infections.
- The GIT involvement is characterized by diarrhea, nausea and food intolerance.
- Other organs may also be involved in GVHD, e.g. ocular GVHD is manifested by hemorrhagic conjunctivitis and pseudomembrane formation.[12]

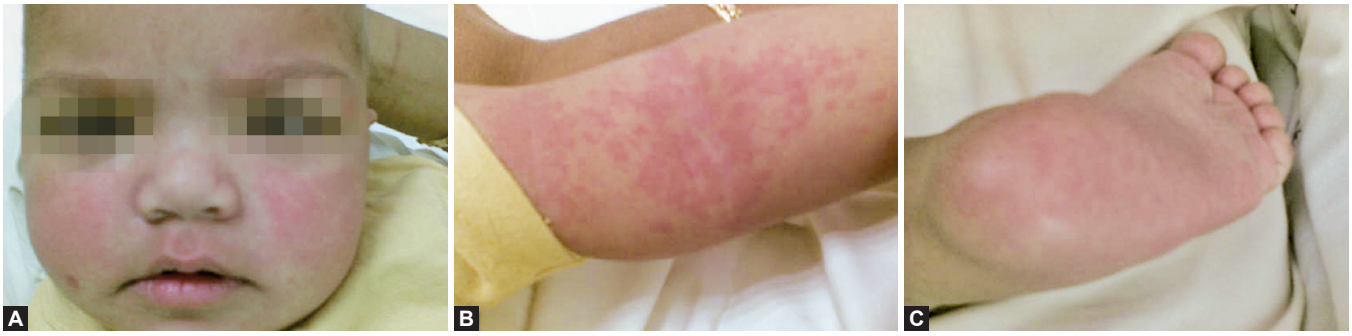
MANAGEMENT OF GVHD

Over the years, there have been several strategies developed for management of GVHD. Prophylactic measures include methotrexate, steroids and cyclosporine. Cyclosporine is a calcineurin inhibitor that interferes with T lymphocyte functions. Agents used for treatment include steroids, agents like tacrolimus, mycophenolate mofetil, monoclonal antibodies like Infliximab (TNF alpha inhibitor) and photopheresis. The principal risk factor for aGVHD is HLA mismatch, but it can occur despite a full HLA match. The incidence of GVHD can also be reduced by in vitro T cell depletion of the graft before transplantation.[12]

Table 3 Grading of graft versus host disease

Clinical staging	*Stage I*	*Stage II*	*Stage III*	*Stage IV*
Skin	Rash <25% BSA	Rash 25–50% BSA	Rash 50–100% BSA	Desquamation and bulla formation
GIT	Persistent nausea or Diarrhea 5–10 mL/kg/day	Diarrhea 10–15 mL/kg/day	Diarrhea > 15 mL/kg/day	Pain +/– ileus
Liver	Bilirubin 2–3 mg/dL	Bilirubin 3–6 mg/dL	Bilirubin 6–15 mg/dL	Bilirubin >15 mg/dL

Clinical grading	*Skin*	*GIT*	*Liver*	*Functional impairment*
0	0	0	0	0
I	I–II	0	0	0
II	I–III	I	I	I
III	II–IIII	II–III	II–III	II
IV	II–IV	II–IV	II–IV	III



Figs 5A to C Skin rash in GVHD

Interstitial Pneumonitis

Transplantation associated lung injury usually occurs within four months of the procedure, and the mortality exceeds 60 percent. Risk factors include TBI, allogeneic transplantation and acute GVHD suggesting that donor lymphocytes target the lung. Treatment with etanercept that blocks tumor necrosis factor, combined with corticosteroids may reduce the injury promptly.[9]

Infections

Transplant related infections result from damage to the mouth, gut and skin from preparative regimens as well as from catheters, neutropenia and immunodeficiency. Prolonged neutropenia, GVHD and the administration of corticosteroids predispose patients to fungal infections.[13] Cytomegalovirus is an important cause of morbidity during this period. The various infections which occur in the course of transplantation are given in Figure 1.

DELAYED EFFECTS

Chronic GVHD: The risk of chronic GVHD increases with recipient and donor age. Chronic GVHD is associated with loss of self-tolerance and often resembles Sjögren's syndrome or scleroderma. Chronic GVHD can cause bronchiolitis, keratoconjunctivitis sicca, esophageal stricture, malabsorption, cholestasis, hematocytopenia, and generalized immunosuppression. Treatment with corticosteroids may be needed for two years or longer.[1]

Growth and development are impaired in children who undergo transplantation as a result of myeloablative preparative regimens. Growth hormone therapy increases height in these children.

Fertility in adulthood may be impaired in children undergoing transplantation. Young men may recover their fertility later in life. If sperms are present before transplantation, semen can be cryopreserved and used later. Women can also go in for cryopreserved oocytes.

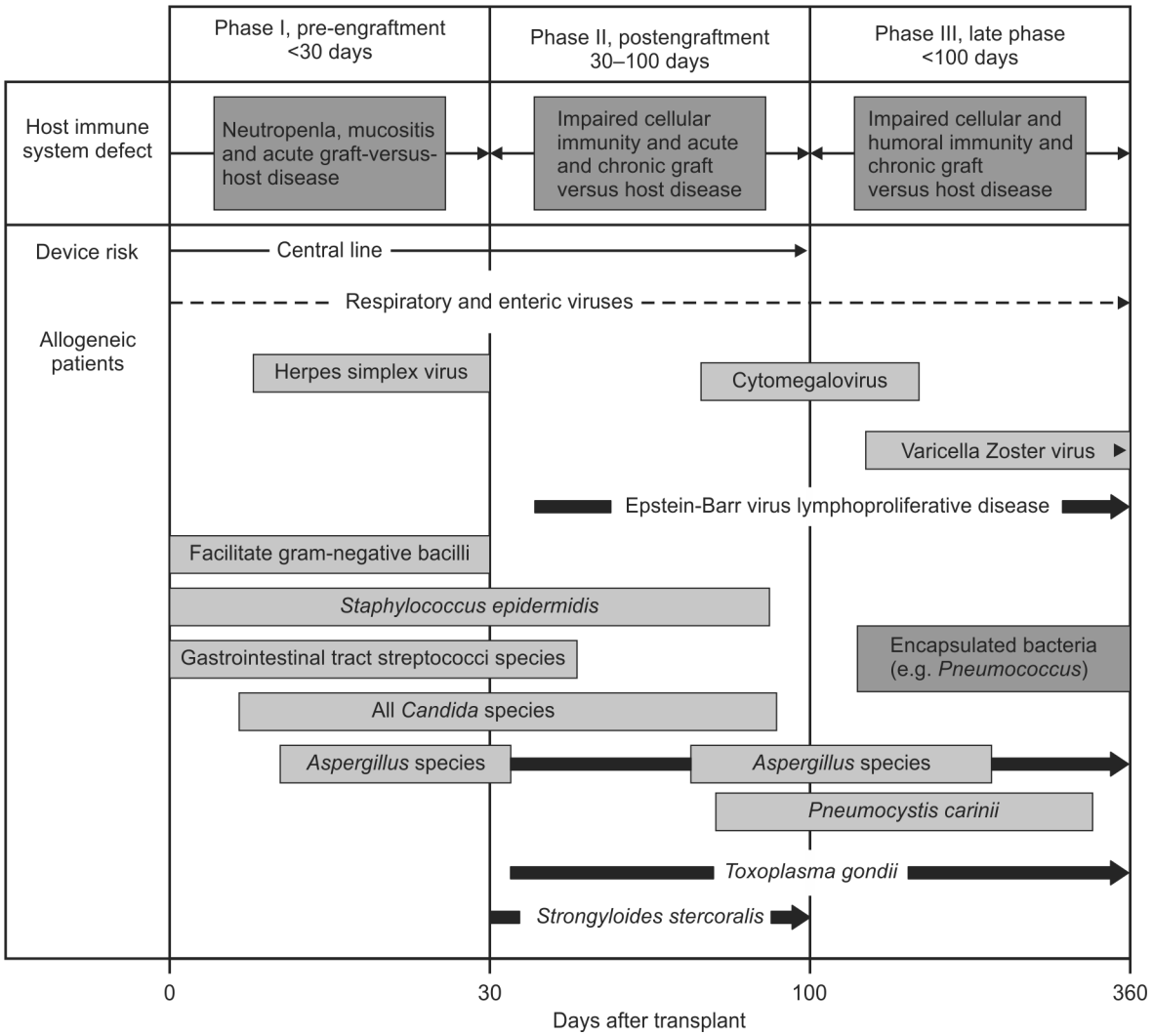


Fig. 6 Timeline of infections in a patient undergoing transplantation

Secondary cancers increase after transplantation: Myelodysplasia and acute leukemia are complications of autologous transplantation for Hodgkin's and non-Hodgkin's lymphoma. Survivors of transplantation should be followed indefinitely to detect early cancer or precursor lesions.

Beta Thalassemia

The median survival of patients with beta thalassemia major who are on regular transfusion and chelation program is around 35 years. By the fourth decade of life most of the patients succumb to complications of the disease. At present, the only curative approach in this illness is allogeneic stem cell transplant. The first transplant was performed in 1982 and ever since, the transplant group from Pesaro, Italy headed by Dr Guido Lucarelli has led the subsequent way. The Pesaro group with their experience, has divided patients with thalassemia into 3 major risk classes based on the presence of hepatomegaly, inadequate chelation and portal fibrosis.[14]

Pesaro Thalassemia Risk Classification

Risk Factors

- *Hepatomegaly:* Determined by physical examination
- *Hepatic fibrosis:* Liver biopsy
- *Inadequate chelation:* History, ferritin value, liver iron quantitation.

Risk Classification

- *Class 1 risk:* No risk factors present
- *Class 2 risk:* 1 or 2 risk factors present
- *Class 3 risk:* All 3 risk factors present.

The conditioning regimen used was Bu 14 to 16 mg/kg and Cy 200 mg/kg.

On analysis of their data of 1003 patients, the overall thalassemia free survival observed was 68 percent (Class 1: 87%, Class 2: 84%, Class 3: 58%). The major problem observed was with Class 3 patients, as many could not tolerate the toxicity of the regimen. In 1997, a new preparative regimen was developed that used myelosuppression and immunosuppression with azathioprine, hydroxyurea and fludarabine followed by conditioning with Bu 14 mg/kg and Cy 160 mg/kg. This strategy named Protocol 26 has shown good results. In 33 class 3 thalassemics <17 years of age, the thalassemia free survival has increased to 85 percent and the rate of rejection has dropped from 30 to 8 percent.

Thus allogeneic transplantation is increasingly becoming a feasible option across the globe. The availability of HLA matched sibling donor is the limiting factor as it is seen only in 25 to 30 percent of cases. Recently, there have been reports of use of alternative donors like umbilical cord blood, HLA mismatched related donors, etc. such techniques need to be perfected before they can be accepted as a standard of care.[15]

APLASTIC ANEMIA

Severe aplastic anemia is defined as ANC <500/μL, Absolute reticulocyte count <20,000/μL and platelet count <20,000/μL. Currently, the frontline therapy consists of either immunosuppressive therapy or matched sibling transplantation. Stem cell transplantation provides curative therapy in SAA. Initial reports of success have been with the use of syngeneic donors. The first successful allogeneic transplantation was done in 1972. The combination of cyclophosphamide with ATG was shown to be a successful conditioning in these patients. Survival for HLA matched sibling transplants has increased from 48 percent in the 1970s to 66 percent in the late 1980s and to 70 to 90 percent in recent data. The rate of graft rejection has fallen since cyclophosphamide with ATG has become the standard conditioning regimen.[16]

The comparison of hematological response rates, as well as long-term responses strongly supports SCT as the treatment of choice in SAA, in cases where an HLA matched related donor is available. However, transplantation is also associated with risk of early mortality. Data indicates that in patients younger than 40 years of age, SCT is always superior to immunosuppressive therapy. In older subjects immunosuppression may be a more feasible option considering the comorbidities. In patients >40 years of age, the decision should be made on an individual basis. The response to immunosuppressive therapy may take as long as 6 to 12 months and also carries a 10 percent risk of evolution of clonal hematopoietic disorders like PNH and myelodysplasia.[17]

LEUKEMIA

Allogeneic stem cell transplantation is used for pediatric patients with acute lymphoblastic leukemia (ALL), acute myeloid leukemia (AML) as well as for chronic myeloid and juvenile myelomonocytic leukemia. In addition to the stem cells, the donor graft also has T cells and NK cells. They populate in the recipient's hematopoietic system and give rise to a new immune system. This can help in eliminating the remaining leukemia cells that escape the conditioning regimen. This is called the Graft versus Leukemia effect. The GVL effect is exerted through T cell mediated allo reactivity.[18]

Acute lymphoblastic leukemia is the most common indication for stem cell transplantation in pediatric age group. In ALL transplantation is done in first remission for patients at very high-risk of a relapse. This includes high risk cytogenetic features like t(9; 22) and t(4; 11). In other patients who relapse while on or after completion of primary treatment, transplantation is indicated in second remission. Conditioning regimens that utilize total body irradiation are associated with better survival than with busulfan/cyclophosphamide alone. The estimated probability for event free survival for patients transplanted in first and second remission is around 65 percent and 50 percent respectively.[1]

Less intensive GVHD prophylaxis is employed to reap the benefits of GVL effect.

Acute myeloid leukemia is another indication for allogeneic stem cell transplantation from an HLA identical donor. Subtypes of AML including acute promyelocytic leukemia and good cytogenetic features like t(8; 21) and inv(16) are no longer considered for transplantation in first remission due to good results with conventional therapy.

In chronic myeloid leukemia, the earlier treatment of choice was allogeneic SCT. With the advent of targeted therapy directed against t(9; 22), i.e. imatinib mesylate, the treatment has been revolutionized. Transplant is indicated only for those patients who fail this treatment or progress to blast crisis.

IMMUNODEFICIENCIES

Bone marrow transplantation for lethal congenital immunodeficiencies has been established as a treatment modality ever since transplants done for severe combined Immunodeficiency and Wiskott Aldrich syndrome were successful in 1968. In contrast to other indications, the goal of transplantation in these patients, is complete recovery of

immune function. Hence 100 percent donor engraftment may not be needed inorder to cure the immunodeficiency. In certain diseases like chronic granulomatous disease, stable donor chimerism of around 10 to 15 percent cells is enough to establish normal host defense mechanisms. Before taking up for transplantation, a detailed evaluation for infections must be undertaken and cure of underlying infections must be attempted whenever possible. Myeloablative chemotherapy conditioning regimens are utilized for patients with non-SCID primary immune deficiency diseases undergoing SCT. Non-myeloablative or reduced-intensity conditioning regimens also have the potential for engraftment without the risk of morbidity and late sequelae associated with standard myeloablative regimens.[20]

INDIAN SCENARIO

The first Bone Marrow transplantation done in India was in 1983 at Tata Memorial Hospital, Mumbai in 1983 for a patient with Acute Myeloid Leukemia. At present more than 15 centers in India have facility for BMT. India caters not only to patients within our country but also to patients from neighboring countries. Since, its humble beginnings, HSCT has progressed to a successful modality of treatment. The BMT Unit at Christian Medical College, Vellore has been designated as center of excellence by ICMR.[19]

As far as our experience of HSCT program at Sir Ganga Ram Hospital is concerned, over a period from January 2006 to August 2009, 39 transplants (16 allogeneic and 23 autologous) were done. The median age of transplant patients was 34 years (11 months-68 years). Children comprised 41.2 percent of the patients. The indications for 16 allogeneic transplants were thalassemia major-6, acute myeloid leukemia (AML)/myelodysplastic syndrome-6, severe aplastic anemia-3 and high-risk acute lymphoblastic leukemia-1. Donors were HLA-matched sibling in 13 cases, HLA-matched relative in 1 and unrelated umbilical cord blood in 2. The source of HSCT was peripheral blood in 8 patients, bone marrow in 6 and umbilical cord blood in 2. Fourteen patients underwent myeloablative transplants and two were given reduced intensity conditioning. Three Donor Lymphocyte infusions were given to two patients. Seven patients (44.8%) are alive and disease free at a median follow-up of 453 days (65–591 days). Thirteen patients showed neutrophil engraftment at a median duration of 14 days (range 11 to 44).

Acute graft versus host disease (GVHD) was seen in 6 patients, grade I-II was seen in 5 patients, which responded to steroids. Steroid-refractory grade IV GVHD was seen in one child; it did not respond to mycophenolate and infliximab. Three patients developed sinusoidal obstruction syndrome. All were given defibrotide and two responded. A total of 7 allogeneic HSCT recipients have died, out of which 2 AML patients died of relapse at 56 and 90 days post-transplant. The main causes of death in 5 other patients were sepsis (3 bacterial and 2 fungal), sinusoidal obstruction syndrome (1 case), acute GVHD (1 case) and chronic GVHD (1 case). Two thalassemic children rejected graft but are alive and transfusion dependent.

The main indications for 19 autologous HSCT were multiple myeloma-9, Non-Hodgkin's lymphoma-4, metastatic neuroblastoma-2, relapsed Hodgkin's lymphoma-1, relapsed rhabdomyosarcoma-1, relapsed primitive neuroectodermal tumor (PNET) of the kidney-1 and rejection post cord blood transplant in thalassemia-1. The source of HSCT was peripheral blood in 16 patients and bone marrow in 3. Ten patients (55.6%) are alive and disease-free at a median follow up of 114 days (range 21-617 days). Seventeen patients engrafted neutrophils at a median duration of 12 days (range 9 to 30).

At a median follow-up of 200 days (range 21 to 1200 days), the estimated overall survival and event free survival for all the transplant population are 67.3 percent ± 8.6 percent and 63.5 ± 8.9 percent respectively. Overall transplant related mortality is 23.5%, with a decrease from 28.6 percent to 22.2 percent after a dedicated HSCT unit with HEPA filtered rooms became functional by mid 2007.[20]

It is remarkable that, through bone marrow and HS-cell transplants, stem-cell therapies have brought about permanent cures for many patients suffering from blood disorders. There are efforts underway to develop therapies using alternative sources of stem cells, such as embryonic stem cells. However, because HS cells are relatively abundant and accessible, alternative sources might be less crucial for treating common blood disorders than for diseases of other organs and tissues. Advances in HS cell based therapies would probably be the answer for the many incurable diseases of today.[21]

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SECTION 7

General

CHAPTERS OUTLINE

CHAPTER 48

Gene Therapy

Aditya Kumar Gupta, Nita Radhakrishnan, Anupam Sachdeva

INTRODUCTION

Normal as well as some defective genes are present in all individuals. The genes usually remain dormant until a disease associated with the gene manifests in a case. Genetic defects can lead to more than four thousand diseases. Apart from the genotype of an individual, the environment in which the individual lives also affect the manifestation of the disease.

Gene therapy is the introduction of a target gene into a cell. Gene therapy can be somatic or germ-line. In somatic gene therapy the genetic make-up of the individual is not altered and it is not transmissible to the off-spring. Somatic gene therapy aims at the introduction of the target gene to correct a defective organ or tissue. Germ-line gene therapy is the introduction of the target gene into the zygote, a change that is transmissible to the offspring. Before the initiation of gene therapy the candidate gene needs to be identified. Diseases that are amenable to probable gene therapy are enumerated in Table 1. The complexity of gene therapy lies in the mechanisms to deliver the therapeutic gene into the target organ in an accurate, controlled and effective way.

In 1990 in NIH, Maryand a four-year-old-boy with severe combined immunodeficiency (SCID) received an infusion of genetically modified stem cells. This was the first instance of gene therapy and the recipient was later known as the bubble boy. Since then the field of gene therapy has targeted many diseases and has expanded its coverage.

Table 1 Diseases for which gene therapy is being explored

Disease	*Underlying defect*	*Target cell for genetic manipulation*
SCID	ADA deficiency	T-lymphocytes
Hemophilia	Factor VIII or IX deficiency	Hepatocytes, muscle fibroblasts or hematopoietic cells
Cystic fibrosis	CFTR gene mutation	Airway epithelial cell in lungs
Hemoglobinopathies	Globin chain defects	Hematopoietic cells
Gaucher's disease	Defect in enzyme glucocerebrosidase	Macrophages or hematopoietic cells
α-1 antitrypsin deficiency	Lack of α-1 antitrypsin	Lung and liver cells
Familial hypercholosterolemia	Lack of LDL receptors	Liver cells
Cancer	Multiple causes	Different cancer cell types
Neurological diseases	Parkinson's/Alzheimer's, etc.	Neuronal cells
Cardiovascular diseases	Arteriosclerosis	Endothelial cells of vessels
Infections	HIV, Hepatitis B	T-cells, liver, macrophages

PROCEDURE OF GENE THERAPEUTICS

Target Tissue

Nature of the disease determines the somatic candidate organ for gene therapy. The target cell needs to be clearly defined. For example in cystic fibrosis the target tissue is the lung where delivery of the therapeutic gene is being tried by the aerosolized route. In a clotting disorders like hemophilia the deficient factor can be provided by introduction of the candidate gene into the myocytes or the hepatocytes. The choice of the target tissue depends upon factors such as protein modification, gene delivery efficacy of the vector and immunological factors.

Vectors

The method of delivery of the target gene into the tissue is of vital importance. Naked genes can be delivered into the cells but the method has low efficiency. Vectors which are usually plasmids or viruses, can move recombinant DNA from one cell to the other. Special synthetic vectors have also been designed for gene transfer.

Retroviruses are RNA viruses that can integrate its nucleic acid into the host cells, using reverse transcriptase enzyme that transcribes RNA into DNA . Other vectors used in gene therapy can be adenoviruses, retrotransposons and liposomes (Fig. 1). Engineered vectors are used for gene therapy where the detrimental gene is removed and the corrective gene is added. The properties of an ideal vector are enumerated in Table 2.

Type of Vectors

- Retrovirus
- Lentivirus
- Adenovirus
- Herpes viruses
- Plasmids, retrotransposons and liposomes.

Low host immunogenicity and allowance for large scale production are advantages of non viral methods over the viral methods. Non viral methods however have the disadvantage of low levels of transfection and subsequent gene expression, but these have been now overcome with modern vector technologies that yield molecules and

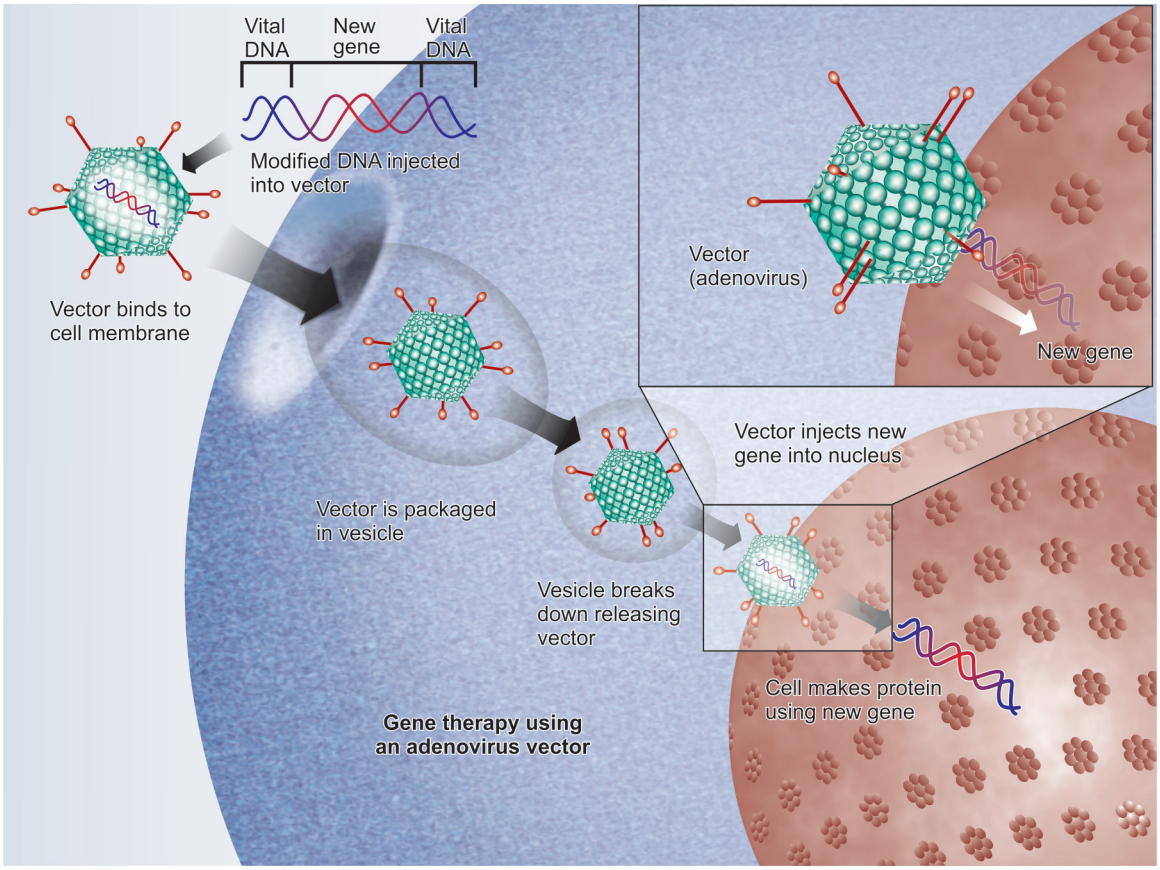

Fig. 1 Gene therapy (a simplified representation)

Table 2 Properties of a good vector for gene therapy

All vectors used in gene therapy—viral or nonviral have certain limitations. The disease type often dictates the type of vector to be employed. Adenoviral vectors are useful in situations where a short term expression of the gene product is needed (e.g. products that may be toxic to malignant cells). In case of sustained gene expression the integrating vector that leads to minimal immunological response is desirable. An ideal vector should have

- A good concentration in minimal amount of injection so that a maximum number of target cells are infected
- Easily reproducibility
- Ability of a stable and site specific integration into the host genome
- Specificity for the target cell
- Minimal immunogenic potential
- Ability of its transcriptional unit to respond to external manipulation.

Presently such a desirable vector that possesses the advantages of both the synthetic and viral vectors is not available. Availability of an ideal vector will make gene therapeutics grow by leaps and bounds.

techniques with transfection efficiencies similar to the viral vectors. Injection of naked DNA, electroporation, the gene gun, sonoporation and magnetofection and the use of dendrimers, lipoplexes, oligo-nucleotides and inorganic nanoparticles are some non viral methods of gene delivery.

GENE THERAPY AND ITS USE IN PEDIATRIC HEMATOLOGY AND ONCOLOGY

As hematopoietic cells can be easily collected and are amenable to *in vitro* manipulation they are ideal targets for gene therapy. Genetic manipulation of the hematopoietic stem cells can be utilized for the cure of many acquired and inherited hematological and oncological diseases. The target cells can be the red cells, leukocytes or any other mature blood element. The desired type of blood cell can be continuously produced for the lifetime of an individual by integrating the desired transgene into the chromatid of the concerned pleuripotent stem cell. For example the sources of deficient clotting factors could be cells such as the hepatocytes and the myocytes.

Gene Therapy for Hemophilia

In mice, expression of factor VIII in blood cells and platelets has been achieved by the use of *ex vivo* transduced hematopoietic stem cells. *In vivo*, transposons expressing factor VIII can be transferred into the endothelial cells or hepatocytes. In canine models the neonatal administration of reteroviral vectors expressing the canine factor VIII completely corrected hemophilia in dogs. Similarly in dogs with hemophilia B factor IX levels that were 28 times more were achieved using double stranded adenoviruses in comparison to the single stranded ones. The factor IX expression however was short lived due to probable immune destruction of the modified cells. Stable phenotypic correction is often hampered by neutralizing antibodies. *In vivo* certain promoters undergo inactivation hampering long term factor VIII or factor IX expression. Several phase I clinical trials are underway presently for hemophilia and some subjects have reported lower bleeding episodes and detectable clotting factor activity.

Gene Therapy for Hemoglobinopathies

A lentiviral vector has been used for inserting the gene for a normal hemoglobin expression in hemoglobinopathies into stem cells from the bone marrow, in mice cells cultured *in vitro*. These cells have then been re-introduced into the mice. The mice that have received this genetic treatment are no different from the normal counterparts. As the stem cells used here were autologous, their rejection has been avoided. Although this therapy is still untested in humans it is possible that the mutations in the β-globin gene be corrected by gene targeting in induced pluripotent stem cells (IPS) derived from somatic cells. Skin fibroblasts have been differentiated into IPS cells. The IPS cells in future could be utilized to create hematopoietic stem cells that synthesize hemoglobin.

Gene Therapy for Immunodeficiencies

In mice models the restoration of lymphocyte function in SCID mice secondary to JAK deficiency has been reported as early as 1998. The same has however proved challenging in larger animals. The transduction efficiency of human hematopoietic stem cells is being optimized by use of alternative envelope proteins to pseudotype vector particles including hat derived from Gibbon ape leukemia virus (GAVL). Cytokine combinations and use of fragments of fibronectin, retronectin to co-localize vector particles and target cells also help the cause.

In chronic granulomatous disease success has been achieved in two patients where genetically modified hematopoietic cells have been successfully engrafted after

partial myeloablation. In these two patients the vector used was the spleen focus forming virus (SFFV) as its enhancer-promoter region is active in myeloid cells.

Gene Therapy for Hematological Malignancies

Inhibitory signals to the host immune response by tumor cells helps in their survival. This can be blocked by the use of gene products or cytokines released from the site of vaccination (i.e. the therapeutic tumor vaccine). The methods used could rely either on the use of genetically modified autologous tumor cells or allogenic tumor cells which may provide a paracrine stimulus. Skin fibroblasts expressing IL-2 and CD-40 ligand have been mixed with irradiated tumor cells and injected into patients of refractory leukemias thereby inducing T cells reactive against the blast cells.

Chimeric antigen receptors (CAR) are single chain antibodies with specificity for the antigen expressed on the human tumor cell is linked to an internal kinase domain which mediates cell activation when the antibody is engaged by the target antigen. In mice CARs targeting CD-19 have been used to cure B cell leukemias. LMP-2 protein of the EBV which is expressed in some human lymphomas can be generated by gene transfer on population of lymphoid cells. These can then be used to yield a population of T cells with potent antitumor property.

Suicide Gene Therapy for Graft Versus Host Disease (GVHD)

A suicide gene can be inserted into a target cell making it susceptible to drug induced cell death. The target cell can be the donor lymphocytes and this can be used to control alloreactivity as seen in allogenic hematopoietic transplants. As shown in Figure 2 , a suicide gene is introduced into the allogenic donor lymphocytes and this in turn has the potential to convert a prodrug into an active drug. Following HSCT the donor receives these genetically modified lymphocytes for immune reconstitution. In event of a GVHD occurring the prodrug can be administered resulting in the ablation of these alloreactive lymphocytes.

CHALLENGES WITH GENE THERAPY

- Short-lived nature—As many cells are rapidly multiplying ones, long term benefits after gene therapy is difficult. Many rounds of gene therapy are needed to achieve a substantial amount of benefit.
- Immune response to the inserted DNA material or the virus *per se* could lead to fatal complications.
- Viruses are the vectors of choice in most gene therapy studies. They however have a variety of potential problems to the patient including toxicity, immune

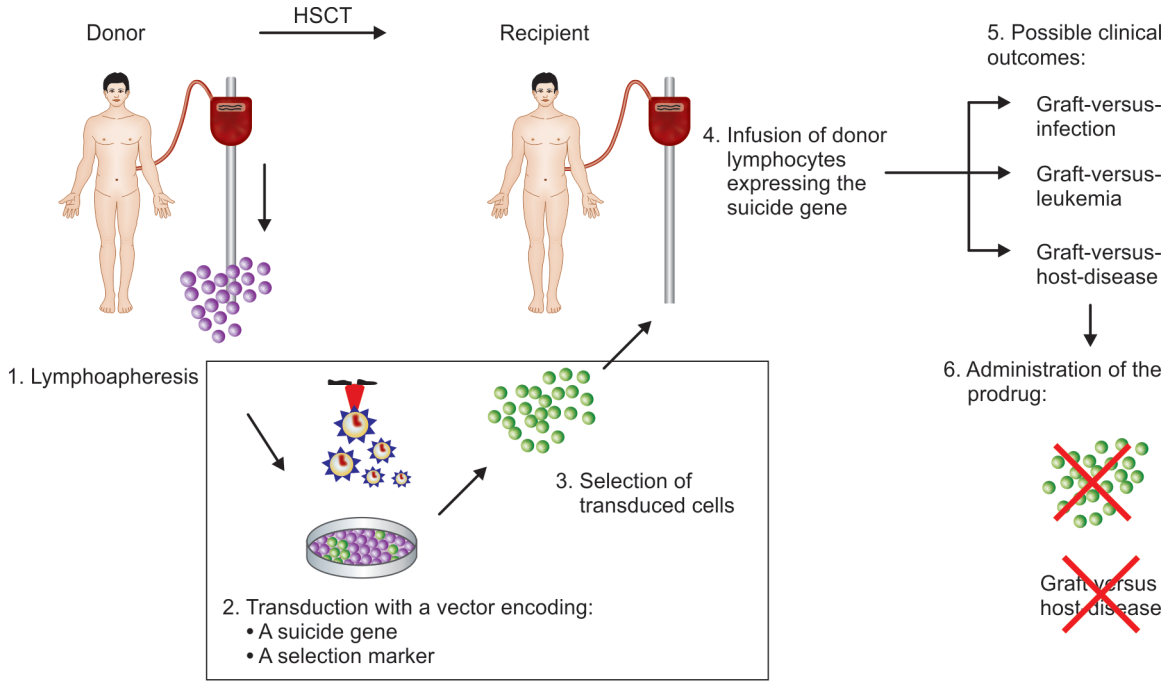

Fig. 2 Gene therapy and its use in HSCT

and inflammatory response. With these viral vectors gene control and targeting issues are also a problem. The possibility of the viral vector regaining its virulence once inside the host cell is always a danger.

- Multigene disorders are poor candidates for gene therapy.
- The novel DNA may inadvertently impact the germline by breach of the somatic-germline barrier against the intentions of the therapy.
- Insertional mutagenesis can occur whereby a tumor can be induced if the DNA is integrated in a wrong place (e.g. in a tumor suppressor gene). Gene therapy in SCID has resulted in T cell leukemias in 3 out of 20 patients due to insertional mutagenesis.

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CHAPTER 49

Monoclonal Antibodies in Pediatric Hematology and Oncology

Saroj P Panda, Girish Chinnaswamy

Despite the tremendous progress in the treatment of pediatric cancers in the past decade, current therapies are associated with wide range of toxicities, which leads to treatment associated mortality and substantial morbidity in long-term survivors. Novel approaches are needed to overcome resistance and to decrease adverse effects of standard treatment. Targeted therapies, which include monoclonal antibodies (MoAbs) have significantly changed the treatment of adult cancers.[1] During the last few years, progress has been made in the therapeutic use of MoAbs in specific groups of pediatric cancers and hematologcal disorders.

In 1975, Kohler and Milstein demonstrated for the first time that monoclonal antibodies (MoAb) could be generated from hybridomas.[2] Because MoAbs can bind to antigens expressed on the surface of malignant cells, it was proclaimed that these agents could be used as chemoimmunotherapy to specifically target and destroy these cells. Moreover, by offering cytotoxic mechanisms different from conventional chemotherapy, MoAb therapy could potentially reduce the risk of tumor cell resistance to the common chemotherapeutic agents. By 1979, the first patient was treated using MoAb therapy and in the ensuing decade over 100 patients with hematologic malignancies have been similarly treated.[3] Although progress in this field has proceeded much slower than was initially anticipated, recent clinical trials have also demonstrated antitumor activity in a variety of pediatric malignancies.

MONOCLONAL ANTIBODY THERAPY

- Effective MoAb therapy for cancer requires the identification of appropriate tumor—specific targets expressed on the surface of the cancer cells.
- Ideally, the antibody should have minimal cross-reactivity with normal tissues and specifically target the tumor cell. Nonspecific binding will reduce effective drug delivery. Moreover, any cross-reactive tissues that are damaged may compromise patient's function and survival.
- The target of the MoAb should not be shed from the tumor following MoAb binding; rather, the antigen-MoAb complex should be internalized by the tumor cell.

The ideal antigen should not undergo modulation (in which the antigen is no longer expressed on the cell surface after antibody binding). Its disappearance from the cell membrane can limit the effectiveness of treatment.

Despite the fact that many of the patients are inherently immunosuppressed secondary to their malignancies and extensive prior chemotherapy, development of neutralizing antibodies have been seen in few patients. Its development can preclude the efficacious administration of MoAbs because of enhanced clearance of the antibody from the circulation, the formation of antigen-antibody complexes with subsequent end organ damage. Hence, they must be rendered sufficiently nonimmunogenic to prevent development of neutralizing antibodies.

Recent advances in genetic engineering have allowed the development of chimeric antibodies and fully humanized MoAbs which limit the likelihood of neutralizing antibody development.

Mechanism

The overall success of MoAb therapy in cancer is determined by the ability of antibody binding to result in

tumor cell death. A variety of mechanisms are thought to play important roles in mediating the observed anti-tumor effects.[4]

- Antibody-dependent cellular cytotoxicity (ADCC) and complement-mediated cytolysis. Binding the antibody to the tumor cell recruits cells with Fc receptors like NK cells and macrophages to the site of the tumor, which then kill the tumor cell, or complement is fixed and the tumor cell is killed.
- *Direct killing:* Key process include interruption of a critical cell signaling cascade by inhibition of ligand binding; downregulation of a receptor tyrosine kinase, which transmits a necessary life signal; and induction of an apoptotic signal following ligation of the target by the MoAb.
- Targeting via a conjugated antibody of antibody receptor (e.g. radionuclide, immunotoxin, cell-based genetic fusion). This targets a lethal "hit" to the tumor cell.

The following gives an overview of the various MoAb which are now in clinical use or in various phases of clinical trials.

- *Rituximab:* It is a chimeric unconjugated MoAb directed to CD20, which is expressed on the surface of malignant and normal B-cells, but not hematopoietic stem cells. It has been shown to significantly increase the response rate and survival of adult patients with CD20-positive B-cell lymphomas. It appears that rituximab has efficacy in children with high-grade B-lymphoma/B-cell acute lymphocytic leukemia. The mechanisms of action include inhibition of B-cell proliferation, antibody-dependent cellular cytotoxicity, complement-dependent cytotoxicity and possible induction of apoptosis.[5] The Children's Oncology Group is currently researching the efficacy of rituximab in recurrent and refractory CD20 lymphomas in children (NCT01230788). With rituximab, a 96 percent overall response rate was reported in one phase II trial for lymphocyte-predominant Hodgkin lymphoma, with 75 percent remaining in remission after one year.[6] Another phase II trial by Ekstrand et al, 2003 showed 100 percent overall response rate (n = 22) with complete response (CR) in 41 percent, unconfirmed complete response in 5 percent, and partial response in 54 percent.[7] A phase II pilot study is underway through the Children's Oncology Group to assess the toxicity of adding rituximab to upfront chemotherapy for B-cell leukemia and lymphoma (NCT00324779). Children with refractory chronic immune thrombocytopenic purpura (ITP) have been treated with rituximab in various series with response rates of 30 to 70 percent. Most of the responses were obtained within 4 weeks and were maintained for a year.[8,9] International consensus report on the investigation and management of primary immune thrombocytopenia recommends a dose of 100 mg or 375 mg/m^2/week administered for four times as standard treatment strategies for children with chronic ITP.[10]

Rituximb has also been proved to be effective in treatment of various benign hematologic conditions like autoimmune hemolytic anemia (refractory to steroids immune-suppressants and splenectomy),[11] AIHA in setting of Evan's syndrome, SLE and autoimmune lympho-proliferative syndrome[12] and in patients with hemophilia who develop inhibitory antibodies to factor VIII and IX.[13]

- *Gemtuzumab ozogamicin (GO):* It is a recombinant humanized MoAb (IgG4) directed against the CD33 antigen that is conjugated to the derivative of the cytotoxic antibiotic calicheamicin.[14] In pediatric acute myeloid leukemia (AML), the differentiation antigen CD33 is expressed in almost all patients. Following binding of GO to the CD33 antigen, the antibody-antigen complex gets internalized into the AML cells. The calicheamicin conjugate is released inside the cell through hydrolysis and subsequently binds to the minor groove of DNA, inducing double strand breaks and leukemia cell apoptosis.

Currently, the agent is being tested both as a single agent and in combination with chemotherapy in children with AML. Results of phase I and II clinical trials indicate promise, with an overall remission response rate of 45 percent and a 1-year event-free survival and overall survival estimates of 38 percent and 53 percent, respectively.[15,16] Several earlier case reports found responses to anti-CD33 in pediatric ALL and in a few cases of relapsed adult ALL.[17] Patients responding to gemtuzumab had very high (>90%) CD33 expression.[17.]

The US Food and Drug Administration (FDA) approved anti-CD33 conjugated with calicheamicin (gemtuzumab [Mylotarg]) for treatment of adult AML in 2000, but the agent was withdrawn from the US market on June 21, 2010. Apart from some infusional allergic reactions, the primary toxicity has been bone marrow suppression caused by binding the MoAb-toxin conjugate to normal hematopoietic precursors that express CD33. Another still unexplained toxicity of anti-CD33–calicheamicin conjugates is hepatic damage, which is characterized by transient increases in liver enzymes in approximately 25 percent of patients and, occasionally, a more severe complication consistent with veno-occlusive disease.

- *Epratuzumab:* It is a humanized anti-CD22 MoAb that binds to the extracellular domain of CD22. Epratuzumab appears to modulate B-cell activation and signaling. Proposed mechanisms of action include antibody-dependent cell-mediated cytotoxicity, comp-

lement dependent cytotoxicity and direct induction of apoptosis. CD22 is widely expressed in B-cell lymphomas and B-precursor ALL. It is rapidly internalized after antibody binding and re-expression on the cell surface is slow, occurring over the period of several days. Internalization of CD22 has been shown to directly induce apoptosis in malignant cells.

Epratuzumab has recently been studied by Children's Oncology Group in pediatric patients with first relapse of pre-B ALL (n = 15). The addition of epratuzumab to re-induction chemotherapy was well tolerated, with no apparent significant increase in toxicity.[18] Although, it did not improve the second remission rates, among patients who attained CR, postinduction MRD-negative rates were higher in comparison with those of historical controls treated with chemotherapy alone (42% versus 25%).[11]

- *Bevacizumab:* It is a humanized murine MoAb that binds to vascular endothelial growth factor-A (VEGF-A) with high affinity and neutralizes its activity. VEGF is one protein that plays a big role in the process of angiogenesis. By cutting off the blood supply to the tumor, it is predicted that the tumor cells should die. Bevacizumab is an antiangiogenesis agent approved for the treatment of colon cancer in adults and has shown activity in carcinoma of the kidney, adenocarcinoma of the rectum and nonsmall-cell lung cancer. VEGF is overexpressed in a number of solid tumors seen in children (NCT01218867), including Ewing sarcoma and glioblastomas, and is currently being investigated in these and other pediatric solid tumors.[19]
- *Alemtuzumab:* It is a humanized MoAb active against CD52; a cell surface co protein expressed by most T and B lymphoblasts. CD52 is neither shed nor internalized, making it ideal for antibody directed immunotherapy. Most malignancies of B-cell origin and almost all T-cell malignancies strongly express the antigen. Binding of alemtuzumab induces the lysis of lymhocytes, while monocytes and their precursors are less sensitive.

Alemtuzumab has shown antitumor activity in chronic lymphocytic leukemia, T-prolymphocytic leukemia, T-cell non-Hodgkins lymphoma.[17] In a few cases, clinical effects were observed in patients with single-drug treatment in relapsed adult ALL.[20] It is being studied by COG in children with ALL in second or greater relapse or primary induction failure after two different regimens (PMC3120889).

Other antibodies that have demonstrated activity in T-cell leukemias, either *in vitro* or *in vivo*, include anti-CD7-ricin, CD25 antigen (IL-2 receptor), anti-CD7-PAP, anti-CD2, OKT3, and a humanized anti-CD3 MoAb. Overall experience with MoAbs in T-cell ALL—with the exception of anti-CD52 alemtuzumab is scarce.

Neuroblastoma cells have been characterized for the expression of tumor associated antigens recognized by antibodies. The identification of GD2 as a major target for MoAb therapy has led to the production of both murine and chimeric anti-GD2 MoAbs in combination with granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-2 (IL-2). The mechanisms whereby anti-GD2 MoAbs kill tumor cells are likely related to complement activation and antibody-dependent cell-mediated cytotoxicity (NCT00026312). The COG is currently investigating the efficacy of a humanized MoAb in combination with a human recombinant interleukin-2 (Hu14.18-IL2) for the treatment of refractory neuroblastoma.

Alice et al. 2010 compared between two treatment groups in patients with high-risk neuroblastoma, the first group received the standard therapy of six cycles of isotretinoin. The second group received the new immunotherapy treatment: six cycles of isotretinoin plus five cycles of the monoclonal antibody ch14.18, in combination with alternating GM-CSF and interleukin-2. Study results after two years showed, the rate of survival without relapse or disease progression was 20 percent greater in the children who received immunotherapy (66% versus 46%).[21]

In a recent phase II trial, Memorial Sloan-Kettering Cancer Center has shown promising result by the use of anti-GD2 monoclonal antibody 3F8 and granulocyte-macrophage colony-stimulating factor in neuroblastoma resistant to intensive induction therapy.[22]

- *Trastuzumab:* In a retrospective review of 53 osteosarcoma patients treated on the Memorial Sloan-Kettering Cancer Center T12 protocol, higher frequencies of HER2/erbB-2 expression were correlated with metastatic disease at presentation, poor histologic response to chemotherapy and significantly decreased event-free survival (47% versus 79% at 5 years, P = .05).[23] Trastuzumab, a MoAb directed against the human epidermal growth factor receptor 2 (HER2), is being attempted as a therapy for osteosarcoma.[24,25] Although, it suggested that trastuzumab can be safely delivered in combination with anthracycline-based chemotherapy and dexrazoxane, the actual therapeutic benefit still remains uncertain.

Adverse Effects of MoAbs

Most reports of adverse events are from adult phase I and II trials, since data for children are limited.

Acute infusion reactions are frequent, which can often be managed with antipyretics, antihistamines, and/or corticosteroid. Immunosuppression and increased risk of infection is not uncommon due to depletion of healthy

hematopoietic cell (rituximab, alemtuzumab). Rapid malignant cell kill can cause tumor lysis syndrome. Hepatitis B virus reactivation with fulminant hepatic failure has also been reported with rituximab. Other adverse effects of monoclonal antibodies documented are hemolytic uremic syndrome, vascular leak syndrome, hypoalbuminemia and transaminitis, veno-occlusive disease, pulmonary infiltrates and acute respiratory distress syndrome (ARDS).[26]

Late effects include congestive heart failure, cardiomyopathy, pericardial effusion, pericarditis, pulmonary fibrosis and nephrotic syndrome.

Monoclonal antibodies that contain high amounts of mouse protein result in various immunogenic responses, including infusion-related reactions. More advanced humanized antibodies contain only 5 to 10 percent of mouse protein sequences in an attempt to overcome the potential for dose-limiting or fatal hypersensitivity reactions. Recently, B-cell epitope mapping has been conducted to identify immunogenic amino acids, with the goal to modify immunotoxin sequence to generate a less immunogenic protein. Other techniques like coating immunotoxin with high molecular weight polyethylene glycol (so called PEGylation) is also under investigation.[27]

Future

Modern recombinant techniques have made it possible to rapidly produce both chimeric and humanized antibodies. Identification of surface receptors that are integral to proliferation and apoptosis has also provided more targets for monoclonal antibodies. At present, there are more than 100 monoclonal antibody based biologic drugs under clinical trials and the optimal agents, dose, schedule, and combination regimens have yet to be defined.

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CHAPTER 50

Biological Response Modifiers

Anupama S Borker, Narendra Chaudhary

Rapid advances in standard management approaches (surgery, chemotherapy and radiotherapy) for cancer, have led to remarkable cure rates in children with cancer. Certain limitations include unresectability of the tumor, resistance to chemotherapeutic agents, intolerability of vital structures to radiotherapy, and critical effect of radiotherapy on growth of pediatric patients. Consequently, new therapeutic approaches are being explored, including the use of biologic response modifiers.

Cancer cells express a wide range of different proteins that act as antigens. Some of these may be a result of oncogenic transformation and are relatively specific to cancer cells. These tumor-associated antigens are delivered to the immune system by antigen-presenting cells (APCs) through major histocompatibility complex (MHC) class I or class II pathways. In the class I pathway, the phagocytosed tumor cells are processed by proteosomes and converted to short peptide fragments, which are then presented on class I MHC molecules. These are recognized by CD8+ cytotoxic lymphocytes, which have direct cytotoxic effects leading to tumor cell lysis. In the class II pathway, the secreted products from tumor cells enter the APCs, which are then processed and presented to MHC class II molecules. These processed antigens are recognized by CD4+ helper lymphocytes, which enhance the CD8+ cytotoxic responses as well as the humoral response to surface antigens present on tumor cells. Lowered expression or lack of MHC antigens on the tumor cells may allow tumor cells to escape the host immune surveillance.[1] The goal of biologic response modifiers is to stimulate the body's own immune system to help eradicate tumor cells. In 1884, Cooley[2] observed absence of postsurgical recurrence of round cell sarcoma in one patient who had erysipelas infection at surgical site. He directly inoculated an infectious agent to a tumor site in hopes of stimulating an immune response against tumor. Immunotherapy has evolved considerably since these early days.

DEFINITION

Biological response modifiers (BRMs) are natural or synthetic substances used to boost or restore the ability of the immune system to fight cancer, infections, and other diseases or used to lessen certain side effects that may be caused by some cancer therapies. These substances are also called as biological therapy, biotherapy or immunotherapy.

CLASSIFICATION OF BIOLOGICAL RESPONSE MODIFIERS

Newer molecularly targeted drugs and biotherapeutic agents have made the classification of anticancer drugs more complicated. Xiong-Zhi Wu[3] tried a simpler classification based on mechanism of action of the drugs. For descriptive purpose, BRMs can be classified as below:

- Monoclonal antibodies
- Nucleic acid based agents
- Small molecule agents
- Cytokines
- Tumor vaccines.

The two sections that follow address monoclonal antibody and nucleic acid-based therapeutic approaches with relevance to pediatric oncology. Examples of small molecule inhibitors directed at specific targets are discussed in later sections. Cytokines and tumor vaccines are discussed briefly thereafter.

Monoclonal Antibodies

Monoclonal antibodies directed against unique tumor antigens have efficacy against neoplastic cells. After binding to their target antigen, monoclonal antibodies have multiple mechanisms of anticancer effect, including:

- Antibody-dependent cell–mediated cytotoxicity (ADCC).
- Complement-dependent cytotoxicity (CDC).
- Interfering with ligand-receptor interactions, including down-regulation of receptor expression on the cell surface.
- Modification of signaling pathways to produce apoptosis.
- Delivery of toxic substances to cancer cells. The antitumor activity of monoclonal antibodies can be increased by:
  - Enhancement of ADCC by priming of effector cells with cytokines like GM-CSF, interleukin (IL)-2 and IL-12;
  - Enhancement of CDC mediated by binding of β-glucan to complement receptor-3 on neutrophils;
  - Conjugation with cytotoxic entities such as radionuclides, toxins, chemotherapy agents, and enzymes. Significant progress has been made in the past few years in the area of antibody drug conjugates (ADCs) for the selective delivery of cytotoxic drugs to tumors. These include SGN-35, an ADC directed against the CD30-positive malignancies such as Hodgkin's disease and anaplastic large cell lymphoma, and trastuzumab-DM1 which has shown activity in metastatic breast carcinoma.[4]

The toxicities associated with monoclonal antibodies can generally be attributed to the action of the antibody on normal cells expressing its target antigen. For example, skin rash with epidermal growth factor receptor (EGFR) targeted antibodies, severe pain with GD2 ganglioside targeted antibodies and first dose reaction with CD52 targeted alemtuzumab.[5]

Clinical Significance of Monoclonal Antibodies[5]

- Rituximab is an anti-CD20 chimeric IgG1 antibody having cytotoxic effect, both through complement activation and ADCC. Rituximab may also induce apoptosis through down-modulation of Lyn kinase. The later may contribute to the chemosensitizing activity of rituximab. Rituximab is the first US food and drug administration (FDA) approved monoclonal antibody for cancer therapy. Clinical trials evaluating the addition of rituximab to standard chemotherapy have documented improved outcome for both indolent lymphomas and aggressive lymphomas. It is being studied in children with CD 20 expressing tumors like Burkitt's lymphoma, DLBCL, and post-transplant lymphoproliferative disease.
- Anti-GD2 antibodies tried in neuroblastoma patients had shown limed success. These include murine monoclonal 3F8, ch14.18 with GM-CSF and IL-2 and ch14.18-IL-2 fusion protein. Radiolabeled 3F8-murine antibodies have been used to deliver up to 40 Gy radiation to tumor. The molecule ch14.18 is being evaluated in the setting of minimal residual disease and in adjuvant setting.
- Epratuzumab is an anti-CD22 antibody under clinical trial for CD22 expressing B-cell ALL and NHL.
- Alemtuzumab is an anti-CD52 antibody, being evaluated in children with recurrent ALL expressing CD52.
- Trastuzumab is an anti-HER2 antibody, being evaluated in children with osteosarcoma.
- Anti-CD30 antibodies have entered phase I/II evaluation in adults with Hodgkin's lymphoma and ALCL expressing CD30, with some evidence of antitumor activity observed.
- IGF-1R blocking antibodies are of potential pediatric interest in rhabdomyosa coma and Ewing's sarcoma.

Nucleic Acid Based Agents/Antisense Agents

The antisense agents are single-stranded DNA-like molecules that modify expression of specific genes by targeting based on complimentary base pairing. Antisense molecules can inhibit production of functional protein by their target mRNAs through several distinctive processes:

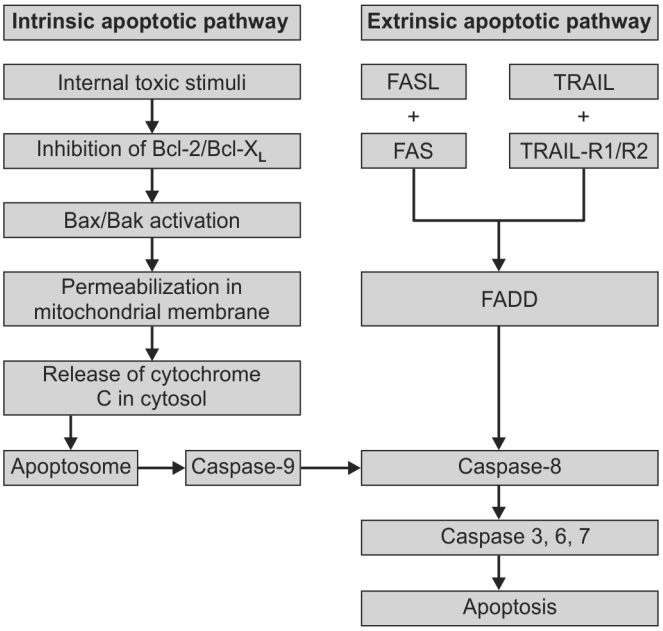
- RNA cleavage by RNase H, an ubiquitous endonuclease
- Inhibition of translation machinery
- Alteration in RNA splicing
- Degradation of homologous RNA by small interfering RNA (RNA interference)

Fomivirsen was approved by the FDA in 1998 for CMV retinitis in AIDS.[6] Bcl-2 antisense agent, oblimersen, is a phosphorothioates, that can induce RNase H cleave of BCL-2 mRNA leading to activation of apoptotic pathways. It is being studied in pediatric population in neuroblastoma (phase1 trial), as higher BCL-2 expression is associated with unfavorable histology and N-Myc gene amplification. Prolonged aPTT and thrombocytopenia are major side effects due to polyanionic backbone structure.

Therapies Targeted to Apoptotic Pathways

The ability of cancer cells to evade apoptosis, provide a survival advantage during tumorigenesis and can also provide cancer cells with increased resistance to treatment. An obvious approach to enhance the effectiveness of cancer therapy is by manipulating the dysregulated cancer cell apoptosis pathways to favor cell death. The two primary apoptotic pathways, the extrinsic (death receptor) pathway and intrinsic (mitochondrial) pathway, are described in Flow chart 1.

Flow chart 1 Extrinsic and intrinsic apoptotic pathways[5]



Extrinsic pathway is initiated by ligation and clustering of members of death receptor superfamily (e.g. tumor necrosis factor receptor-I, Fas, and the TNF-related apoptosis inducing ligand receptors TRAIL-R1 and TRAIL-R2) followed by recruitment of adaptor proteins and caspase-8, which can then activate downstream effector caspases leading to apoptosis. The intrinsic (mitochondrial) pathway is responsive to internal toxic stimuli (e.g. DNA damage, disruption of microtubules, etc.). Inhibition of Bcl-2 and Bcl-x_L function or direct activation of Bax and Bak in mitochondrial membranes results in the release of cytochrome C and other proapoptotic factors into the cytoplasm. Subsequent formation of apoptosome in cytoplasm results in production of active caspase-9 and which then activates downstream effector caspases. Inhibitors of apoptotic pathways are evolutionarily conserved cytoplasmic proteins that can inhibit caspases.

These pathways can be targeted at Bcl-2 family proteins, inhibitors of apoptotic pathways (IAPs), and the death receptor pathway.

Inhibition of Bcl-2 Family Proteins

Oblimersen is a Bcl-2 antisense agent. Gossypol, a natural product derived from cottonseed extract, is a small molecule inhibitor of Bcl-2 and Bcl-x_L. Preclinical studies demonstrated *in vitro* and *in vivo* anticancer activity for this agent.

Molecules Targeting IAPs

Inhibitors of apoptotic pathways (IAPs) are cytoplasmic proteins that can inhibit caspases. Survivin, X-IAP, c-IAP1, and c-IAP2 have been most studied for their association with cancer. XIAP overexpression appear to be associated with poor prognosis in children with AML.[7] XIAP antisense molecules have entered clinical evaluation in AML. Survivin is of particular pediatric interest because of association between survivin over expression and poor prognosis in neuroblastoma. Survivin maps to chromosome band 17q25, a region that is often represented by chromosome gain and poor prognosis in neuroblastoma.[8]

Survivin antisense molecule (LY2181308) has entered clinical evaluation. The molecule inhibited tumor growth in xenograft models and sensitized cancer cells to radiation therapy and chemotherapy.[9]

TNF-related Apoptosis Inducing Ligand (TRAIL) Receptor Agonism

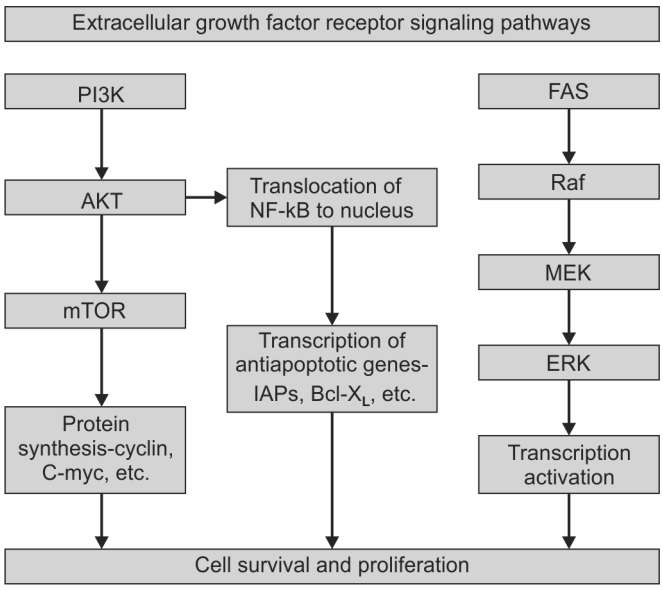
By death receptor pathway, TRAIL induces apoptosis in many cancer cell lines but does not do so for most normal cells. TRAIL-induced apoptosis does not require p53 function, which could allow TRAIL to remain effective against cancer cells that are resistant to chemotherapy and radiation therapy due to loss of p53 function. A recombinant soluble version of human TRAIL, and agonistic antibodies directed towards TRAIL receptors (e.g. mapatumumab) have entered clinical evaluation. Of note, TRAIL induced apoptosis in pediatric-relevent cancer cell lines including those of Ewings sarcoma, rhabdomyosarcoma, and high grade glioma.

Therapies Targeted to Extracellular Survival Signaling Pathways

Growth factor receptor activation can initiate signaling along multiple intracellular pathways that promote proliferation and survival (Flow chart 2). These pathways include:

- Mitogen activated protein (MAP) kinase pathway or extracellular signal regulated kinases (ERKs) cascade is initiated when Ras activation leads to Raf membrane recruitment and activation, followed by activation of MEK and ERK. Activated ERK can translocate to the nucleus and phosphorylate specific transcription factors.
- Growth factor receptor signaling also leads to PI3K and AKT activation. PTEN can reduce the extent of AKT activation, whereas mutations of PTEN resulting in absence of PTEN can lead to constitutive AKT activation.

Flow chart 2 Extracellular survival signaling pathways[5]



AKT plays a central role in promoting survival by phosphorylating multiple proteins involved in survival/apoptosis pathways. AKT activates mTOR, which leads to phosphorylation of eukaryotic initiation factor 4E (eIF4E)-binding protein 1 (4E-BP1) and ribosomal protein S6 kinase 1 (S6K1). Phosphorylation of 4E-BP1 by mTOR frees eIF4E and promotes translation of mRNAs with complex secondary structures in their 5′-untranslated region (e.g. cyclin D1 and c-Myc). Activation of S6K1 leads to increased mRNA translation.

- Growth factor receptor signaling can also lead to NF-κB pathway activation, in part through AKT phosphorylation and activation of IkB kinase (IKK), which leads to IkB phosphorylation and freeing of NF-κB to translocate to the nucleus.
- STAT pathway can also promote proliferation and survival of following growth factor receptor activation.

The importance of these signaling pathways in cancer cells is indicated by the variety of activating mutations reported for members of the receptor tyrosine kinase family, including EGFR mutations, translocations resulting in platelet-derived growth factor receptor (PDGFR) activation, KIT mutations in gastrointestinal stromal tumors, and FMS like tyrosine kinase-3 (FLT3) mutations in AML. Activating mutations in cancer cells also occur in downstream signaling pathways, as exemplified by mutations of PTEN (which results in AKT activation) in many cancer types and mutations of B-Raf in melanoma.

EGFR Inhibitors

EGFR family includes four members: EGFR/human epidermal growth factor receptor 1(HER1), HER2 (ErbB2), HER3 (ErbB3), and HER4 (ErbB4). Binding of either EGF or transforming growth factor alpha (TGF-α) to EGFR activates kinases and initiate signaling cascades.

Small molecule inhibitors (gefitinib and erlotinib) and monoclonal antibodies (cetuximab) are the EGFR blocking agents most evaluated clinically. Gefitinib is a well-tolerated oral EGFR-tyrosine kinase inhibitor, improved disease-related symptoms and induced radiographic tumor regressions in patients with NSCLC persisting after chemotherapy.[10] Use of erlotinib in advanced nonsmall cell lung cancer demonstrated survival advantage.[11] A phase 1 study of gefitinib by COG[12] demonstrated that the drug is well tolerated in pediatric patients at oral doses 150 to 500 mg/m^2. Skin rash, anemia, diarrhea, nausea, and vomiting were common side effects. Preliminary evidence of activity was noted in Ewing's sarcoma, CNS tumors and Wilm's tumor. Gefitinib was shown to have synergistic action with topotecan or irinotecan and additive action with cyclophosphamide in neuroblastoma cell lines.[13]

Cetuximab was approved by FDA for use in combination with irinotecan (or as a single agent if patients cannot tolerate irinotecan) for patients with advanced EGFR-expressing colorectal cancer that is refractory to irinotecan-based chemotherapy.[14] A case report by Grisanti S et al[15] showed anticancer activity of cetuximab against recurrent and metastatic mucoepidermoid carcinoma of salivary gland. The report also revealed inability of cetuximab to cross the blood-brain barrier and the consequent development of CNS metastases during treatment.

Lapatinib is a small molecule reversible tyrosine kinase inhibitor of both EGFR and HER-2. Objective responses have been observed in patients with HER-2 overexpressing breast cancer.[16]

Trastuzumab is a humanized antibody that targets the extracellular domain of HER-2 over expressing breast cancer and newly diagnosed metastatic osteosarcoma. Trastuzumab is approved by FDA for use as monotherapy in patients with HER-2-overexpressing metastatic breast cancer. A concern with the use of trastuzumab is increased incidence of cardiac dysfunction.

KIT and PDGFR Inhibitors

KIT (CD117) and PDGF receptors, along with FLT3, are members of PDGF receptor subfamily of receptor tyrosine kinase. Binding to their ligand activate tyrosine kinase and subsequent signaling pathways.

Imatinib, sunitinib and masitinib are small molecule inhibitors of KIT and PDGFR, with imatinib having more widely accepted additional activity as bcr/abl kinase inhibitor. Imatinib has a well established role in CML and Ph+ ALL with bcr/abl kinase activation. Tyrosine

kinase activating mutations are important as predictors of single agent response to imatinib. Gastrointestinal stromal tumors typically express KIT with gain of function mutation and imatinib is effective in blocking signaling from these mutant receptors. Sunitinib may be active in imatinib resistant GISTs. Masitinib (AB1010), is a novel, potent and selective tyrosine kinase inhibitor targeting KIT that is active, orally bioavailable *in vivo*, and has low toxicity.[17] PDGFR and KIT signaling may play roles in the growth and survival of some pediatric cancers.[18] Approximately 60 percent of pediatric AMLs express KIT. High grade gliomas, sarcomas and neuroblastoma also express PDGF subfamily receptors, but the concentrations required for growth inhibition exceeded those required for KIT and PDGFR inhibition by 20-fold, suggesting that targets other than KIT and PDGFR may be responsible for imatinib's effect against these cell lines.

FLT3 Inhibitors

FLT3 is expressed primarily on hematopoietic and neural tissues. Activating mutations in FLT3 have been observed in adult and childhood AML, in children with hyperdiploid ALL and in infants with ALL with MLL rearrangements.[19] CEP-701, PKC-412, sunitinib and sorafenib are small molecule tyrosine kinase inhibitors which already entered clinical evaluation. Although the clinical responses to FLT3 inhibitors are somewhat encouraging, true clinical benefit will require additional measures to enhance the magnitude of these responses, including evaluation of regimens that combine an FLT3 inhibitor either with standard chemotherapy agents or with other cell signaling inhibitors.[20]

Src Family Kinase Inhibitors

Src family kinases (SFKs) have a critical role in cell adhesion, invasion, proliferation, survival, and angiogenesis during tumor development. Dasatinib is an orally available small-molecule multikinase inhibitor. It potently inhibits BCR-ABL and SFKs, but also inhibits c-KIT, PDGFR, and ephrin receptor kinase. Dasatinib is about 300 times more potent than imatinib in cells expressing unmutated BCR-ABL *in vitro*, and have good CNS penetration. It effectively inhibits the growth of leukemic clones harboring all known imatinib-resistant BCR-ABL kinase domain point mutations, with the exception of V299L, T315I, and F317L mutations. Dasatinib is approved for the treatment of patients with BCR-ABL-positive CML and ALL, resistant or intolerant to imatinib. It has been used at doses of 100 mg/m^2/day in chronic phase of CML, 140 mg/m^2/day in ALL and accelerated/crisis phase of CML in adults, and 60 to 160 mg/m^2/day in children. Porkka et al[21] demonstrated promising therapeutic potential of dasatinib in managing intracranial leukemic disease and substantial clinical activity in patients who experience CNS relapse while on imatinib therapy.

Mammalian Target of Rapamycin (mTOR) Inhibitors

Mammalian target of rapamycin (mTOR), a serine/threonine kinase that is ubiquitously expressed in mammalian cells, is an important regulator of cell growth and proliferation in response to external factors (e.g. growth factors) and nutritional conditions, through its downstream effectors, 4EBP1 and S6K. Inappropriate mTOR activation has been implicated in the pathogenesis of numerous tumor types. The largest body of clinical experience with mTOR inhibitors is in the solid organ transplant setting. Rapamycin (sirolimus), temsirolimus, ridaforolimus, and everolimus are the mTOR inhibitors, which are being studied in cancer patients. Temsirolimus is a pro-drug, and its primary active metabolite is rapamycin (sirolimus). Temsirolimus is approved by the FDA for the treatment of advanced renal cell carcinoma (RCC). It is administered intravenously on a once-weekly schedule. Ridaforolimus is also administered intravenously on an intermittent schedule, although an oral formulation is currently being evaluated in sarcoma. Everolimus is an orally available mTOR inhibitor that is typically administered on a continuous daily schedule or on a weekly schedule in combination regimens. Everolimus has recently obtained FDA approval for the treatment of advanced RCC after failure of treatment with sunitinib or sorafenib.[22] Reversible leukopenia, thrombocytopenia, and dose-dependent hyperlipidemia have been the principal toxicities associated with rapamycin and everolimus in the transplant setting. Preclinical studies in pediatric setting have shown activity of rapamycin against a number of pediatric malignancies, including ALL, rhabdomyosarcoma, osteosarcoma, medulloblastoma, and Ewing's sarcoma. Preclinical observations that the combination of rapamycin and tyrosine kinase inhibitors (e.g. FLT3 or Bcr-Abl inhibitors) showed enhanced activity *in vivo* against leukemias provide rationale for exploring such combinations in the pediatric setting.[23]

Histone Deacetylase Inhibitors

Histones are a family of nuclear proteins that interact with DNA, resulting in DNA being wrapped around a core of histone octamer within the nucleosome. Acetylation of selected lysine residues plays a key role in controlling the function of many proteins, including histones. The level of protein acetylation is maintained by the counterbalancing actions of histone acetyltransferases (HATs) and histone

deacetylases (HDACs). Histone acetylation alters chromatin structure and induces a local chromatin environment conducive with gene transcription, whereas histone de acetylation is commonly associated with repression of transcription.

Through histone hyperacetylation-mediated changes in chromatin conformation and gene expression, histone deacetylase (HDAC) inhibitors induce differentiation, cell cycle arrest, apoptosis, growth inhibition and cell death, which are more pronounced in transformed cell-lines than in normal cells. Additional anti-cancer effects of HDAC inhibitors include inhibition of migration, invasion and angiogenesis *in vivo.*

Preclinical data have demonstrated the efficacy of various HDAC inhibitors as anticancer agents, either as monotherapies or in conjunction with other treatments such as chemotherapy, biologic therapy, or radiation therapy. Vorinostat and depsipeptide, two actively studied HDAC inhibitors, were recently approved by the FDA for the treatment of refractory cutaneous T-cell lymphoma. Other inhibitors, for example, belinostat (PXD101), PCI-24781, ITF2357, MGCD0103, MS-275, valproic acid and panobinostat (LBH589) have also demonstrated therapeutic potential. It is noteworthy that ITF2357 showed significant anti-Hodgkin's lymphoma activity. Panobinostat showed consistent antileukemic effects. Belinostat appears to be promising for treating low malignant potential ovarian tumor. The combination of demethylating agents, valproic acid, and all transretinoic acid (ATRA) has significant clinical activity in leukemia and MDS. Epigenetic agents in combination regimens for cancer therapy are being actively studied.[24]

Role of valproic acid in infant spinal glioblastoma needs further evaluation as a case report showed decrease in the size of the tumor and improvement of symptoms with the use of sorafenib plus valproic acid.[25] Preclinical studies have shown activity of HDAC inhibitors in some pediatric tumors including neuroblastoma (with ATRA), medulloblastoma, Ewings sarcoma and Burkitt's lymphoma.

Protein Farnesyl Transferase Inhibitors

Ras is an important anticancer target, but intracellular ras signaling requires its association with the cell membrane, which in turn requires a post-translational addition of farnesyl or geranyl group at carboxy terminal cysteine residue, a process known as prenylation. There are two forms of prenylation-farnesylation by farnesyltransferase, and geranylation by geranylgeranyl transferase. Farnesylation is the dominant class of post-translational modification required for proper intracellular localization of RAS to the inner surface of the cell membrane. Farnesyl transferase inhibitors (FTIs) were developed to inhibit this process and thus interfere with the function of RAS. One such FTI is tipifarnib (R115777), which is currently being evaluated in acute leukemias, juvenile myelomonocytic leukemia, pediatric brain tumors, and neuroblastoma. Goemans et al identified T-cell ALL and AML-M5 as the most sensitive subset of pediatric acute leukemia.[26] Oral tipifarnib is well tolerated in children receiving the drug twice daily for 21 days and a continuous dosing schedule at 200 mg/m^2/dose, which is equivalent to the maximum tolerated dose (MTD) in adults. The pharmacokinetic profile of tipifarnib in children is similar to that in adults.[27]

Proteosome Inhibitors

The 26S proteosome regulates the degradation of many proteins involved in cell cycle control, apoptosis, and tumor growth. The inhibition of the proteosome by specific inhibitors, which results in stabilization of tumor suppressor proteins IkB, p21 and p53, is a viable target for antitumor therapy. Most prominently, the proteosome inhibitor bortezomib was approved by the FDA for the treatment of relapsed or refractory multiple myeloma in adults, and is presently considered for pediatric malignancies such as leukemias, lymphomas, neuroblastoma, rhabdomyosarcoma, and Ewing's sarcoma. The first clinical trials by the Children's Oncology Group (COG) were conducted with bortezomib for the treatment of refractory solid tumors and refractory leukemia. Bortezomib is well tolerated in children with recurrent or refractory solid tumors and leukemia. The recommended phase II dose of bortezomib for children was 1.2 mg/m^2/dose, administered as an intravenous bolus twice weekly for 2 weeks followed by a 1-week break. Thrombocytopenia was dose limiting toxicity.[28]

Angiogenesis Inhibitors

Although angiogenesis is a complex process involving many factors, VEGF appears to be rate limiting in normal and pathologic blood vessel growth. Therapeutic approaches to targeting this angiogenic pathway include antibodies directed against VEGF, antibodies directed against VEGF-R2, small molecule inhibitors of VEGF-R2, and interference of integrin-matrix interactions. Trials are currently underway to evaluate several antiangiogenesis agents, including SU5416, bevacizumab, TNP-470, thalidomide, SU6668, ZD4190, ZD6474, and PTK787. Bevacizumab is a monoclonal antibody that inhibits a single isoform of the VEGF ligand, VEGF-A. It has FDA approval for administration as second line treatment of metastatic carcinoma of the colon or rectum. It is also approved in the USA and Europe for the first-line treatment (in combination with interferon) of advanced RCC.[29]

Thalidomide has antiangiogenesis and other biologic activities. It was approved by FDA for erythema nodosum leprosum and multiple myeloma. Lenalidomide is active against multiple myeloma and is being tried in childhood refractory solid tumors/brain tumors. Sunitinib and sorafenib are VEGFR small molecule inhibitor which significantly prolonged progression free survival in patients with advanced RCC.

Cytokines

Active immunotherapy with cytokines such as interferons (IFNs) and interleukins (ILs) is a form of nonspecific active immune stimulation. The cytokines have been tested as therapies for many hematologic and solid neoplasms and have demonstrated therapeutic benefits in various cancers. To date, only two cytokines have achieved approval for cancer. IL-2 for the treatment of metastatic melanoma and renal cell carcinoma, and IFN-alpha for the adjuvant therapy of stage III melanoma.[30]

Interferons

Interferons (IFNs) are a group of glycoproteins that are produced by a variety of cells stimulated by viral antigens and mitogens. There are three types of interferons produced by a variety of cells. IFN-alpha is produced by macrophages and lymphocytes, IFN-beta is produced by fibroblasts and epithelial cells, whereas IFN-gamma is produced by $CD4^+$, $CD8^+$, natural killer (NK) cells, and lymphokine-activated killer (LAK) cells. IFNs have antiproliferative, immunomodulatory, apoptotic inducing, and antiangiogenesis activities.[31]

In a cooperative group multi-institutional clinical trial, stage III melanoma patients were treated with 1 year of IFN-alpha-2b. An overall improvement in median relapse-free survival from 1 to 1.7 years and in median overall survival from 2.8 to 3.8 years was reported.[32]

Based on initial clinical trials data, IFN-alpha was approved by the FDA for the treatment of hairy cell leukemia (HCL). Despite, the initial enthusiasm, a large number of patients developed relapse after discontinuation of therapy. The introduction of nucleoside analogues, with a complete response rate close to 90 percent, has relegated IFN therapy to second-line treatment in patients who have refractory disease or in those with contraindications to nucleoside analogs.

Interferon-alpha has also been tested in patients who have CML, and preliminary trials suggested that complete hematologic responses were possible in more than half of patients who had CML, with complete cytogenic responses in nearly 25 percent. Prospective randomized trials documented the superiority of IFN-alpha over chemotherapy.[33] Patients with Kaposi's sarcoma with mucocutaneous or asymptomatic visceral involvement and patients with follicular lymphoma have also been shown to benefit from IFN-alpha.

Constitutional symptoms are quite common in patients receiving IFN therapy, and are likely to occur in 80 percent or more of patients. These typically consist of fever, fatigue, headaches, and myalgias. More serious are the neuropsychiatric issues, which include depression (45%), confusion (10%), and mania (1%). Close monitoring of patient mental status or prophylactic use of antidepressants can reduce the risk for these side effects. Gastrointestinal side effects, myelosuppression, autoimmune thyroid dysfunction are among the other significant toxic effects.[34]

Interferon-gamma has been shown to enhance DNA fragmentation and cytotoxicity caused by tumor necrosis factor.[35] Daily subcutaneous recombinant gamma-IFN can be easily administered on an outpatient basis with minimal local skin toxicity, results in prolonged serum levels, and is associated with immunological changes of potential antitumor significance.[36]

Interleukins

IL-2 is a glycoprotein produced by mature T-lymphocytes during an immune response after receiving a signal from an antigen-presenting cell (APC). IL-2 increases HLA-restricted cytolytic activity of cytotoxic T-lymphocytes and NK cells. Furthermore, the activation and expansion of lymphocyte-activated killer (LAK) cells, which are a mixture of NK cells and CD4/CD8 T cells, is responsible for HLA-unrestricted killing of all tumor cell lines. The IL-2 also have regulatory effect on immune response through activation of regulatory T cells. The balance between effector T cells and regulatory T cells may be critical for influencing the rejection or acceptance of tumors.

IL-2 is a promising immunotherapeutic agent for the treatment of metastatic melanoma, acute myelogenous leukemia, and metastatic renal cell carcinoma. While high-dose IL-2 regimens have shown clinical benefit in the treatment of melanoma and renal cell carcinoma, serious dose-limiting toxicities have limited their clinical use in a broader group of patients. The toxicity profile of IL-2 is largely associated with a capillary leak syndrome. In addition, IL-2 can cause constitutional symptoms (e.g. fever, chill, fatigue) and gastrointestinal side effects, pulmonary edema, cardiac arrhythmias, myocarditis, reversible renal and hepatic dysfunction, pruritus, electrolyte abnormalities, thrombocytopenia, anemia, and coagulopathy. Although early studies with IL-2 reported a 2 percent mortality rate that was generally

related to gram-positive sepsis, current IL-2 centers that routinely use prophylactic antibiotics report no mortality. Low dose and combination regimens have been tried to reduce the toxicity, but these attempts were mostly disappointing. The addition of IL-2 to chemotherapeutic regimens (biochemotherapy) has been associated with overall response rates of up to 60 percent in patients with metastatic melanoma, but this has yet to be translated into a confirmed improvement in survival. It remains to be determined whether further modifications of IL-2-based regimens or the addition of newer agents to IL-2 will produce better tumor response and survival.[37]

IL-12, IL-15, IL-18, IL-21, IL-23 and GM-CSF are among the other cytokines under investigation for their antitumor activity.

Tumor Vaccines

Current cancer vaccines aim to improve tumor-antigen presentation and host T-lymphocyte activation. This is done by enhancing antigenic peptide-MHC molecule stability, by restoring costimulatory signals, and by amplifying recruitment of the host's immune effector cells. Some clinical trials used autologous or allogeneic tumor cells genetically modified to express immunostimulatory molecules, and some of the trials used tumor cells admixed with nonspecific adjuvant or soluble cytokines (adjuvant-specific immunotherapy) like BCG, IFN, or GM-CSF. Other approaches have used vectors encoding immunomodulatory gene products or tumor antigens directly administered *in situ* in the tumor, with limited efficacy. Overall, the toxicity of these approaches is minimal. Some studies report a rise in the frequency of precursors to cytotoxic T cells specific to the tumor cells, but the relevance of this finding is not established. Cytotoxic T cells capable of lysing tumor cells *ex vivo* have been isolated from fresh peripheral blood in some patients after vaccination, but the correlation of these findings to the clinical response is not obvious.[38]

The major advance in T-cell based immunotherapy in last few years has been the molecular definition of a number of tumor antigens that can be targeted by T cells. One family of tumor antigens can be described as differentiation antigens, with most defined being melanoma associated differentiation antigens. Several new differentiation antigens have been called "universal" tumor antigens because they are expressed in vast majority of tumors, including childhood cancers. For example, telomerase expression in Ewing's sarcoma, and survivin expression in Ewing's sarcoma, osteosarcoma and neuroblastoma. Another family of tumor antigens called as cancer-testis antigens (so called because of their expression in cancer cells and normal testis), have widespread expression in pediatric cancers including gliomas, medulloblastoma, neuroblastoma and Ewing's sarcoma. The forth group of tumor antigens include mutated forms of normal "self" molecules. For example, breakpoint region of translocation in leukemias represent noval epitope that do not exist in normal tissues and hence, may be susceptible to immune targeting. Finally, viral antigens also provide potential tumor targets particularly relevant to EBV associated tumors in children.[39]

In summary, although cancer vaccines have shown limited success so far, the barriers to inducing effective tumor immunity are rapidly being defined. Future challenges include investigating whether immunotherapy is most effective as a combinatorial therapy with surgery, chemotherapy and/or radiation therapy.[30]

SUMMARY

Although current treatment approaches in children with cancer are highly successful, we need new treatment approaches for a significant number of unresponsive and relapsed cases. On the other hand, it is very difficult, rather unethical, to introduce experimental therapy in an already successful standard regimen. Furthermore, effect of a new modality is difficult to quantify in a patient receiving established treatment regimen or in a heavily pretreated patient. Because of these problems, most of the studies have been done in adult cancer patients. Till date, the success of biological therapy is limited but significant. With more and more understanding of molecular pathology, biological response modifiers may have more defined role in pediatric cancer treatment.

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