Thalassemia and Other Hemolytic Anemias

Edited by Isam AL-Zwaini

THALASSEMIA AND OTHER HEMOLYTIC ANEMIAS

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Contributors

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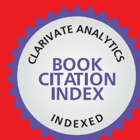

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Meet the editor

Prof. Isam Jaber AL-Zwaini was born on January 4 1963, Baghdad, Iraq. After graduating from AL-Mustansiryia College of medicine in 1987, he worked as a house officer in different hospitals in Baghdad for 15 months followed by military service for 3 years. He started his pediatric study in 1991 and gained the Fellowship of Iraqi Commission for Medical Specializations in 1996. He started his work as a lecturer in the department of pediatrics, AL-Anbar Medical College from 1996 to 2001 when he upgraded to the degree of assistant professor. In 2005, he started working in the department of pediatrics, AL-Kindy Medical College, University of Baghdad, and upgraded to the degree of professor in 2008. He received the associate membership of the Royal College of Paediatrics and Child Health, UK, in 2007. He has been head of the pediatric department in AL-Anbar and AL-Kindy Medical College for many years. He published more than 30 scientific papers in different pediatric fields. He has a special interest in pediatric hematology, neurology and nutrition.

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Preface

Thalassemia is a very common disease first described by pediatrician Thomas Benton Cooley in 1925 who described it in a patient of Italian origin. At that time, it was designated as Cooley's anemia. A Nobel prize winner, George Hoyt Whipple, and a professor of pediatrics, Bradford, at the University of Rochester in 1936 coined the name thalassemia, which in Greek means anemia of the sea (Thalassa means sea and emia means blood) because it is very common in the area of the Mediterranean Sea. This name is actually misleading because it can occur everywhere in the world. Thalassemia is not a single disease; it is rather a group of hereditary disorder of the production of globulin chain of the hemoglobin. Throughout the world, thalassemia affects approximately 4.4 of every 10,000 live births. It represents a major social and emotional impact on the patient and his family and a major burden on health services where the prevalence is high. I am, therefore, proud to present this book that includes very interesting chapters that cover various aspects of thalassemia.

The first chapter is an introductory chapter by the editor in which an overview of thalassemia is given. Epidemiology, genetic bases of thalassemia, and types of hemoglobin, consequences of thalassemia, and the future hope for the cure are overviewed.

The second chapter by Dr. Özgür Aldemir presents a framework for understanding the genetic aspect of thalassemia from diagnosis to treatment. The recent genetic studies have successfully characterized the key variants and pathways involved in HbF regulation, providing new therapeutic targets for HbF reactivation. According to the current literature, using *Lentivirus* vector for gene therapy and genome editing-based treatment strategies for b-thalassemia and SCD is discussed and well documented. New treatments are struggling with developing gene therapy of b-thalassemia include pharmacological or disruption of BCL11A erythroid enhancer by CRISPR-CAS9 technology besides zinc finger or transcription activators like effector nuclease or attempts at repairing the defective b-globin gene in HSC by genome editing. These approaches are successful; gene addition has the advantage of making use of a single product applicable to all cases of b-thalassemia.

The third chapter by Dr. Lingwen Zeng and Dr. Luxin Yu is dedicated to the new technique for thalassemia gene detection using isothermal nucleic acid amplification, which is a simple process that rapidly and efficiently accumulates nucleic acid sequences at a constant temperature such as 37°C and 42°C. Isothermal nucleic acid amplification approach offers several advantages over temperature circle methods (such as PCR) including rapid assay results, cost-effectiveness, and portability. Two detection approaches based on circular strand-displacement polymerization reaction (CSDPR) were presented in this chapter for sensitive and specific thalassemia gene detection. One is a lateral flow strip biosensor based on CSDPR for

semiquantitative detection of thalassemia DNA. The other is a spectrophotometric DNA detection approach based on CSDPR for quantitative detection of thalassemia DNA.

The fourth chapter by Dr. Abdullah Tuli and Dr. Ebru Dündar Yenilmez deals with invasive and noninvasive approaches in prenatal diagnosis of thalassemia. Prenatal diagnosis of thalassemia is the essential part of preventive medicine and is currently dependent on the use of invasive diagnostic tests within the first 2 months of pregnancy by molecular diagnostic methods using PCR techniques. Alternative method using cell-free DNA from a maternal blood sample is available, and it eliminates the small but significant risk of fetal loss of about 1% associated with the invasive diagnostic test.

The fifth chapter by Dr. Alice Ioana Albu and Dr. Dragos Albu highlights the problem of hypogonadism in female patients with beta-thalassemia. Thalassemia is a systemic disease with two major problems, namely, anemia and iron overload. Every system in the body might be affected including the endocrine glands. Hypogonadotropic hypogonadism is the most frequent endocrine complication, presenting as amenorrhea, delayed puberty, and infertility. Recent progress in the treatment of BTM dramatically improved life expectancy and quality of life of these patients. As a consequence, the concern for fertility and pregnancy gained importance and became a subject of interest. The chapter gives a review of the available data regarding the prevalence, physiopathology, consequences, and treatment of hypogonadism in female patients with BTM.

The sixth chapter by Dr. Mohamed Ramadan El-Shansory et al. discusses the problem of hepatitis C in thalassemia. As thalassemic patients are blood transfusion dependent, they are subjected to the high rate of blood-borne infections especially hepatitis C virus infection. Seroprevalence rates of 10–20% have been reported among children who received blood products for conditions such as thalassemia. All persons with positive anti-HCV antibody tests must undergo additional testing for the presence of the HCV itself using polymerase chain reaction (PCR). Recently, the FDA approved the first direct-acting antiviral agents (DAAs) for children that included sofosbuvir and ledipasvir/sofosbuvir in the adult dose, 90 mg/400 mg, to treat HCV in children and adolescents aged 12 years and older or weighing at least 35 kilograms. Similarly, giving half the adult fixed dose of ledipasvir/sofosbuvir, 45 mg/200 mg, to children aged 6–11 years is still under clinical trials with promising results.

The seventh chapter by Dr. Karen Cordovil gives an overview on sickle cell disease, the second important disorder of hemoglobinopathies. A structural and monogenetic genetic disorder due to a mutation that occurs in the globin β-chain results in the formation of hemoglobin S (Hb S), a protein composed of two normal and two β-type mutant chains. All over the world, the prevalence of sickle cell disease is 4.4%. The difficulty in circulating the sickle cell, its interaction with endothelial cells, leukocytes, platelets, endothelial dysfunction, and the abnormal expression of adhesion molecules permeate the beginning of the blood vessel occlusion process as well as pathophysiological aspects of SCD. Among the secondary complications are stroke, pulmonary hypertension, leg ulcer, renal disorders, and all complications associated with vascular dysfunction. Clinical and biochemical markers of disease severity can be used to predict risk, prevent complications, and increase the expectation and quality of life of the SCD population.

Dr. Takeshi Sugimoto in the *eighth chapter* discusses anti–red blood cell antibody and antiglobulin test. The direct antiglobulin test is a useful clinical examination tool for the diagnosis of autoimmune hemolytic anemia; however, false-positive or false-negative results are

sometimes detected. Some pitfalls about diagnoses of autoimmune hemolytic anemia are presented in this chapter.

Finally, I hope this book with its interested chapters will shed light on some of the interesting aspects of this important disease. I would like to thank all authors who contributed with their chapters and for their patience and cooperation throughout the processing of the book. Also, I would like to present my in-depth thanks and gratitude for the InTech personnel, especially Ms. Kristina Kardum who offered me the opportunity to be the editor of a book that deals with a subject in the field of my interest and that I spend many years of my life as a head of its center in one of the governorates in my country, Iraq.

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Section 1

Introduction

Chapter 1

Introductory Chapter: Thalassemia - An Overview

Isam Jaber Al-Zwaini

Additional information is available at the end of the chapter

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1. Introduction

Thalassemias are a heterogenous group of genetic disorders, transmitted as autosomal recessive inheritance, in which the rate of hemoglobin production is partially or completely suppressed due to the reduced rate of synthesis of α- or β-chain, the two chains of adult hemoglobin (Hb A) [1]. The molecular defects that cause thalassemia are located within the human globulin gene which encodes for alpha and beta globulin polypeptide chain of hemoglobin on chromosome numbers 16 and 11, respectively. These genetic molecular defects in thalassemia lead to reduced or no production of one globulin chain with the excess of the other resulting in alpha thalassemia when the alpha chain is affected and beta thalassemia when the beta chain is affected [2]. In thalassemia, the imbalance of globin chain synthesis leads to red cell damage resulting in destruction of red cells in the marrow (ineffective erythropoiesis) and peripheral circulation (hemolysis) [3]. In addition to pediatrician and hematologist, family physician and general practitioner need to know what thalassemia is, how it is diagnosed and differentiated from other hypochromic microcytic anemias, and what are the principles of treatment and prevention.

2. Epidemiology

Classically, thalassemia affecting population across thalassemic built which extends from the Mediterranean region through Middle East and Sub-Saharan Africa to South and Southeast Asian countries. Nowadays, thalassemia occurs all over the world because of continual migrations of population from these areas to western countries. Thalassemia affects both sexes equally, occurring approximately in 4.4% of every 10,000-live birth [4] and accounting for about 60,000–70,000 children each year who born with different types of thalassemia [5].

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3. Genetic bases and hemoglobin types

In the embryonic life, blood in the blood islands of the yolk sac produces specific types of hemoglobulin called Gower 1 composed of two zeta chains and two epsilon chain (ξ2ε2), Gower 2 composed of two alpha chains and two epsilon chains (α2ε2), and Portland hemoglobulin composed of two zeta chains and two gamma chains (ξ2γ2). In the first trimester, definitive hematopoietic stem cell emerges from the ventral wall of the dorsal aorta which then migrates to fetal liver where fetal hemoglobin is produced (Hb F). Around the time of birth, hematopoietic stem cell migrates to the bone morrow which will become the main site of adult hemoglobin production for the rest of life. The switching from embryonic to fetal and then to adult hemoglobin occurs as a result of well-coordinated developmental stage due to specific expression of globin genes in Alpha and Beta globin loci [6]. Each type of hemoglobin composes of four globin chains. Fetal hemoglobin has two alpha and two gamma chains ($\alpha_2\gamma_2$) accounting for approximately 80% of hemoglobin at birth, while adult hemoglobin (Hb A1) has two alpha and two beta chains ($\alpha_2\beta_2$) constituting the remaining 20%. While the

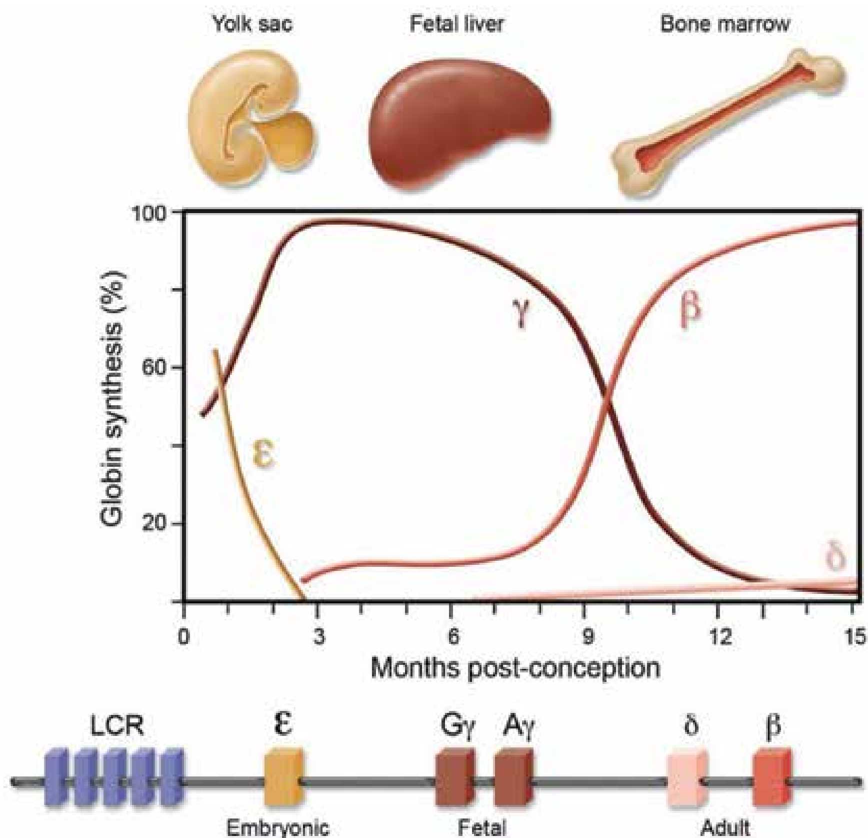

Figure 1. Embryonic, fetal, and adult hemoglobin [7].

switch on transition from gamma chain to beta chain production after birth, the hemoglobin A1 gradually replaces hemoglobin F, and at 6 months of age, Hb A1 constitutes about 97% and Hb F about 1%. The remaining small amount is of Hb A2 composed of two alpha and two delta chains ($\alpha_2\delta_2$) (**Figure 1**).

In alpha thalassemia, reduced or no production of alpha globin chains is usually caused by deletion of one or more of the genes responsible for its production. Deletion of one gene results in alpha thalassemia carrier state in which the patient is asymptomatic with normal hematological findings. Deletion of two genes results in alpha thalassemia trait or alpha thalassemia minor in which the patient has no or very mild anemia but with microcytosis. On the other hand, deletion of three genes results in significant production of hemoglobin H consisting of four beta chain (β_4), also called alpha thalassemia intermedia, which usually presents with hemolysis, microcytosis, and splenomegaly, while the deletion of the four genes results in the production of significant amount of hemoglobin Bart which consists of four gamma chain (γ_4); Bart's hemoglobin, also called alpha thalassemia major, definitely will result in hydrops fetalis.

Regarding the more common type of thalassemia, B thalassemia, more than 200 mutations, and rarely deletions, of the two genes responsible for the production of B chain can be criminated. In B thalassemia, the production of beta chain can range from normal to completely absent resulting in variable clinical presentations. In the one-gene defect, the patient has thalassemia minor, also called trait, in which the patient is asymptomatic apart from mild anemia (hemoglobin level is 2–3 g below the average of his age) and microcytosis. This is commonly mistaken as the more common iron deficiency anemia. If both genes are affected resulting in no or reduced production of beta chain, the patient has thalassemia major (when it is absent) or thalassemia intermedia when it is reduced. Thalassemia major is usually asymptomatic at birth because at this age, normally, the hemoglobin F is predominant. At 6 months of age, the patients present with severe anemia and hepatosplenomegaly. In patients with reduced production of beta chain, clinical spectrum will be beta thalassemia intermedia in which the patient will suffer less severe symptoms with the occasional requirement for blood transfusion and he enjoys long life survival.

4. Consequences

The two major consequences of thalassemia major are severe anemia, resulting from ineffective erythropoiesis, and iron overload resulting from regular blood transfusion and also increasing the rate of iron absorption in small intestine. Almost all complications of thalassemia major can be attributed to these two factors. Examples of these complications include skeletal changes, hepatosplenomegaly, growth retardation, multiple endocrine failure, and cardiac problem. Additionally, blood transfusion for anemia and chelation therapy for iron overload represent a major burden on the patient and his family and on the health resources where its prevalence is high. The only hope for children implicated with thalassemia major is bone marrow transplantation when it is done early in life before complications occur.

5. Hope for cure

Hematopoietic stem cell transplantation represents the only curative approach for those unlucky patients with thalassemia. Hematopoietic stem cells from a healthy donor replace the affected bone marrow and restore the normal hematopoiesis. The success rate is inversely correlated with the degree of iron overload and hepatic damage. The availability of healthy acceptable donor is another problem especially in societies with small family size as in China [8].

In areas where thalassemia rate is high, determination of carrier rate by population screening and prenatal diagnosis associated with genetic counseling is very useful by allowing couples at risk to decide about their reproductive choice after explaining the nature of the disease and the associated risk of having an affected child. It is clear that the only way to stop thalassemia is to prevent the birth of affected child by making the premarital screening tests for thalassemia carrier state compulsory for the general population and providing counseling for the affected families. Implementation of effective program for thalassemia control adopted by health authority with the help of international agencies like World Health Organization is fundamental for effective control of thalassemia in countries where its prevalence is high.

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Section 2

Thalassemia - Genetic Bases

Chapter 2

The Genetic Aspect of Thalassemia: From Diagnosis to Treatment

Özgür Aldemir

Additional information is available at the end of the chapter

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Abstract

Hemoglobinopathies are a group of red blood cell productivity disorders, including α-thalassemia, β-thalassemia, and sickle cell disease (SCD), that are autosomal recessive and very common in Mediterranean, Middle Eastern, East Asian, and African countries. Thalassemia presents with the following clinical signs and symptoms: fatigue, weakness, yellowish skin, facial bone deformities, and abdominal swelling. Genetic studies have successfully characterized the key variants and pathways involved in hemoglobin F (HbF) regulation, providing new therapeutic targets for HbF reactivation. According to the current literature, using lentivirus vector for gene therapy and genome-editing-based treatment strategies for β-thalassemia and SCD have been discussed and well documented. According to current studies, novel treatments are becoming more important for thalassemia patients, because the consequences of supportive treatments are not sufficient for patients and their families. Supportive treatment does not have a positive effect on the survival rate of β-thalassemia patients. New treatments are empowering to develop develop a gene therapy for β-thalassemia and include pharmacological or disruption of BCL11A erythroid enhancer by CRISPR-CAS9 technology in addition to zinc-finger or transcription activator-like effector nuclease, and attempts at repairing the defective β-globin gene in hematopoietic stem cells by genome editing. These approaches are needed to improve for being more successful; gene addition has the advantage of making use of a single product applicable to all cases of β-thalassemia.

Keywords: α-thalassemia, β-thalassemia, SCD (sickle cell disease), HSC (hematopoietic stem cell), lentiviral and CRISPR-CAS9 technology, HSPCs

1. Introduction

β-Thalassemia is the most common inherited hemoglobinopathy in the world and is on the increase in Mediterranean, Middle Eastern, and Asian populations. β-Thalassemia is a common

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genetic disease caused by the coinheritance of two mutant β-globin alleles [1]. The β-thalassemias are inherited in an autosomal recessive disease. After fertilization, each patient has a 25% chance of being affected, a 50% chance of being an asymptomatic carrier, and a 25% chance of being unaffected and not a carrier. Heterozygotes (carriers) may have moderate phenotype and anemia but they clinically have no symptoms. Three clinical and hematological forms of increasing severity are recognized: β-thalassemia carrier, β-thalassemia intermedia, and β -thalassemia major (B-TM). The β-thalassemia carrier form, which results from heterozygosity for β-thalassemia, is clinically nonasymptomatic and is described by specific hematological findings. B-TM is a mild transfusion-dependent anemia. Thalassemia intermedia is known in terms of a clinical and genotypical heterogeneous group of diseases, varying in severity from the asymptomatic carrier to B-TM. The β-globin (HBB) gene maps in the short arm of chromosome 11, in a region including the δ-globin gene, the embryonic ε-gene, the fetal A-gamma and G-gamma genes, and a pseudogene. βThalassemia in the group of thalassemia syndromes is molecularly heterogeneous. Especially, genetic testing can provide important information for diagnosis, treatment, and prevention. There are some limitations to genetic testing in all types of thalassemia; the most important issue is to find the right patient on which to perform genetic testing [2].

Clinical management of B-TM involves routine long life red blood cell transfusions and iron chelation therapy to remove iron introduced in excess with transfusions. β-Thalassemia therapy modalities include curative allogeneic bone marrow or stem cell transplantation and symptomatic iron chelation therapy such as using deferoxamine. Therapies under investigation are the induction of hemoglobin F (HbF) with pharmacologic compounds and stem cell gene therapy [3]. The novel therapies are based on clustered regularly interspaced short palindromic repeats/CRISPR-associated protein (CRISPR-Cas9) system therapy and stem cell gene therapies.

The β-like globin genes are each expressed at distinct stages of development through a process referred to as Hb switching (embryonic → fetal → adult). At 6 months after birth, -HbF ($\alpha2\gamma2$), which comprises <5% of the total Hb, continues to fall reaching the adult level of <1% at 2 years of age, when adult Hb becomes the major Hb, and mutations affect the adult HBB gene. The HBB locus is a paradigm for tissue- and developmental stage-specific regulation; expression of the individual globin genes relies on a timely and direct physical interaction between the globin promoters and the β locus control region (β-LCR), the interaction being mediated through binding of erythroid-specific and ubiquitous transcription factors. The double mechanism has been suggested for developmental expression: (1) gene competition for the upstream β-LCR, presenting an advantage for the gene closest to the LCR [4], and (2) transcriptional suppression of the preceding gene. The ability to take part in β-LCR and transcriptional repression depends on the change in the abundance and list of different transcription factors that favor promoter–LCR interaction. While the ε- and γ-globin genes are autonomously silenced at the proper developmental stage, expression of the adult β-globin gene depends on the absence of competition from the upstream γ-gene for the LCR sequences. According to integrating molecular studies (such as mutational and epigenetic analyses), researchers have characterized a number of cis-regulatory genomic regions in the β-globin locus with a potential role in Hb switching. Naturally occurring deletions encompassing β- and γ-globin genes in the β-globin gene cluster and point mutations in the promoters of global

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genes result in increased HbF expression and a benign situation called hereditary persistence of fetal Hb (HPFH). HPFH point mutations may disrupt binding sites for γ-globin silencers or generate new binding sites for γ-globin activators [5].

β-Thalassemia is characterized by reduced synthesis of the Hb β-chain that results in microcytic hypochromic anemia, an abnormal peripheral blood smear with nucleated red blood cells and diminished amounts of HbA on Hb analysis. Patients with T-BM have profound anemia and enlarged liver and spleen; they mostly come to medical therapy within the first 2 years of life [1]. β-Thalassemia is caused by a spectrum of mutations that results in a quantitative reduction of β-globin chains that are fundamentally normal. β-Globin is encoded by a structural gene found in a cluster with the other β-like genes on chromosome 11. The cluster includes five genes, δ (HBD), β (HBB), ε (HBE), Gγ (HBG2), and Aγ (HBG1), which are organized along the chromosome in order of their development expression to synthesize different Hb tetramers: embryonic Hb Gower-1, Hb Gower-2, Hb Portland, fetal Hb, and adult Hb. Expression of the globin genes is dependent on local promoter sequences as well as upstream β-globin LCR, which consists of five hypersensitive sites distributed between 6 and 20 kb 5′ of HBE gene. All these regulatory regions bind a number of key erythroid-specific transcription factors, remarkably GATA-1, GATA-2, NF-E2, KLF-1, and SCL, as well as various cofactors and factors that are more ubiquitous in their tissue distribution, such as Sp1 [6].

Molecular genetic reports have been effective in describing the crucial variants and pathways involved in HbF regulation, furnishing novel therapeutic molecules for HbF reactivation. BCL11A has been well known as a quantitative repressor, and progress has been made in manipulating its expression using genomic and gene-editing strategies for therapeutic benefits. Studies and understanding in the mechanisms of ineffective and abnormal erythropoiesis have also provided further therapeutic molecules, two of which are now being tested in clinical experiments. BCL11A cooperates with SOX6, GATA1, FOG1, and NuRD complex to repress the expression of γ-globin genes in adult erythroblasts. The expression of BCL11A is regulated by KLF1 that favors fetal-to-adult Hb switching by directly activating β-globin gene expression. KLF1 is a key molecule involved in the γ- to β-globin Hb switching process by different mechanisms. In addition, KLF1 is a direct activator of genes that code repressors targeting recovery from the severity of β-thalassemia. The transcription factor lymphoma-related factor silences γ-globin expression through the NuRD complex. GATA-1 is the main gene regulating erythropoiesis and positively regulates specific erythroid genes such as erythropoietin receptor (EPoR), glycophorin (GpA), and globin chains, and is necessary for megakaryocyte and erythrocyte differentiation [7]. While the interaction of FOG1 with GATA-1 helps maintain erythroid homeostasis and control HBB transcription, KLF1 controls β-globin expression and switching between fetal and adult globin expression [8–11]. Several nuclear factors are believed to provide the Hb switching mechanism, including KLF1, MYB, the stage selector protein, and the nuclear receptors TR2/TR4 and COUP-TFII. The zinc-finger transcription factor BCL11A has a major role in the silencing of δ-globin expression in human cells. BCL11A is thought to exert this function by interacting with the erythroid master regulator GATA1, SOX6, FOG-1, and the NuRD complex (**Figure 1**). BCL11A is essential for the proper development of B cells, and murine BCL11A-deficient hematopoietic stem cells (HSCs) show defects in cell cycle, engraftment, and multilineage differentiation [11].

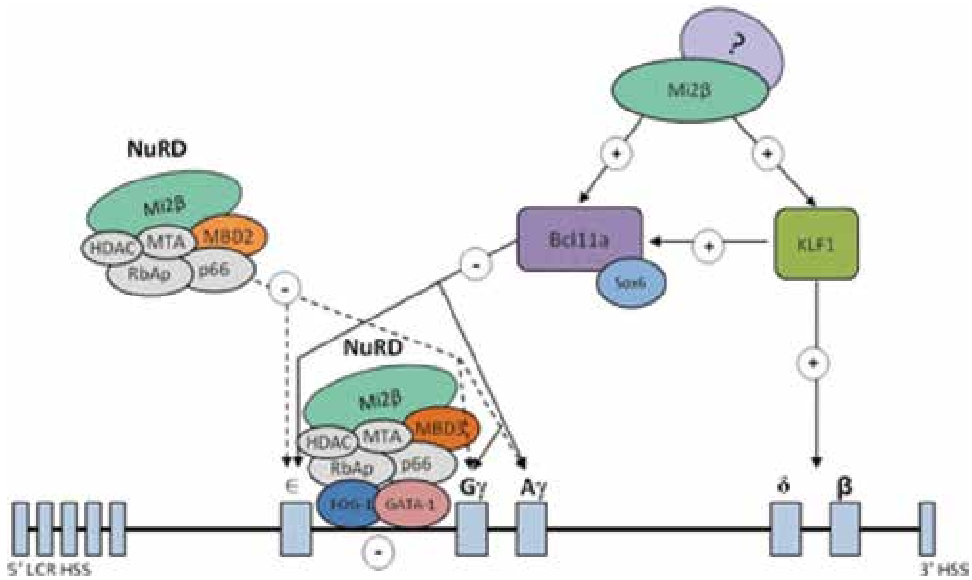

Figure 1. Major players in fetal-to-adult globin switching.

The manifestation of inherited mutations in KLF1 make HPFH suggest that this factor is a practical target for gene therapy and might be achieved by RNA interference technology to design a haploinsufficiency situation. Promising key molecules such as KLF1 and BCL11A for therapeutic efforts aimed at increased HbF level have been determined; however, further preclinical data are necessary before manipulation of transcription factors can be translated into therapeutic choices. According to some study groups, significant advances in the field by defining mechanisms through which BCL11A repressed γ-globin expression hold promise for the development of genome-based therapy in the future [12].

Hb is a tetramer comprised of both α- and β-like polypeptide subunits. According to ontogenesis, the constitution of these subunits varies, leading to assembly of Hb molecules with various physiologic assets. In humans and Old World monkeys, two developmental switches take place for the synthesis of the β-like subunits of the Hb molecule. The initial switch is present in all mammals and involves a switch from Hb subunits expressed entirely in the transiently produced embryonic primitive wave of erythrocytes to the Hb subunits produced in the earliest definition wave of erythrocytes arising from the fetal liver. The Hb switch is well known as the primitive-to-definitive switch at the β-globin locus. It is important to note that at the α-globin locus in humans a similar switch from an embryonic Hb, which is normally restricted to primitive erythrocytes, to the adult α-globin subunits occurs [13].

2. Molecular diagnosis of β-thalassemia

Molecular diagnostic methods are mostly beneficial in giving genetic counseling and during the application of prenatal diagnosis. PCR-based tests are adequate for finding carriers of

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α-thalassemia deletions, and applicable multiplex PCR and multiplex ligation-dependent probe amplification (MLPA) tests are suitable for diagnosing α- and β-thalassemia caused by deletions [14]. Diagnosis of β-thalassemia is more challenging, because of detecting the variety of diseases caused by mutations., Direct DNA sequencing might be the most practical

I. Promoter regulatory elements
(1) CACCC box
(2) ATA box
(3) 5′ UTR (CAP +1 to +45)
II. RNA processing splice junction
(1) IVS1-position 1 and 2
(2) IVS1–3′ end (minor deletion of 17 to 44 bp and insertion of 22 bp)
(3) IVS2-position 1 and 2
(4) IVS2–3′ end
Consensus splice sites
(5) IVS1-position 5
(6) IVS1-position 6
(7) IVS1-positions −3, 128, 129
(8) IVS2–5
(9) IVS2–3′ end cryptic splice sites
(10) IVS1-positions 110, 116
(11) IVS2-positions 654, 705, 745, 837
(12) CD10 (GCC → GCA)
(13) CD19 (AAC → AGC) Hb Malay (Asn → Ser)
(14) CD24 (GGT → GGA)
(15) CD26 (GAG → AAG) (Glu → Lys, HbE)
(16) CD26 (GAG → GCG) (Glu → Ala, Hb Tripoli)
(17) CD27 (GCC → TCC) (Ala → Ser, Knossos)++RNA cleavage—poly A signal.
(18) AATAAA—single base substitutions, minor deletions; others—in 3′ UTR.
(19) Term CD +6, C → G.
(20) Term CD +90, del 13 bp
(21) Term CD +47 (C → G)
III. RNA translation initiation codon
(1) ATG—single base substitutions, 45 insertion nonsense codons
(2) Numerous examples of single base substitutions, all leading to premature termination codons frameshift
(3) Numerous examples of minor insertions, deletions, shifting reading frame, and leading to premature termination codons

Table 1. Categories of the mutation in β-thalassemia [6].

method for detecting all mutations associated with β-thalassemia in the United States, due to having different ethnic populations. There is less heterogeneity in β-thalassemia mutations in populations in some parts of the world, and a more targeted strategy may be used to screen for β-thalassemia carriers in these areas of the world. Recently, reverse dot-blot hybridization, restriction fragment length polymorphism, and denaturing gradient gel electrophoresis have been used as the most commonly mutation screening methods. These tests are labor intensive with low throughput, need expensive high-technology equipment, and may give results that are difficult to interpret.

The most common mutation in β-globin gene is IVS-I-110 in Mediterranean countries, including Turkey, Italy, Egypt, and Greece. The Aldemir et al. study has shown the spectrum of β-thalassemia mutations in Hatay province, Turkey, and it established a foundation for prenatal genetic testing that will be a part of an effective prevention program for β-thalassemia disease. The spectrum of β-thalassemia mutations in 93 unrelated affected patients was determined by Aldemir et al. A large amount of β-thalassemia mutations was identified by them using a direct sequencing method. The results are different from the other parts of Turkey. The most common mutations were: IVS-I-110 (G > A), IVS-I-6 (T > C), IVS-I-1 (G > A), frameshift codon (FSC)8(−AA), codon 39 (C > T), and IVS-II-745 (C > G). They reported that many Syrian and Iraqi immigrants presented with a prevalence of thalassemia traits that were different from other studies [2]. β-Thalassemia is caused by more than 300 different β-globin chains (http://www.ithanet.eu/db/ithagenes;http://globin.bx.psu.edu/hbvar) but only about 40 account for 90% or more of β-thalassemia cases worldwide [6].

A wide spectrum of mutations interferes with the processing of the primary mRNA transcript. Those that affect the invariant dinucleotide GT or AG sequences at intervening sequence (IVS) prevent normal splicing altogether, causing β^0-thalassemia. Both exons and introns also contain "cryptic" splice sites, which are sequences very similar to the consensus sequence for a splice site but are not normally used. Mutations can occur in these sites producing a sequence that looks like the normal splice site; however, it is not normally used. During RNA processing the created site is used differently, leading to unusual splicing; inadequately spliced mRNA is not functional because a frameshift and a premature termination codon are created by spliced intronic sequences. Such mutations in codon 26 in exon1 of HBB gene result in the HbE variant. Clinical manifestation of compound HbE/β-thalassemia heterozygotes looks like those with two β-thalassemia alleles, extending between severe anemia and transfusion dependency and nontransfusion-dependent states (i.e. thalassemia intermedia or nontransfusion-dependent thalassemia (NTDT)), depending on the non-HbE/β-thalassemia allele and other genetic factors. Other RNA processing mutants affect the polyadenylation signal and the 3′ UTR. These are mild β-thalassemia alleles [5]. Those mutations are categorized in three different regulatory sites (shown in **Table 1**) [6].

3. Genetic factors on clinical severity, classical therapy, and novel treatments: gene therapy for β-thalassemia

The clinical severity of the β-thalassemia syndromes relies on the extent of α-globin chain/non-α-globin chain imbalance. The erythroid precursors in the bone marrow and in the spleen are

damaged by the α-globin chain, causing ineffective erythropoiesis. However, bone marrow examination is usually not needed for diagnosis of patients. The bone marrow is very cellular, mainly as a result of marked erythroid hyperplasia, with a myeloid/erythroid ratio reversed from the normal (3 or 4) to 0.1 or less. Clinical presentation of B-TM occurs between the ages of 6 and 24 months. The usual presentation is progressive pallor in the second half of the year with abdomen distention due to hepatosplenomegaly. After age 10–11 years patients are at risk of developing severe complications related to iron overload, depending on their obedience with chelation therapy [3].

Heterogeneity in the clinical expression of β-thalassemia diseases may occur from the nature of β-globin gene mutations, α-thalassemia gene interaction, or differences in HbF production. This study was undertaken to define whether these genetic determinant factors can predict the phenotypic severity of patients with β-thalassemia and to evaluate the relationship between the phenotype and genotype. According to the current literature, coinheritance of types of β-thalassemia mutation and α-thalassemia in a patient who has at least one allele of the genotype of β-thalassemia is predictive of the clinical severity of the disorder. Conversely, a mild clinical finding in some patients with β_o/β^+-thalassemia or β_o-thalassemia /HbE is that these individuals do not have an α-thalassemia haplotype.

The β-thalassemia phenotype was found to be modified by different factors. β-Thalassemia with coinheritance of α-globin gene deletion may have moderate clinical features while deletion of both α-globin genes is characteristically related to thalassemia intermedia. HbF is the major modifier of disease severity in individuals with β-thalassemia. Because the severity of homozygous β-thalassemia is associated with the disproportion between α- and β-globin chains, even the low levels of γ-globin in F cells decrease the relative excess of α-globin and provide a selective survival of cells producing HbF at the time of ineffective erythropoiesis in the most common forms of β-thalassemia. Any factors that can decrease the degree of disproportion may develop the clinical feature. Thus, this selective survival might account for high levels of HbF in homozygous β-thalassemia. The presence of genetic variants continues to produce HbF, hence it has a strong influence on the clinical feature of development [12]. Higher levels of expression of HbF in adulthood have been suggested to improve the morbidity and mortality in sickle cell disease (SCD). Nemati et al. have studied the incidence of Xmn1 polymorphic sites in B-TM patients from western Iran. The study detected that having this polymorphic site caused a positive effect on HbF production and the G-γ ratio, which could improve the clinical findings of β-thalassemia patients [15].

4. Classical treatments in β-thalassemia

Classical treatments are classified into five subgroups. We discuss the benefits of these therapies and their long-term side effects below.

4.1. Transfusion treatment

Increasing evidence shows the advantage of transfusion therapy in decreasing the occurrence of complications such as pulmonary hypertension and thromboembolic events. Thus, although

the common practice was to start transfusion when complications arose, it may be valuable to initiate transfusion therapy earlier as preventive strategies arise, which will help ease the increased risk of alloimmunization with delayed start of transfusion. Although earlier introduction of blood transfusions will increase the ratio of iron accumulation, current approaches of iron chelation are available now. Transfusion becomes necessary when the sense of well-being of the patient diminishes to a level insufficient to enjoy the activities of a normal life. Problems such as chronic hypoxia for levels of Hb below 70 g/L usually develop in patients. The patient's general situation such as regular growth, growth velocity, size of the spleen, bone age, periods of rapid growth, bone deformities, and pregnancy must be considered. Transfusion sometimes becomes necessary during infection-induced aplastic crises. Heart disease is also an indication to transfusion therapy. The transfusion treatment should be similar to the one generally adopted for B-TM, when the decision to transfuse is made [4, 16, 17].

4.2. Splenectomy

The present indications for splenectomy in Thalassemia Intermedia (TI) involve growth retardation, increased transfusion requirement, leukopenia, thrombocytopenia, and splenomegaly [16]. Splenectomy, however, can contribute to a rising predisposition to thromboembolic events and pulmonary hypertension in TI. The presence of a chronic hypercoagulable situation could be due to the procoagulant influence of the anionic phospholipids exposed on the surface of the impaired circulating red blood cells [4]. Devastating postsplenectomy sepsis is a sudden event, and it can be mortal. The most frequent bacteria in postsplenectomy patients are *Streptococcus pneumoniae*, *Hemophilus influenzae*, and *Neisseria meningitidis* [18]. Iron-loaded macrophages lose the ability to kill intracellular pathogens by the interferon-γ-mediated pathways. The loss of this ability is associated with reduced formation of nitric oxide in the presence of iron. Splenectomy generally stops transfusion in the majority of patients. However, it does not generally modify the high output state and the increased pulmonary artery pressure that often characterizes thalassemia intermedia. Partial dearterialization of the spleen and partial splenectomy have a crucial and beneficial effect, but are not long-lived [19].

4.3. Iron chelation therapy

After one decade, iron chelation therapy was begun for preventing iron accumulation in some organs, but iron chelation may not prevent the development of clinical cardiac disease. A direct calculation of liver iron concentration is suggested, either by biopsy or by a noninvasive method such as R2 MRI. Iron chelation therapy should generally be started if liver iron concentration exceeds 7 mg/g dry weight of liver tissue. Threshold serum ferritin values of 400–500 ng/mL could be considered as an indicator for starting iron chelation therapy [19, 20].

4.4. Alteration of fetal hemoglobin production

Drugs increase levels of HbF ($\alpha_2\gamma_2$) and could be an advantage in patients with β-thalassemia intermedia because of an improvement in the balance of globin synthesis. Hydroxyurea is accomplished by inducing HbF synthesis and increasing γ-globin production. Both hydroxyurea

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and butyrate by-products have shown only modest increases in Hb. Some patients with β-thalassemia intermedia, who are not transfusion dependent, may respond to hydroxyurea treatment. Hydroxycarbamide has been administered to thalassemia patients according to many different regimens, alone or in combination with other drugs. A significant decrease in the need for blood transfusions was observed in many patients; the need was completely removed in some patients. α-Deletions, the XmnI polymorphism, and HbE/β-thalassemia may be predictive of a good response to hydroxycarbamide. The sense of well-being, almost generally reported, may imitate the significant decrease in ineffective erythropoiesis. Using recombinant human erythropoietin (rHuEPO) in some clinical trials for the treatment of thalassemia showed a significant, dose-dependent increase in thalassemic erythropoiesis, without an increase in HbF, mean corpuscle volume (MCV), and mean Hb content (MHC), and without an alteration in the α/non-α ratio, mostly in splenectomized patients with thalassemia intermedia. Combination therapy with erythropoietin and hydroxyurea in thalassemia patients appears to be more advantageous than either therapy alone with respect to HbF increase and an increased packed cell volume [19].

4.5. Antioxidants

Oxidative damage may be generated by the presence on the cell-free globin chain and labile plasma iron (LPI), a chelatable component of nontransferin-bound iron, which can be reduced by treatment with iron chelating agents. The persistent stress on neutrophils can decrease their antibacterial ability and their respiratory eruption reaction. Combined administration of vitamin E with *N*-acetylcysteine and iron chelators could be more efficient than the use of a unique antioxidant [19].

B-TM patients' prognoses have considerably developed over the last decade with the advent of noninvasive methods to measure organ iron before the occurrence of clinical symptoms, new chelators, and increased blood safety procedures. The novel improvements in medicine have opened the way to an important development in diminishing cardiac mortality, previously reported to cause 71% of deaths in B-TM patients [5]. Studies show that despite ethnic differences, most individuals with transfusion-dependent thalassemia have normal cardiac iron but a significant proportion have simultaneous liver iron overload [21]. The novel and the most promising method for this purpose are gene-editing tools (GETs), which allow for site-specific genome editing and targeted transgene integration in an efficient and accurate situation. Transcription activator-like nucleases (TALEN), zinc-finger nucleases, and CRISPR/Cas9 cooperate with the major GETs. The nuclease systems can be approximately classified into two groups based on their type of DNA recognition: ZFN, TALEN, and meganucleases succeed specific DNA binding via protein–DNA interactions, Cas9 is targeted to specific DNA sequences by a short RNA key molecule that base-pairs directly with the target DNA, and protein–DNA relations have a role in its targeting. CRISPR/Cas9 is a simple and efficient GET for in vitro and in vivo systems. In recent decades, a great amount of research has been conducted for the management of β-thalassemia. One of the novel therapeutic strategies, β-globin gene targeting, is then differentiated into HSCs and returned to the patient. When using induced pluripotent stem cells (iPSCs), the most urgent topics to be considered are the

elimination of transcription factors not needed after induction and reestablishment of correct reprogramming in a way that the iPSCs are not developing into tumors [8, 22, 10].

Gene therapy strategies for β-thalassemia are so versatile; the common goal in these strategies is corrected β-globin gene defects in iPSCs and $CD34^+$ hematopoietic stem pluripotent cells (HSPCs). There are two alternative ways to use these treatments: the first one is to use $CD34^+$ HSCs and subpopulations may be corrected by gene therapy. The second one is to isolate and reprogram somatic cells (shown in **Figure 2**) [23]. The emergence of gene-editing technology, which enables precise genome application, offers a new strategy for treating β-hemoglobinopathies. Site-specific double strand breaks (DSB) can be induced with zinc-finger nucleases, TALENS, meganucleases, and more recently with the CRISPR/Cas9 system. CRISPR/Cas9 has revolutionized gene targeting. Unlike other nucleases, which use a protein dimer for target sequence recognition and require a novel protein to be engineered for each new target site, CRISPR/Cas9 technology uses a short guide RNA (gRNA) with a 20 bp sequence complementary to the DNA sequence to be targeted [24].

Gene therapy by either gene insertion or editing is a curative therapeutic option for hereditary blood cell disorders such as SCD and β-thalassemia. The safety and efficacy of gene transfer techniques has improved by using lentiviral vectors. This technology has developed perfectly, although there are some limitations, including number of engraftment-transduced HSCs and

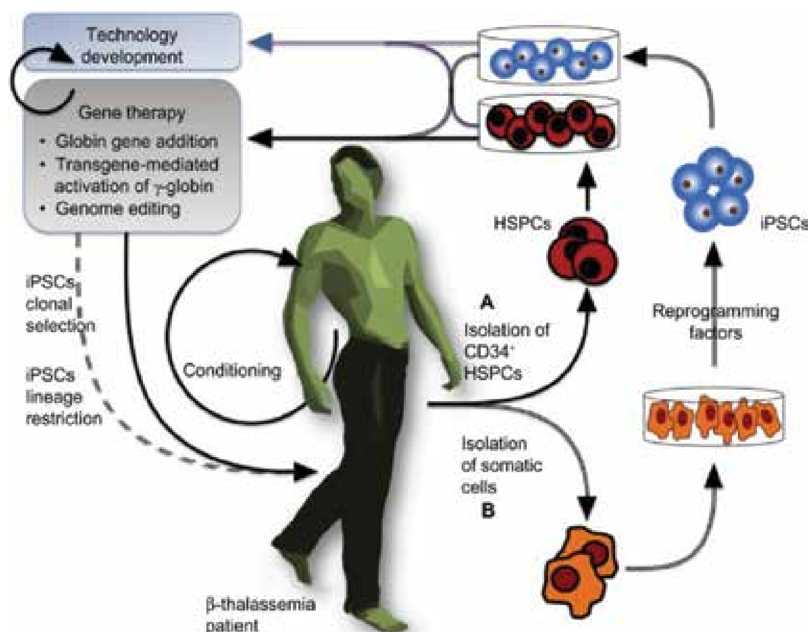

Figure 2. Gene therapy strategies for beta thalassemia. (A) The commonly used $CD34^+$ HSPCs and subpopulations may be corrected directly by gene therapy. (B) Alternatively, somatic cells can be isolated and reprogrammed to pluripotency, with the resulting iPSCs then being a patient-specific substrate for gene therapy, clonal selection, and lineage-specific differentiation. Excepting circular arrows, solid arrows indicate procedures for HSPCs and hollow arrows those for iPSCs [23].

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sufficient transgene expression that results in complete correction of B^0-TM. This underlines the need to classify and address factors that might be contributing to the in vivo survival of the transduced HSCs or find means to develop expression from current vectors. According to recent knowledge, specific gene therapy methods for hemoglobinopathies are needed to be reviewed in the light of the success rate of preclinical studies and clinical trials. The preclinical trials will be needed to correct the side effect of viral vectors such as HIV-1 (LV), and murin Moloney leukemia virus (RV). The main problems are removing the genetic elements responsible for their virulence and pathogenicity, and adding the β-globin gene and its LCR. LV has been most useful at correcting hemoglobinopathy animal models and has resulted in their clinical translation. Initial studies looking at RV-mediated human β-globin gene transfer without inclusion of the LCR showed variable and low levels of gene expression. Following a number of studies, nearly 10 years of effort to develop RV for expressing effective globin gene expression was found to be inadequate. RVs utilizing the enhancer/promoter sequences of LTR to drive transgene expression of genes other than globin genes were the first ones to be used in clinical trials. Despite their clinical success in immunodeficiencies of patients' gene therapy, concerns about their safety emerged following reports of vector-mediated insertional mutagenesis. The RV vector insertions give rise to immortalization of primary hematopoietic progenitor cells. While the RV-LTR is a strong enhancer and upregulates transgene expression to very high levels compared to relatively weaker enhancers from the HIV-LTR and cytomegalovirus, it concurrently activates cellular protooncogenes flanking insertion sites. Moreover, methylation of the LTR can cause inactivation of the integrated transgene promoter and prevent long-term transgene expression. The structure of a self-inactivating (SIN) vector design deletes the LTR promoter/enhancer and allows the transgene expression to be driven by internal cellular promoters, reducing LTR enhancer-mediated genotoxicity and its methylation-induced inactivation [25].

The preclinical LV-mediated human studies were shown to rescue mouse models of β-thalassemia intermedia and B-TM. Imren et al. showed correction of β-thalassemia mice using a vector carrying βT87Q gene, where a point mutation in the β-globin gene confers it with antisickling properties. However, multiple copies were required for adequate correction of the mouse thalassemia phenotype [25]. Miccio et al. used an LV vector carrying the β-globin gene linked to a minimized LCR HS2/HS3. It showed that the thalassemia phenotype in th3/+mice can be fully corrected by transduced hematopoietic cells with 30–50% frequency having an average vector copy number of 1. Moreover, it was shown that genetically corrected erythroblasts having an in vivo survival advantage were necessary to investigate the utility of reduced intensity transplant regimens for clinical gene therapy studies. According to Malik et al., gene therapy for hemoglobinopathies is a reality now, with several patients cured of their β^0/βE-thalassemia or with significant enhancement from β^0/β^0-thalassemia and one patient with SCD, while others are showing modest transgene expression [26].

The severity of thalassemia disease tries to limit the current curative capacity of gene transfer technology. The challenges to effective clinical translation in hemoglobinopathies cover the dose of engraftable-transduced HSCs, the intensity of the preconditioning transplant regimen, and expression of the transgene. In vivo selection models can provide expansion of the few genetically modified engrafted HSCs. Improving vector potency will increase gene expression.

Efforts to promote differentiation of iPSC technology to produce engraftable HSCs can expand the HSC source, and gene editing can circumvent the need for high transgene expressing LV and potential, insertional mutagenesis of LV.

New technologies that can restore the future of gene therapy are gene editing using CRISPR-Cas9 and development of HSCs from iPSCs with long life repopulating potential, although this may need much work in reality. With scientific improvements in stem cell biology and genetic manipulation, it is considered that a future is possible where a child prenatally diagnosed with hemoglobinopathy can have her/his genetically modified cord blood stem cells transfused even before the fetal-to-adult Hb switch. This therefore will prevent the presence of any disease manifestations [25]. Lin et al. worked on a study to raise fetal hemoglobin for the treatment of β-thalassemia and SCD. They used CRISPR-Cas9 to modify normal bone marrow HSCs and HSPCs to the deletional HPFH genotype. The erythroid cells derived from such modified HSPCs showed significantly higher γ-globin expression compared with the nondeletion-modified $CD34^+$ HSPCs, iPSCs. They made use of this clinical invention and modified $CD34^+$ HSPCs to have part of the β-globin locus removed and repaired the genome by nonhomology end joining to create a genotype mimicking HPFH that produced a high level of γ-globin expression when differentiated into erythroid cells. The study showed that the CRISPR-Cas9 system is a new potential approach to autologous transplantation therapy for the treatment of homozygous β-thalassemia and SCD [27]. The current gold standard procedure for β-thalassemia patients involves a combination of granulocyte-colony stimulating factor (G-CSF) and plerixafor, a bicyclam molecule that antagonizes the binding of stromal cell-derived factor-1 expressed by bone marrow stromal cells to the chemokine CXC-receptor-4 located on the surface of HSPCs. G-CSF plus plerixafor has been shown to provide very high numbers of $CD34^+$ cells by single apheresis in mobilized thalassemic patients, despite the currently reported highest enrichment in HSCs using plerixafor alone [18]. In addition, two or three bone marrow harvests are required to collect an adequate dose of HSPCs from SCD patients; this is mainly because the HSC harvest is negatively influenced by (1) the inflammatory SCD bone marrow microenvironment and (2) the formation of cell aggregates during the isolation of bone marrow mononuclear cells. In patients with SCD, the administration of G-CSF led to severe adverse events and is therefore contraindicated. They suggested that plerixafor is potentially a safer mobilizing drug for SCD patients (http://www.clinicaltrials.gov identifier NCT02212535) [18].

In 2012, a phase 1 clinical study in β-thalassemic patients in the United States used the TNS9.3.55 vector expressing the wild-type β-globin transgene and a reduced intensity busulfan-based conditioning method. The result of this method was limited, and the four treated patients did not show sufficient clinical positive effect. A second phase clinical study is planned using a TNS9.3.55 variant vector, patient disease-symptoms free, although results need to be reproduced in a large number of patients and over a longer follow-up time (**Table 2**). For $β^0$-thalassemia and possibly SCD, further improvements in manufacturing, cell processing, and protocol design are needed for clear clinical benefit. Several groups have proposed alternative LV and genome-editing-based strategies for raising endogenous γ-globin expression or correcting the disease causing mutations. However, LV vectors are regularly and safely used to genetically modify patient HSCs, and the clinical application of genome-editing approaches must be confirmed in terms of editing efficiency of long-time HSCs for the possibility of delivering associated toxicity

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Vectors trans gene	Erythroid enhancer	Key findings	Key problems and possible solutions
TNS9 β-globin	HS2–3-4	Correction of anemia in thalassemia intermedia mice and rescue of lethality in mouse model	Variable human β-globin expression in thalassemia.
Beta[87] β^{T87Q}-globin	HS2–3-4	Correction of anemia in thalassemia intermedia mice. High-level expression of antisickling globin in erythroid cells	Multiple copies are required for correction; gene expression surrounding the integration sites was not analyzed
D432β γ-globin γ	HS2-HS3-HS4	Correction of anemia in thalassemia intermedia mice	Variable phenotypic improvement in thalassemia intermedia mice due to chromosomal position effects
LARβΔγ γ-globin V5	Extended HS2-HS3-HS4	Correction of anemia in thalassemia intermedia mice	Improved βγ-globin expression and reduced position effects
BG-I β-globin	HS2-HS3-HS4	Correction of human thalassemia major phenotype in vitro and in immune deficient mice [28]. Reduced position effects and uniform expression Identification of minimal regions of cHS4 necessary for optimal insulation	Low viral titers with full-length cHS4 insulator. Identification of regions of cHS4 that impart optimal insulation and have minimal effect on vector titers
T10 β-globin	HS1-HS2-HS3-HS4	Correction of anemia in thalassemia intermedia mice with lower vector copies	Low viral titers with full-length cHS4 insulator. Identification of regions of cHS4 that impart optimal insulation and have minimal effect on vector titers
HS40–11 γ-globin	HS-40	Partial correction of mouse γ-thalassemia intermedia with high transduction levels	γ-Globin expression insufficient for correction of thalassemia major
GLOBE β-globin	HS2-HS3	Correction of β-thalassemia major by gene transfer in murine [29] and human thalassemia hematopoietic progenitors	High vector copies are required for correction of thalassemia major mouse model

Table 2. Comparisons of different β-or γ-globin vectors studied successfully in mouse and human models of β-thalassemia.

and the potential off-target activity of the specific genome-editing tools [18]. Current clinical studies discovering alternate strategies such as matched distinct donors and development of novel regimens using haploidentical donors will enhance the transplant option for the majority of SCD patients in the future [28]. The other strategy to cure β-thalassemia is efforts to develop gene therapy. Two β-thalassemia patients with an altered β-globin LV-based vector (gene therapy) had been treated successfully. This improvement holds potential for SCD [30–32].

β-Thalassemia is the second common hereditary hemoglobinopathy after SCD. The discovery of gene therapy for β-thalassemia has been right by HLA-identical donors, the narrow window of application of HSC transplantation to the youngest patients, and recent advances in HSC-based gene therapy. A large number of publications on this topic show that gene therapy has the potential to become the therapy of choice for patients who lack either an HLA genoidentical sibling or an alternative, medically acceptable donor. Genetically modified HSCs are a good alternative to allogeneic hematopoietic stem cell transplantation (HSCT) for treating

β-hemoglobinopathies. It supersedes the need for a matched donor and thus avoids the risk of graft versus host disease and graft rejection after HSCT. Furthermore, the conditioning regimen required for the engraftment of genetically modified cells, because of their autologous origin, does not include immunosuppressive drugs. The worldwide application of this treatment may be possible nowadays, thanks to better safety and the absence of treatment-related mortality in gene therapy trails to date [18]. In terms of the current literature, using novel LV vectors for gene therapy and genome-editing-based treatment strategies for β-thalassemia and SCD is discussed and well documented. Novel therapeutic strategies are based on the use of LV vectors and/or genome-editing tools to reactivate endogenous HbF expression [18, 27].

Several approaches can be used to genetically manipulate HSCs and correct the genetic defect underlying β-hemoglobinopathies. A large number of current clinical trials (based on the transplantation of autologous β-thalassemia and SCD HSCs) are investigating the use of LV vectors that express β-like globin transgenes. Their results support further evaluation of integrase defective lentiviral vectors (IDLV) as a new HIV-1 vaccine delivery platform [18, 26, 29]. Clinical trials of globin-expressing LVs are now under way at several sites in Europe and the United States. The initial results are encouraging with regard to the accomplishment of transfusion independence. Despite these promising results, some issues deserve further investigation. The purpose of the conditioning method is to eliminate diseased HSCs effectively while avoiding untargeted toxicity in other organs [26]. Novel treatment strategies are based on the use of LV vectors and/or genome-editing tools to reactivate endogenous HbF expression.

Traxler et al. used the CRISPR/Cas9 system to regulate a 13 bp HPFH deletion in the γ-globin promotors via microhomology-mediated end joining. This region is thought to contain a binding site for HbF repressor BCL11A. In their study, HbF reactivation was associated with reduced levels of sickle β-globin and ameliorated the SCD cell phenotype in vitro. Recreating levels of HPFH requires the nonhomolog end joining (NHEJ)-based excision of long genomic fragments containing the β-and δ-globin genes and putative 3.5 kb D-gamma-intergenic HbF silencer targeted by BCL11A. This approach takes advantage of the NHEJ repair pathway, which might decrease the overall efficiency of genome editing. Nonetheless, if the proof of principle works, this approach reactivated HbF, constantly disrupted the β-globin gene, and ameliorated the SCD cell phenotype [18, 33, 34]. The prestige of potential clinical studies in gene therapy is upcoming and an open clinical study for β-thalassemia is included in **Table 2**. The reprogramming of somatic cells into induced pluripotent stem cells opens a new approach for treating β-thalassemia. Ye and coworkers reprogrammed the skin fibroblasts of a patient with homozygous β^0-thalassemia into induced pluripotent stem cells, which produced Hb. These reports that induced pluripotent stem cells could offer a new approach for the treatment of β-thalassemia. These strategies are at an in vitro level at the present time [35]. Epigenetic therapy approaches for β-thalassemia are promising for the cure for this disease.

This chapter outlined the key molecules of the molecular mechanism underlying β-thalassemia in relation to the development of novel treatments and an update is given both at clinical and preclinical trials. Gene therapy has reached an important point and phase 1 clinical trials have been launched to discover the effectiveness and particularly long-term safety [36].

Finally, the efficiency of genome editing and potential side effects (toxicity) of this strategy need to be further tested in well-intentioned HSCs. The different strategies must be compared in terms

of efficiency, efficacy, and safety to provide patients suffering from β-hemoglobinopaties with the best therapeutic option. Recently, several research groups have discovered genome-editing-based methods for correcting β-thalassemia mutations [18]. Ultimately, innovative trials have developed an enthusiasm that fully differentiated somatic cells can be reprogrammed to make stimulated pluripotent stem cells [32]. Subsequent trials showed alteration of a mouse model of SCD using this original strategy, and hence have paved the way to using these cells to treat hemoglobinopathies with a few restrictions: one restriction was the inability to repair all hematopoietic lines with induced pluripotent stem cells, which prevents using human treatment [37–39]. The future of gene therapy has exhibited remarkable advances. Phase 1 trials are already recruiting individuals with the goal to examine the effectiveness and mostly the long life safety of transplantation of autologous $CD34^{+}$ erythroid progenitor cells transduced ex vivo with LV β-globin vectors [36].

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Chapter 3

Emerging Techniques for Thalassemia Gene Detection

Lingwen Zeng, Luxin Yu and Yinghui Zhang

Additional information is available at the end of the chapter

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Abstract

Isothermal nucleic acid amplification is a simple process that rapidly and efficiently accumulates nucleic acid sequences at constant temperature such as 37 and 42°C. Isothermal nucleic acid amplification approach offers several advantages over temperature circle methods (such as PCR) including rapid assay results, cost-effectiveness, and portability. Two detection approaches based on circular strand-displacement polymerization reaction (CSDPR) were presented in this chapter for sensitive and specific thalassemia gene detection. One is a lateral flow strip biosensor based on CSDPR for semi-quantitative detection of thalassemia DNA. The other is a spectrophotometric DNA detection approach based on CSDPR for quantitative detection of thalassemia DNA.

Keywords: emerging techniques, isothermal amplification, thalassemia gene detection

1. Introduction

Thalassemia is a heterogeneous group of inherited disorders characterized by the reduced synthesis of one or more normal hemoglobin chains leading to imbalanced globin chain synthesis, which results in microcytic anemia [1–3]. Based on genetic disorders, thalassemia is divided into two main types, alpha-thalassemia and beta-thalassemia. Alpha- and beta-thalassemia occur when there is a reduction in alpha-globin chains and beta-globin chains, respectively. Symptom of anemia is variable ranging from none to severe.

Because the structural hemoglobin variants and thalassemias occur at high frequencies in some populations, both types of genetic defect can be found in the same individual such as $-^{SEA}/-\alpha^{3.7}$. The different genetic varieties of thalassemia and their combinations with genes for abnormal hemoglobins produce a series of disorders defined as thalassemia syndromes [4].

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Until now, marrow transplantation is the only way in which they can be cured. Unfortunately, expensive fee and restricted marrow sources have hindered its application in thalassemia patients. Therefore, thalassemia carrier screening in premarital checkup is an effective way for defective child birth. Carrier screening for thalassemia can reduce the burden on individuals by identifying those at increased risk, thereby enabling individuals to receive information about their health, future health, and/or potential health of their offspring, so that they are informed and understand their reproductive risks and options [5].

Traditional diagnosis methods include osmotic fragility test and hemoglobin electrophoresis techniques. The decrease of osmotic fragility indicates the decrease of hemoglobin production. Although it is simple and convenient, osmotic fragility test is not specific for thalassemia. Hemoglobin electrophoresis was used to initially identify samples for common hemoglobinpathies such S and C. More complex electrophoresis techniques were then used to identify a great number of hemoglobin subtypes. Nevertheless, electrophoresis techniques are very complicated in experimental procedures, low in sensitivity, and difficult for quantitation.

Along with the advances in molecular biology, a variety of novel nucleic acid detection methods have been invented and applied in thalassemia gene detection, such as gap-PCR [6], reverse dot blot (RDB) [7], and DNA microarray [8]. However, these methods for thalassemia detection rely solely on PCR to amplify target genes. They are not only expensive and complicated in procedure, but also liable to contamination. On the other hand, as they operate at a constant temperature (mostly at 37°C), the isothermal nucleic acid amplification approach offers several advantages over temperature circle methods including rapid assay results, cost-effectiveness, and portability.

2. Emerging techniques for thalassemia gene detection

2.1. Types of globin

Globin, the protein portion of the hemoglobin molecule, is a tetramer consisted of two α and two non-α-globin chains. The structure and ontogeny of the hemoglobins are listed in **Figure 1**. The α-globin chains are encoded by two closely linked genes ($\alpha 2$ and $\alpha 1$) located on chromosome 16. The non-α genes, β, γ, and δ, are encoded by a cluster of genes on chromosome 11. All types of hemoglobins have similar protein structures. Each hemoglobin has a quaternary structure composed of two separate pairs of the same globin chains. The embryonic hemoglobins consists of two ζ or α chains and two ε or γ chains; all normal human hemoglobins consist of two α chains and two non-α chains. In hemoglobin A, the chains are composed of β chains ($\alpha_2\beta_2$); in hemoglobin A2, they are composed of δ chains ($\alpha_2\delta_2$); and in hemoglobin F, they are composed of γ chains ($\alpha_2\gamma_2$).

There are two main classes of thalassemia, and, in which α- and β-globin genes are involved. **Table 1** summarizes the different classes of α/β-thalassemia mutations. When only 1 of the 4 α-globin chain genes is defective, the patient has little clinical symptoms and this silent condition can only be detected at birth by the presence of a small amount of Bart hemoglobin.

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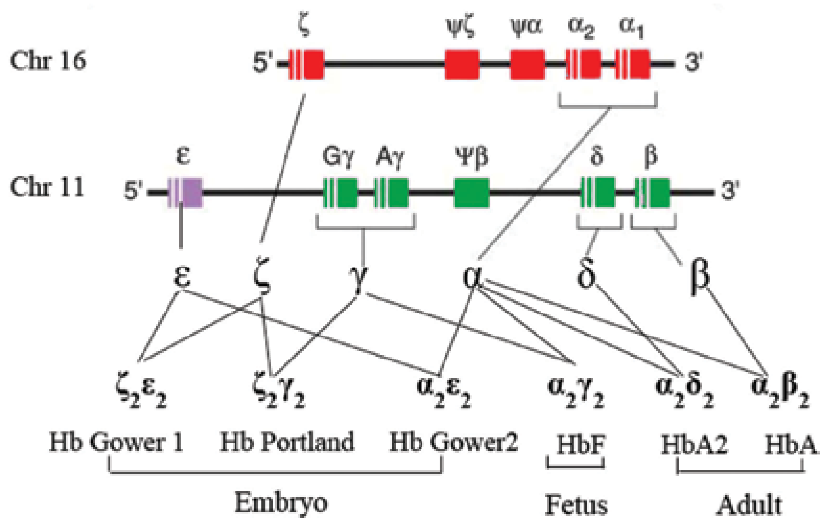

Figure 1. Schematic illustration for genetic control and protein structures of hemoglobin. The α- and β-globin are controlled by gene clusters located on chromosomes 11 and 16, respectively. Each hemoglobin has a quaternary structure composed of two separate pairs of the same globin chains.

α^0-thalassemia
Deletions involving both α-globin genes
Deletions downstream from α_2 gene
α^+-thalassemia
Deletions involving both α-globin genes
Point mutations involving α_2 or α_1 genes
mRNA processing
β^0-or β^+-thalassemia
Transcription
Deletions
Insertions
Promoter
5′UTR

Table 1. Classes of mutations that cause α/β-thalassemia.

α hemoglobin is made up of 4 γ-globin chains. Current high performance liquid chromatography (HPLC) techniques are relatively insensitive to the very low level of Bart hemoglobin with a single chain defect and are, therefore, not useful for the diagnosis of α-thalassemia carriers. Heterozygous α^0 and homozygous α^+ patients show microcytosis, hypochromia, and a

mild anemia, suggesting thalassemia minor. Patients with β-thalassemia minor due to either a gene deletion (β^0) or mutation (β^+) can be identified by the slight elevation in fetal hemoglobin and an increase in the hemoglobin A_2 level to between 4 and 7%.

2.2. Detection of thalassemia genes with traditional methods

Carrier detection approaches must be able to detect α- and β-thalassemias. The results of complete blood cell count (CBC) including the values of MCH, MCV, HbA_2, and HbF can provide clues to the hematological characteristics of the different types of thalassemia genes and their interactions. The most common thalassemia mutations and abnormal hemoglobins can be detected by PCR-based techniques such as RDB and gap-PCR. The main applications of molecular detection for carrier analysis are summarized as follows. 1. Heterozygous α-thalassemia and homozygous α-thalassemia can be discriminated by the analysis of α-thalassemia mutations using gap-PCR; 2. The identification of β-thalassemia mutations for patients requires prenatal diagnosis to predict the severity of the clinical phenotype of homozygous β-thalassemia; 3. The discrimination between δβ-thalassemia and hereditary persistence of fetal hemoglobin (HPFH) deletions can be carried out by gap-PCR [9].

Routine detection strategies for the detection of thalassemia gene are mainly based on PCR. Gap-PCR was developed for the detection of deletion types of thalassemia gene mutations. More than eight β-thalassemia deletions and two common α^+-thalassemia deletion genes, the -$\alpha^{3.7}$ and -$\alpha^{4.2}$ alleles, and five α^0-thalassemia deletion genes, the three Southeast Asian deletions ($-^{SEA}$, $-^{THAI}$ and $-^{FIL}$) and two Mediterranean deletions ($-^{MED}$ and -$\alpha^{20.5}$), can be detected by gap-PCR.

Meanwhile, allele-specific oligonucleotide hybridization (ASO) and RDB are applied to non-deletional α- and β-thalassemia mutations. ASO is used to detect single base mutations without the need of PCR or gel electrophoresis. For mutation analysis, a panel of ASO probes is employed to detect mutations in patients. For prenatal diagnosis and for genotyping homozygous patients, two labeled probes are employed to detect the mutation sequence, one complimentary to the normal gene DNA sequence and the other complimentary to the mutant DNA sequence at the same position. Generally, there is only one nucleotide difference in the two ASO probes. The genotype of the sample is observed by the absence or presence of the hybridization signal of two ASO probes.

In the RDB assay, the amplified target DNA is first fixed to a nylon membrane to form a filter-fixed DNA dot, the dot is then hybridized to ASO probes whose 5′ terminal was conjugated with either P-labeled deoxynucleoside triphosphates, biotin or horseradish peroxidase. The technique has been successfully applied in many laboratories, especially for populations with just one common mutation and a small number of rare ones. However, RDB was not suitable for the screening of a large number of different mutations because the method is time-consuming as it needs separate hybridization and washing steps for each mutation.

All these routine methods for thalassemia detection are tedious, expensive, and liable to contamination. To improve these methods, many PCR-derived methods have been developed

and applied to thalassemia gene assay, such as real time PCR, dissociation curve analysis, and droplet digital PCR. These methods are simple; however, expensive apparatus and need for experienced personnel hinder their broad application in basic level laboratories.

Recently, many amplification strategies have been developed to improve the sensitivity of DNA detection. Two commonly used methods are target amplification and signal amplification. PCR as well as several isothermal amplification techniques such as rolling circle amplification (RCA) and helicase-dependent amplification (HDA) are typical target amplification strategies. However, these conventional amplification methods for sequence-specific DNA detection suffer from the limitations of complicated procedures, easy contamination, and high cost, which have been hampering their application in many laboratories. Signal amplification techniques that are commonly applied to directly amplify the detection signal have been developed to overcome the disadvantages of traditional target amplification strategies. These amplification approaches including nicking enzyme signal amplification (NESA) and CSDPR [10], which are most commonly used for sequence-specific DNA amplification, provide several advantages over target amplification approaches including ease of use, portability, rapid assay results, and cost-effectiveness. Particularly, CSDPR combined with different detection platforms have attracted great attention due to its robustness and simplicity. CSDPR amplifies the DNA signal at an isothermal temperature that yields a large amount of DNA signal products that can be detected with different platforms.

2.3.1. Detection thalassemia gene with emerging techniques lateral flow biosensors

Based on CSDPR and gold nanoparticles (AuNPs), we have developed a lateral flow biosensor (LFB) for the visual detection of thalassemia genes [11]. This sensor is highly sensitive (the detection limit is 0.01 fM of nucleic acid), highly specific, and easy to use. It does not require the use of complex and expensive instrumentation. The development of this biosensor represents an important step toward point-of-care testing for genetic disorders. The Southeast Asia (SEA) deletion of α-thalassemia was detected with this lateral flow biosensor. The method has been successfully validated for the detection of the SEA type of α-thalassemia (100% concordance with the results obtained using PCR). In addition, this test can distinguish SEA from other types of thalassemia, such as -$\alpha^{3.7}$, -$\alpha^{4.2}$, and β-thalassemia.

As depicted in **Figure 2**, a CSDPR reaction comprising biotin-modified hairpin DNA, digoxin-modified primer, and DNA polymerase is initiated by target mutant DNA strand. The hairpin probe forms a stem-loop structure with biotin at the 5′ end of the stem region. The stem is a 10-nt sequence, and the loop is a 20-nt sequence that is complementary to the mutant target DNA. The 8-nt primer includes a digoxin tag at the 5′ end and is complementary to the stem region of the probe at the 3′ end. The biotin-modified hairpin recognizes and hybridizes with SEA mutant DNA when present, causing the hairpin probe to undergo a conformational change that leads to stem separation (1). The primer then anneals with the open stem (2) and triggers a polymerization reaction in the presence of dNTPs and polymerase Klenow exo- (3). During primer extension, the mutant DNA is displaced by the strand displacement activity of the polymerase, permitting the synthesis of complementary DNA and formation of a hairpin-DNA complex. To start the next cycle, the displaced target mutant DNA strand hybridizes with

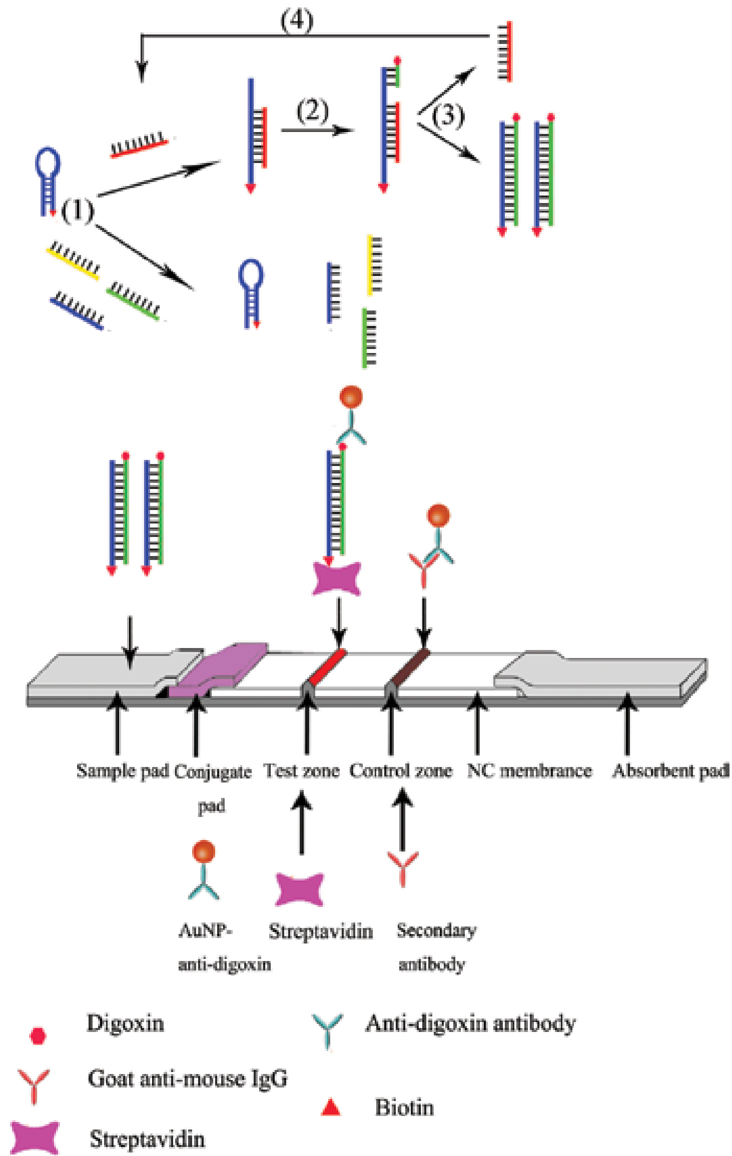

Figure 2. Illustration of the production of sequence tagged duplex DNA based on CSDPR and the structure of a lateral flow biosensor including four steps. (1) the biotin-modified hairpin recognizes and hybridizes with SEA mutant DNA when present, causing the hairpin probe to undergo stem and loop separation; (2) the primer then anneals with the open stem; (3) this triggers a polymerization reaction in the presence of dNTPs and polymerase Klenow exo-; (4) to start the next cycle, the displaced target mutant DNA strand hybridizes with another hairpin probe.

another hairpin probe, initiating another round of polymerization (4). Through this cyclical process, a large number of tagged-duplex DNA complexes are produced from a minute amount of target mutant DNA. The hairpin retains its original stem-loop structure in the presence of

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wild-type or non-complementary DNA due to the weak hybridization between wild-type DNA and the hairpin probe. Therefore, the primer is unable to anneal to the hairpin to induce a polymerization reaction, and no tagged-duplex DNA complex is produced.

The large number of tagged-duplex DNA complexes produced by the CSDPR reaction is then detected using an LFB. As shown in **Figure 2**, the CSDPR products are mixed with a running buffer and migrate along the LFB by capillary action to the conjugate pad, where anti-digoxin–AuNP conjugates are deposited. The digoxin attached to the duplex DNA reacts with anti-digoxin on the AuNP surface to form a complex that consists of biotin-double strand DNA-digoxin–anti-digoxin–AuNP. The resulting complexes can be arrested by streptavidin at the test zone. The accumulation of gold nanoparticles on the test zone can be observed as an obvious red band. Superfluous free anti-digoxin-AuNP complexes keep on moving and accumulate at the control zone to form a second red band. In the absence of mutant SEA DNA or in the presence of wild-type DNA or non-complementary DNA, no duplex DNA is produced in the CSDPR; therefore, no anti-digoxin-AuNP conjugate is captured, and no red band is observed at the test zone. In this case, the observation of a red band at the control zone indicates that the LFB is working correctly.

This assay termed as lateral flow nucleic acid biosensor (LFNAB) can reach a sensitivity of 0.01 fM when using synthetic target DNA. **Figure 3** presents typical images together with the corresponding optical responses of the biosensors when loaded with various amounts of synthetic target DNA.

2.3.2. Spectrophotometric approach

LFNAB can qualitatively detect DNA by naked eye without expensive apparatus. It is fast and convenient. However, LFNAB is a qualitative or semi-quantitative detection method and the color observation may vary between different people. Therefore, we have developed a spectrophotometric DNA detection approach based on CSDPR for sensitive, selective, inexpensive, and quantitative detection of South East Asia (SEA) type of α-thalassemia [12]. As depicted in **Figure 4**, the signal amplification system is composed of a hairpin capture probe, a primer, polymerase, and streptavidin-horse radish peroxidase (SA-HRP). The structure of the hairpin capture probe consists of a biotin at 3′ end, a 11 base pair (bp) stem, a thiol group at 5′ end, and a 20-nt loop. The sequence of the loop is complementary to the target DNA. The primer (8-nt) is complementary to the stem region of the hairpin probe at 3′ end. The hairpin probe was first fixed on a gold nanoparticles (AuNPs) layer, which is coated by γ-globulin on a 96-well microtiter plate.

In the presence of target DNA, the loop region of the hairpin probe recognizes and hybridizes with the target DNA, causing conformational change of the hairpin probe to open the stem of the hairpin. The short primer then hybridizes with the open stem sequence and triggers a polymerization reaction in the presence of polymerase and dNTPs. Along with primer extension, the target DNA is released by polymerase with strand displacement activity, after which a longer

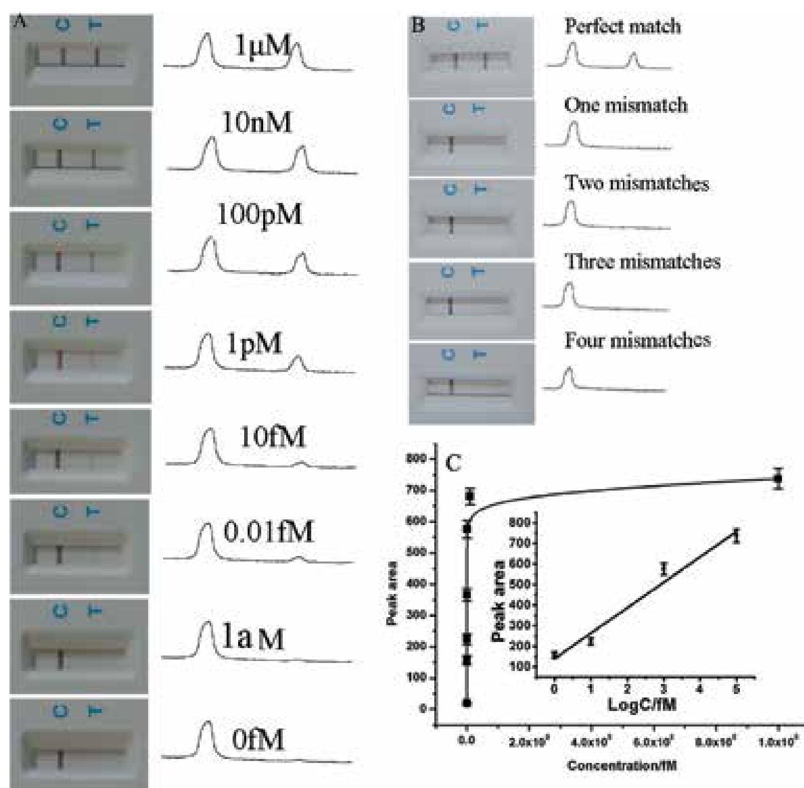

Figure 3. (A) Typical photographic images (left) and corresponding intensities (right) of an LFNAB in the presence of various concentrations of synthetic target DNA and the negative control. (B) Photographic images (left) and corresponding intensities (right) of the LFNAB tested with target DNA and target DNA including one or more mismatched bases. (C) Calibration curve of an LFNAB with various concentrations of target DNA. The error bars represent the standard deviations, $n = 3$.

complementary DNA is produced, forming a hairpin–DNA complex. The displaced target can hybridize with another immobilized hairpin probe to start the next cycle, which triggers yet another polymerization and target DNA release. Through this cyclical process, a great number of duplex DNA complexes with the biotin labeled are produced. The biotin groups on the duplex DNA products can conjugate with the SA-HRP. Finally, the target DNA can be sensitively detected via the HRP catalyzed substrate 3, 3′, 5, 5′-tetramethylbenzidine using a microtiter plate reader.

When the target DNA was absent or non-complementary DNA (such as wild-type DNA) was present, the fixed hairpin probe keeps the stem-loop structure and the reaction of biotin–SA-HRP would not take place as the steric effect. No signal would be detected.

Under optimal conditions, the sensitivity was calculated to be 8 aM and the dynamic range of the assay was from 0.1 fM to 10 nM. **Figure 5** presents the absorption spectrum and calibration curve of the assay.

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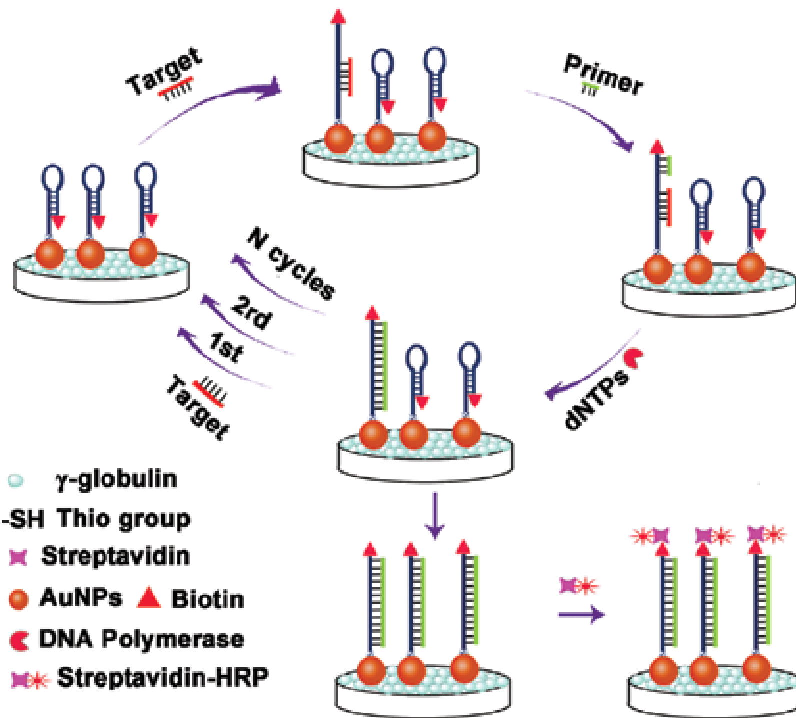

Figure 4. Schematic illustration for DNA detection based on CSDPR.

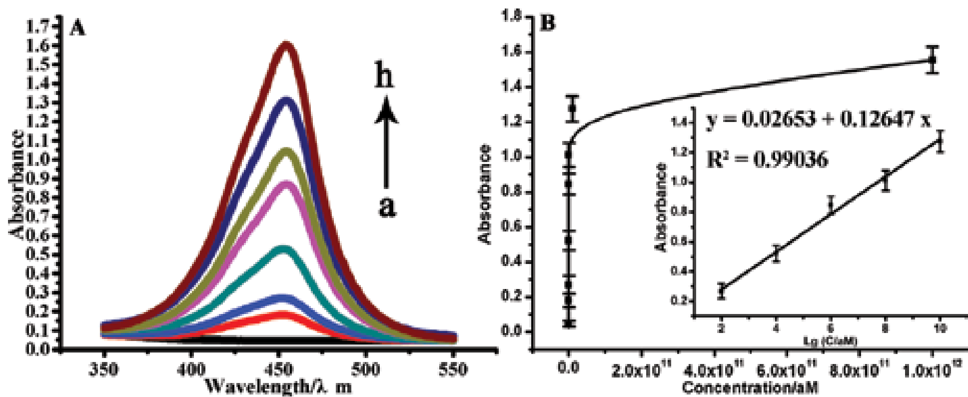

Figure 5. (A) Absorption spectrum response of the assay with different target DNA (0, 0.01 fM, 0.1 fM, 10 fM, 1 pM, 100 pM, 10 nM and 1 μM). (B) Calibration curve of the DNA assay with different concentrations of target DNA. Standard deviation was represent as error bars, $n = 3$.

CSDPR is a promising method for the detection of thalassemia genes. When combined with nucleic acid point-of-care testing methods such as DNA strip and spectrophotometric biosensors, this isothermal DNA amplification based approach could be widely applied in thalassemia carrier screening, especially in community hospitals.

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Section 3

Thalassemia - Prenatal Diagnosis

Chapter 4

Invasive and Noninvasive Approaches in Prenatal Diagnosis of Thalassemias

Abdullah Tuli and Ebru Dündar Yenilmez

Additional information is available at the end of the chapter

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Abstract

Thalassemia is a significant health problem worldwide. There are two main classifications, α- and β-thalassemias, which are usually caused by the defective synthesis of the α-globin, and which are commonly caused by different mutations of the β-globin chain. Different hemoglobin mutations have been identified to date. Thalassemias can result in profound anemia from early life and, if not treated with regular blood transfusions, can lead to death in the first year. Prenatal diagnosis of thalassemia is the essential part of preventive medicine and is currently dependent on the use of invasive diagnostic tests within the first 2 months of pregnancy. These diagnostic techniques carry a small but significant risk of fetal loss up to 1%. Molecular diagnostic methods have been developed for genotyping thalassemias based on PCR techniques and high-throughput technologies. Noninvasive tests using cell-free DNA (cfDNA) from a maternal blood sample is also an alternative method, thus eliminating the risk of miscarriage. This chapter summarizes the current invasive approaches and the noninvasive methods using cell-free fetal DNA as new molecular diagnostic methods for genotypic diagnosis of thalassemia in clinical practice. Prevention strategies that encompass carrier screening, genetic counseling, and prenatal diagnosis are discussed.

Keywords: thalassemia, prenatal diagnosis, noninvasive test, cell-free DNA, molecular method

1. Introduction

Hemoglobin (Hb) is the oxygen carrier molecule in red blood cells. Each adult Hb molecule consists of four subunits: two α-globin and two β- (or β-like) globin chains. The α-globin gene

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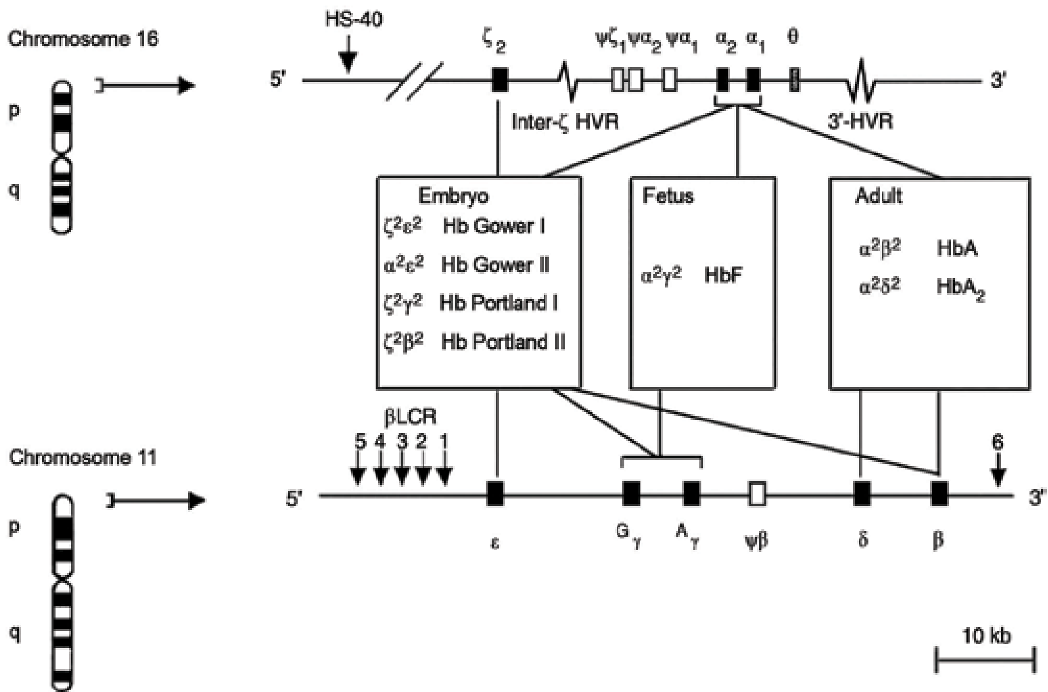

Figure 1. Alpha beta globin gene cluster [4].

cluster maps near the telomere of the short arm of chromosome 16. The human β-globin spans a region of 70 kb on the short arm of chromosome 11 and contains 5 functional genes. Hb A is the predominant form of Hb molecule in an adult human [1–4]. The *α*-globin gene cluster undergoes one developmental "switch" but the β-gene cluster undergoes two "switches". Transcription of the ε gene in the embryonic gene stage switches after the sixth week of gestation to the transcription of the two γ genes in the fetal liver and around the prenatal period, to the δ (minor adult) and β (major adult) genes (**Figure 1**). At 6 months after birth, hemoglobin F (HbF) constitutes less than 5% of the total hemoglobin and continues to fall, reaching the adult level of <1% at 2 years of age [4].

2. Hemoglobinopathies

Inherited hemoglobinopathies are large groups of autosomal recessive disorders [4]. There are more than 700 defects in globin genes that are found to be responsible for hemoglobinopathies [5]. The defective (+) or absent (0) production of one of the globin chains of the Hb tetramer causes autosomal recessive inherited disorders. The type of globin chain involved distinguishes *α*-, β-, and δ-thalassaemias [6]. The meaning of thalassemia comes from the Greek words *thalassa*; sea and *aemia*; anemia. It was observed that thalassemia is prevalent in areas in which malaria was seen or was endemic [7, 8].

The resulting imbalance in the ratio of α:β chains underlies the pathophysiology [3]. Both α-thalassemia and β-thalassemia, however, have a high frequency in many populations; although, β-thalassemia is more prevalent and more widely distributed. β-Thalassaemia is a member of an inherited hemoglobin disorder family that is characterized by reduction of β-globin chain synthesis [9]. The high frequency of thalassemia is due to the protective advantage against malaria that it confers on carriers, analogous to the heterozygote advantage of sickle cell hemoglobin carriers. Thalassemias can result in profound anemia from early life and, if not treated with regular blood transfusions, can lead to death in the first year [7, 10].

3. Prevalence and classification of thalassemias

Thalassaemia was originally thought to be a disease limited to the Mediterranean region, in countries such as Greece, Italy, and Cyprus. There is a characteristic distribution of thalassemias in a band around the Old World—in the Mediterranean, the Middle East, and parts of Africa, India, and Asia [4, 5]. As a result, clinically important interactions may occur among different alleles of the same globin gene or among mutant alleles of different globin genes [6, 8]. The prevalence of β-thalassemia trait varies within the Mediterranean coastal regions in Turkey; Mediterranean (Adana 3.7%); Aegean (İzmir 4.8%); and Marmara (İstanbul 4.5%) [4] (**Figure 2**). In highly prevalent regions, an ideal and effective strategy to decrease the birth rate of thalassemia patients is to identify high-risk couples, who are both carriers, before pregnancy by screening (or carrier testing) and then perform a prenatal diagnosis during pregnancy [11].

Thalassemias are genetically classified according to the particular globin chains that are ineffectively synthesized into α, β, δβ, and εδβ thalassemias. α- and β-thalassemias are important in the public health view [12]. Different hemoglobin mutations have been identified to date, the majority being single nucleotide substitutions, deletions, or insertions of nucleotides leading to a frame shift; rarely does β-thalassaemia result from gene deletions. More than 250 gene defects (more than 1150 mutations have been reported) have been described for different thalassemia phenotypes in different populations [11, 13]. β-Thalassemia have been described, and it has been found that the majority thalassemias can be caused by large deletions or by point mutations. α-thalassemia is often caused by large deletions for single (α^+-thal) or both α-genes (α^0-thal) (**Table 1**) [13].

Thalassemias exhibit a wide spectrum of phenotypes. Depending on the clinical severity, thalassemias are generally divided into three groups: (1) Thalassemia minor/trait: they are carriers who are often asymptomatic and do not need any treatment. (2) Thalassemia intermedia (TI): they have moderate anemia and occasionally require red blood cell transfusion; in α-thalassemia, it is known as Hb H disease. (3) Thalassemia major (TM): they have severe anemia and require transfusions for survival; in α-thalassemia, this clinical form was named Hb Bart’s hydrops fetalis. The fetus usually dies in utero or shortly after birth [11]. According to the degree of quantitative reduction in the synthesis of normal β-globin, β-thalassemia mutations are classified into three groups: (1) β^0-thalassemia mutation, which results in the absence of β-globin; (2) β^+-thalassemia mutation, which severely reduces the output of β-globin; and

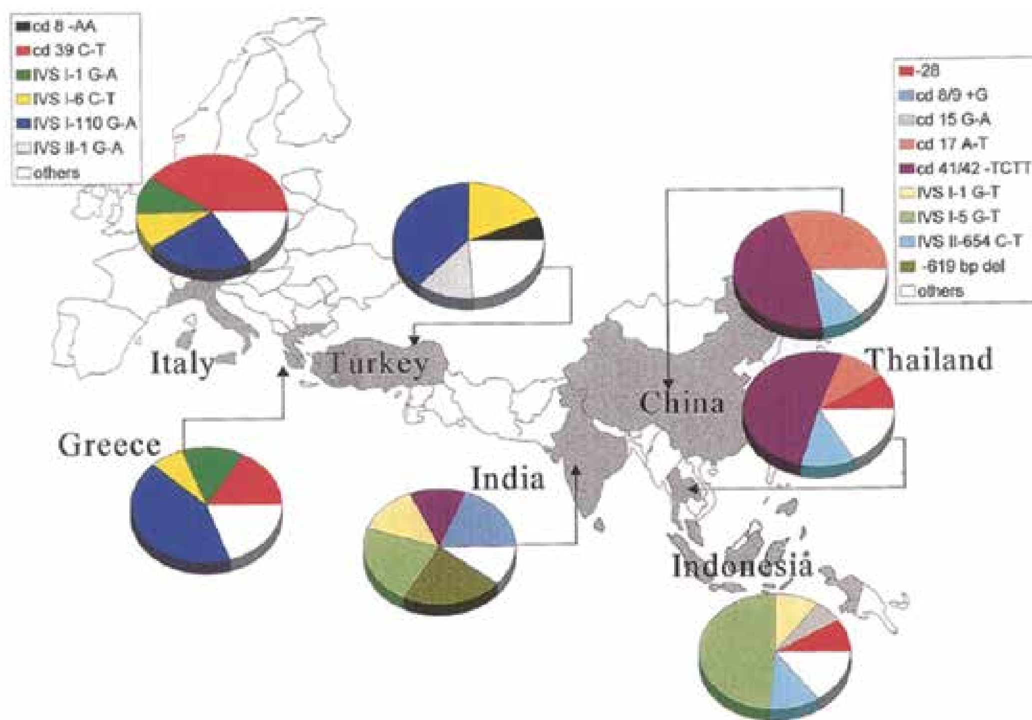

Figure 2. The distribution of common β-thalassemia mutations [7].

Locus	Mutation/deletion types	Common mutations
α-globin	α^0-deletion	$--^{SEA}$ (Southeast Asia), $--^{MED}$ (Mediterranean)
	α^+-deletion	$-\alpha^{3.7}$, $-\alpha^{4.2}$ (worldwide)
	$\alpha^T\alpha$ (α2 gene)	Hb CS (Southeast Asia), $\alpha^{IVS1(-5nt)}\alpha$ (Mediterranean), $\alpha^{PA(AATAAG)}\alpha$ (Middle East Asia)
	$\alpha\alpha^T$ (α1 gene)	Hb Q-Thailand (Southeast Asia)
β-globin	β^{++}-mutation	$\beta^{-101(C>T)}$ (Mediterranean)
	β^+-mutation	$\beta^{IVS1-110(G>A)}$ (Mediterranean), Hb E (Southeast Asia)
	β^0-mutation	$\beta^{CD39(C>T)}$ (Mediterranean), $\beta^{CD41-42(-CTTT)}$ (Southeast Asia)
	deletion (β gene)	619 bp deletion (Asian Indian)
	Deletion (HPFH/ζβ)	SEA-HPFH, Chinese $^G\gamma^+$ $(^A\gamma\delta\beta)^0$ deletion (Chinese)

Deletion (β gene): deletions affecting only the β-globin gene.
Deletion (HPFH/ζβ): large deletions involving part or all of the β-globin gene clusters.

Table 1. The common deletions and mutations of thalassemias [13].

(3) β^{++}-thalassemia mutation, also known as silent β-mutation, which mildly reduces the output of β-globin (**Figure 3**) [11].

Clinical presentation for thalassemia carriers is varied from almost healthy to severe anemia requiring blood transfusions all life [14]. A practical way to prevent thalassemia is identification of carrier couples; genetic counseling and offering prenatal diagnostic services for both carrier couples. Two carrier parents have a 25% chance of having an unaffected child, a 25%

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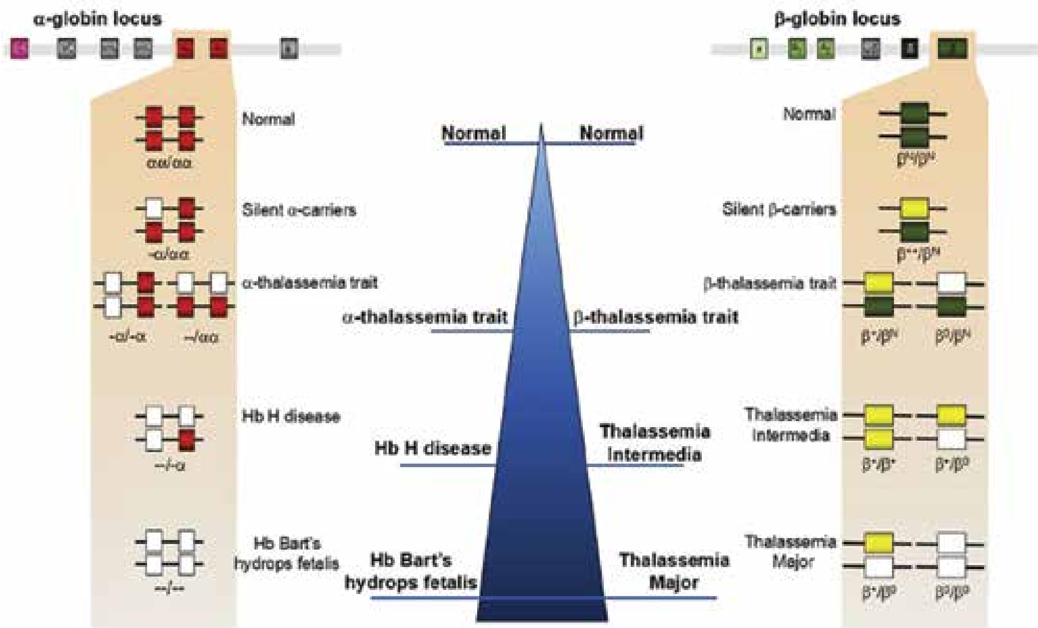

Figure 3. Clinic classification and genotype-phenotype association of thalassemias [11].

chance of having an affected one, and a 50% risk of having a carrier child. Carriers can only be detected using laboratory methods. Laboratories should increase the diagnostic proficiency for prevention of this blood disorder [13, 15, 16].

In this review, we aim to provide an algorithm consisting of biochemical and molecular methods in the screening of thalassemia carriers and evaluate traditional and new prenatal diagnosis methods for the disorder.

4. Identification of carrier and diseased individuals with thalassemias

Carrier detection is a necessity in populations in which both α- and β-thalassemia are prevalent. For laboratory diagnosis of thalassemias, molecular diagnostic algorithms should be produced by screening centers (**Figure 4**). Combining blood hematology/biochemistry and clinical parameters with laboratory analysis, discussing the clinical results with physicians is important as the beginning step for carrier identification. The hematological information obtained from a complete blood count (CBC) is the first essential test for screening thalassemias (**Figure 1**). Specific mutations of hemoglobin will cause hematologic changes.

Hypochromic microcytic parameters and anemia is generally mild in α- or β-thalassemia carriers. In cases with rare β-thalassemia, anemia can be intermediate or hemolytic and even severe because it is due to the reduced expression of the thalassemic gene and depends significantly on the degree of β-α imbalance [13].

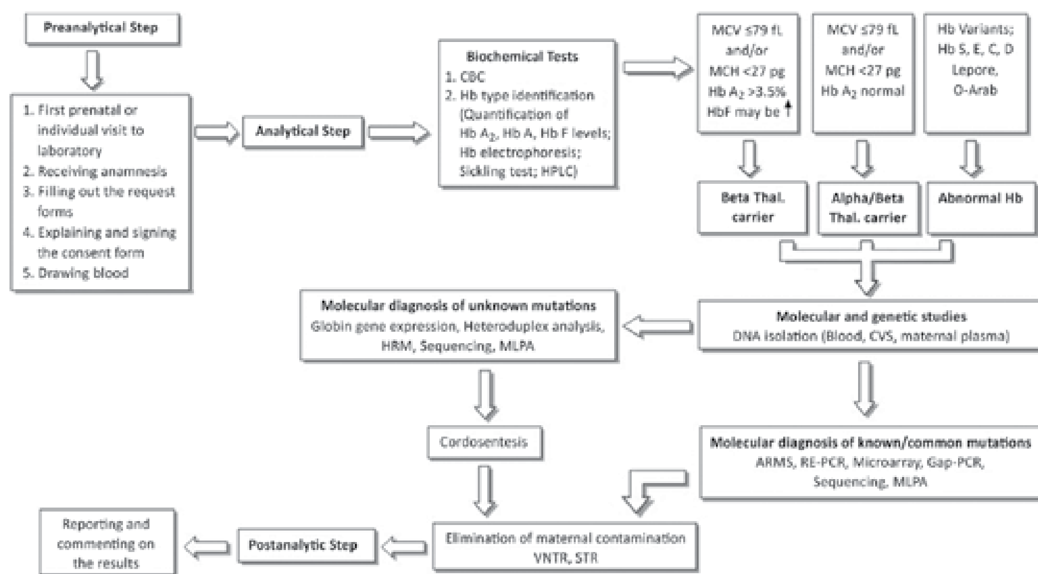

Figure 4. The algorithim of screening and prenatal diagnosis for thalassemias.

The first set of carrier detection procedure is the determination of main erythrocyte indices parameters such as the erythrocyte mean corpuscular volume (MCV), erythrocyte mean corpuscular hemoglobin (MCH) determination and also HbA_2 quantitation. The current screening strategies for thalassaemia carriers are to identify individuals with clinically related mutations. Hypochromic microcytic anemia occurs in both iron deficiency anemia and thalassemias. A decrease in red blood cell count (RBC) and an increase in red cell distribution width (RDW) is accompanied by iron deficiency anemia. Increased RBC count and normal RDW values have been seen in hemoglobin disorders [13]. Serum ferritin levels, the golden standard, will usually be normal or elevated in β-thalassemia carriers but might also be borderline or low in α-thalassemia carriers [13]. Decreased hemoglobin (Hb) concentration and MCV levels, altered shape and size of the RBCs (anisopoikilocytosis) and existence of target cells are additional hematologic phenotypes observed in individuals with hemoglobin abnormalities. When compared to MCV, the MCH is a more stable parameter, and values of <27 pg and <25 pg have been recommended as alternative screening cut-offs for β-thalassemia and α°-thalassemia, respectively (**Table 2**) [17].

4.1. β-Thalassemia

Heterozygote β-thalassemia (β° or β^+) is characterized by high red blood cell count, microcytosis, hypochromia, increased HbA_2 levels, and unbalanced α-globin/non-α-globin chain synthesis. Elevation of HbA_2 (standard cut-off value is above 3.5%) is the most important characteristic in identifying heterozygote β-thalassemia [7, 19, 20]. A number of heterozygotes for β-thalassemia may have normal or borderline HbA_2 levels [21]. Some typical carriers have mild β^+ thalassemia mutation, i.e., IVSI-6 (T-C) mutation. The δ- and β-double heterozygotes exhibit normal HbA_2 level, low MCV and MCH values. These double heterozygote cases should be separated from the α-thalassemia carriers. Carriers of γδβ- and δβ-thalassemias HbA_2 levels are also normal. δβ-thalassemias have specific elevated HbF

Traits	Hb	Ferritin	MCV	MCH	RBC	RCM
Iron deficiency	↓	↓↓	↓	↓	↓	APC, HY
β-Thalassemia trait	↓	↑↑	↓	↓	↑↑↑	APC, HY, TC
α-Thalassemia (-α/αα)	↓or N	↓N↑	↓	↓	↑	Rare TC, HY
α-Thalassemia (--/αα)	↓	N↑	↓	↓	↑↑	APC, HY, TC, rare IB
α-Thalassemia (-α/-α)	↓	N↑	↓	↓	↑↑	APC,HY, TC
α-Thalassemia (--/-α)	↓	↑↑↑	↓	↓	↓	APC, HY, TC, IB

Hb, hemoglobin; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; RBC, red blood cell; N, normal; APC, anisopoikilocytosis; HY, hypochromia; TC, target cells; IB, inclusion bodies [18].

Table 2. The most significant parameters observed from complete blood count, ferritin, MCV, MCH, RBC, and red cell morphology tests in the common traits at risk of intermediate or severe conditions for thalassemias.

levels, which distinguish this group from the others [5, 12]. In the presence of a normal MCH, hereditary persistence of fetal hemoglobin (HPFH) should be considered. Another problematic group difficult to identify is the silent β-thalassemia and the triple α-gene arrangement. In silent β-thalassemia, the MCV and MCH values are usually normal (i.e., β^+-101 C > T mutation). The value of HbA_2 and HbF are also normal (**Table 3**) [21, 22].

4.2. α-Thalassemia

α-Thalassemia mutations are mostly gene deletions. To identify known inherited deletional α-thalassemia mutations, gap-PCR (polymerase chain reaction) is the most common method. A multiplex gap PCR targeting common α gene deletions in a population is most often used as the first step in α-thalassemia genotyping. The sequencing method detects non-deletion α^+-thalassemia mutations. The five commonest α°-thalassaemia deletions can be diagnosed

Phenotype	Genotype
Normal red cell indices	• α- and β-thalassemia interaction
Normal HbA2 level	• Iron deficiency
	• Co-inheritance of δ- and β-thalassemmia
	• Some mild β-thalassemmia mutations
	• γδβ-thalassemia
Normal red cell indices and HbA2 level (silent)	• Silent β-thalassemmia mutations
	• α-globin gene triplication
Severe heterozygote β-thalassemmia	• Hyper unstable hemoglobin
	• Co-inheritance of heterozygote β-thalassemmia and triple α-globin gene

Table 3. Interpretations to consider when the hematologic is consistent with atypical β-thalassaemia trait [7].

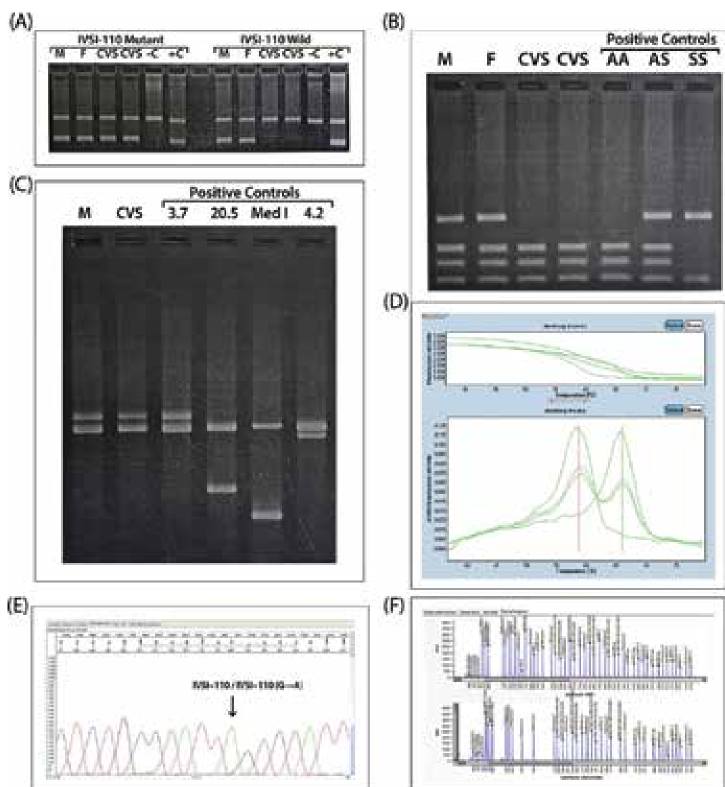

Figure 5. Applications of (A): ARMS-PCR, (B): RE-PCR for, (C): Gap-PCR, (D): HRMA and (E): sequencing analysis, (F): Detection unknown mutations using MLPA for deletional alpha, beta gene mutations (M: Mother, F: Father, C: Control, CVS: Chorionic villus sample).

by gap-PCR: the --SEA allele, the --MED and -(α)20.5 alleles; the --FIL allele, and finally the --THAI allele. The two commonest α^+-thalassemia deletions are also diagnosed by gap-PCR: the -α3.7 and -α4.2 alleles. In our laboratory, the common deletions detected with gap-PCR are -α3.7, and -(α)20.5, --MED and -α4.2 (**Figure 5**).

5. Molecular methods

Blood count is not always consistent with typical β-thalassemia trait. Furthermore, there is no specific screening test for the clear identification of heterozygote α-thalassaemia. Only molecular DNA analysis could give certain results in rare mutations [23, 24].

Molecular methods for DNA analysis of hemoglobinopathies currently in use are based on PCR methods that can be used to detect the globin gene mutations. The PCR-based methods differ in identifying hemoglobin variants. Amplification refractory mutation system (ARMS), denaturing gradient gel electrophoresis (DGGE), restriction endonuclease PCR (RE-PCR), real-time PCR, high-resolution melting analysis (HRM), sequencing analysis (Sanger), pyrosequencing,

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microarrays can be counted among these PCR-based detecting methods [19, 25]. To detect deletions, Southern blot analysis has been replaced by methods including gap-PCR, multiplex ligation-dependent probe amplification (MLPA), and array comparative genome hybridization (aCGH) [19]. Gene scanning methods such as HRM analysis are also useful for locating possible β-globin gene variants [26]. This technique allows the detection of mutations between the primers used in the assay, which is in contrast to more localized techniques such as hybridization based technologies or restriction enzyme-based assays [27]. In diagnostic use, for this method, it is mandatory to characterize any nucleotide variation by automated sequencing because they do not determine nucleotide changes. Small deletions can be detected by polyacrylamide gel electrophoresis of an amplified beta gene product. Polyacrylamide gel electrophoresis can be used to detect small deletions in an amplified β gene product. Gap-PCR and recently MLPA identifies larger deletions from the β-globin gene [10]. MLPA detects all common, rare, and novel forms of deletional α-thalassemia (in contrast to gap-PCR) and provides a reliable alternative screening method for the prenatal diagnosis of α°-thalassemia [28, 29, 30]. This technique can also identify triple and quadruple α-gene re-arrangements [31]. β°-Thalassemia, which is caused by small nucleotide deletions and some larger deletions which the whole β-globin gene removes, can be identified by gap-PCR (Hb Lepore, some δβ-thalassaemia deletions, and the HPFH1/2/3 deletion mutations, etc.).

5.1. Sequencing analysis

The parents' DNA whose mutations were not found by classic PCR methods were analyzed by ABI 3130 automatic sequencer. The DNA of the cord blood samples were sequenced to confirm the cordosentesis results. BigDye Terminator v3.1 Cycle Sequencing kit (ABI) and primers that cover all the exons, introns,and exon-intron boundaries of the β-globin gene were used for sequencing analysis.

6. Prenatal diagnosis of thalassemias

Prenatal diagnosis created a new option to couples at risk of a major hemoglobinopathy and changed the perspective of screening and counseling for thalassemias [7]. The first step to prevent thalassemia is prenatal diagnosis of these hematological disorders. Prenatal diagnosis for thalassemias is still carried out by traditional conventional methods such as amniocentesis, chorionic villus sampling (CVS), and cordocentesis. These conventional methods have a risk of fetal miscarriage risk around 1% [32].

6.1. Blood sampling from parents

Phenotype of parents should be performed by DNA analysis after whole blood count and electrophoresis. Hematology results should be sent to the molecular diagnostic laboratory. Antenatal screening of parents should be performed before the first trimester of pregnancy [17].

6.2. Fetal sampling

Fetal DNA for analysis can be obtained by traditional invasive methods; amniocytes, CVS, and cordosentesis [4, 36].

6.2.1. Chorionic villus sampling

A fetal-derived tissue, genetically reflecting the fetus and easily accessible in the first trimester (up to 11 weeks) can be used for the prenatal diagnosis of hemoglobinopathies. High-quality DNA can be obtained from CVS material to perform DNA analysis. Maternal contamination is low, especially if careful microscopic dissection is performed to remove contaminating maternal tissues prior to DNA extraction and analysis.

6.2.2. Amniocentesis

This is the most commonly used method among invasive prenatal diagnostic methods. Amniotic fluid is the environment of life before the baby is born, and all secretions are in this atmosphere [33]. The prenatal diagnosis result based on an amniocentesis is available later in pregnancy compared to CVS, as amniocentesis is not usually performed earlier than the 15th week [19].

6.2.3. Fetal blood sampling

This sampling method can be used for molecular analysis, globin chain synthesis studies, or high-pressure liquid chromatography (HPLC). It can be useful in women at risk of α-thalassaemia hydrops fetalis. Fetal blood sampling is associated with a higher rate of miscarriage and results are available much later in pregnancy (after 18–20 weeks) [19].

6.2.4. HPLC analysis of cord blood

Cord blood is taken in ethylenediaminetetraacetic acid (EDTA) by an obstetrician at 18–20 weeks of gestation. The hemograms are measured for all samples by using an automatic blood cell counter (Coulter T-890). HPLC analysis were studied from the prepared hemolysate by Agilent 1100 using the thalassemia short program. The levels of HbA, HbF and Hb Barts, and HbS were estimated.

Molecular diagnostic methods that are based on PCR techniques (ARMS, RFLP, GAP-PCR, VNTR, etc.) and high-throughput technologies (Gene expression, HRMA, microarray, MLPA, NGS, etc.) are currently used in prenatal diagnosis. The molecular genetic tests for prenatal diagnosis within the first 2 months of pregnancy is necessary to prevent infants of carrier couples from being thalassemia [19].

6.3. Remove the contamination risk

Maternal cell contamination test is recommended in all prenatal samples to remove the risk of contamination of fetal DNA with maternal DNA. It is important to pay attention to all CVS and AF samples (with or without culture) may have maternal contamination. Maternal contamination should be ruled out after careful dissection of CVS from maternal tissue.

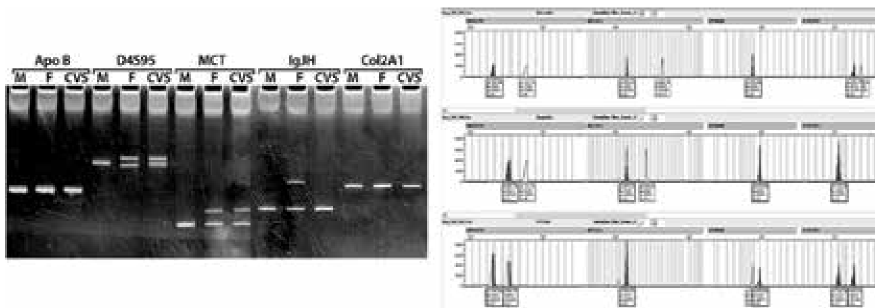

Figure 6. VNTR and STR analysis of a family for checking the maternal contamination in CVS. M: Mother, F: Father, CVS: Choronic Villus Sampling.

Figure 7. Noninvasive prenatal diagnosis using cfDNA from maternal plasma.

Polymorphic DNA sites in fetal and parental samples can be identified to monitor maternal contamination (**Figure 6**).

6.4. New diagnostic tests

The discovery of cell-free fetal DNA (cffDNA) in the maternal plasma give the chance to conduct noninvasive prenatal diagnosis (NIPD) during pregnancy [34]. Some encouraging clinical noninvasive approaches to detect paternally inherited mutations such as the detection of fetal sex and RhD status have been improved successfully [35, 36].

Noninvasive tests using cell-free DNA (cfDNA) from a maternal blood sample is also an alternative method, thus eliminating the risk of miscarriage (**Figure 7**). Cell-free fetal DNA (cffDNA) constitutes approximately 10–15% of the total cfDNA and has been shown to represent the entire fetal genome [35].

6.5. Centrifugation

The noninvasive prenatal detection of paternal mutations in β-thalassemias is achievable using HRM analysis, and next generation sequencing of cell-free fetal DNA [36].

7. Conclusion

β-Thalassemia is an autosomal recessive disorder characterized by hemolytic anemia and micrositosis. It causes decreased synthesis of β-globin chain. This disorder influences 150 million people in large parts of Asia, North Africa, and in the Mediterranean. A wide range of mutations occurs due to the effects of different cultures living together in Turkey. Genetic heterogenity is more in the southern part of Turkey than in other regions.

Premarital screening and genetic counseling are essential for the prevention and control of thalassemia and hemoglobinopathies [9]. Today, prenatal diagnosis by chorionic villus sampling is an accepted method to protect families having children with thalassemia major and to keep the disease under control. The mutation can be identified from samples obtained by chorionic villus sampling (CVS) and amniosentesis methods by classic polymerase chain reaction (PCR).

The molecular diagnostic algorithms should be produced by genetic diagnosis centers for screening of the carriers and prenatal diagnosis of the couples both of whom carriage has been detected before pregnancy for protection against thalassemia.

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Section 4

Thalassemia - Complications

Chapter 5

Hypogonadism in Female Patients with Beta Thalassemia Major

Alice Ioana Albu and Dragos Albu

Additional information is available at the end of the chapter

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Abstract

Beta thalassemia is the most frequent hemoglobinopathy worldwide. In patients with beta thalassemia major (BTM), the consequence of long-term life-saving transfusions is iron overload in liver, heart and endocrine glands. Hypogonadotropic hypogonadism is the most frequent endocrine complication. Recent progresses in the treatment of BTM dramatically improved life expectancy and quality of life of these patients, making the concern for fertility and pregnancy to gain importance. Therefore, we performed a review of the available data regarding hypogonadism in female patients with BTM. We found that hypogonadotropic hypogonadism is still frequently found in female patients with BTM. Pituitary iron overload seems to be the main factor contributing to hypogonadism occurrence, although iron-related damage of the ovaries and the genital tract cannot be excluded. The increased oxidative stress observed in BTM patients was hypothesized as a contributor to pituitary-gonadal dysfunction. Hypogonadism has significant consequences on quality of life, final height, bone health and fertility of the patients. Estro-progestative administration is essential in order to minimize consequences, although the best treatment regimen should be carefully weighted in each patient. Although spontaneous fertility is reduced by the presence of hypogonadism, it seems that ovulation-induction treatment with gonadotropins is effective in achieving pregnancies in majority of patients.

Keywords: beta thalassemia, fertility, infertility, hypogonadism, pregnancy, short stature, osteoporosis

1. Introduction

Beta thalassemia major (BTM) is a severe medical condition that requires long-term life-saving transfusions. As a consequence, secondary hemosiderosis results in significant morbidity due

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to organ injury produced by excessive iron. Most frequently affected organs are heart, liver and endocrine glands. While iron-induced cardiomyopathy is the most common cause of death in BTM patients, endocrinopathies are among the most frequent complications, leading to a low quality of life. In the last decades, increase of iron chelating agents' availability and better compliance to treatment with oral agents was followed by an increase in life expectancy and a decrease of morbidity in BTM patients. However, the prevalence of endocrine complications remained high enough to significantly impact their quality of life. Hypogonadism is the most prevalent endocrine complication in BTM patients and affects patients from various age groups. The early forms of hypogonadism have a clinical impact starting at age of 10–11 years as decreased growth velocity, short stature and delayed pubertal development. In pubertal patients, the appearance of hypogonadism results in arrested pubertal development and lack of sexual maturation. In adult patients with full pubertal development, the occurrence of hypogonadism causes regression of secondary sexual characteristics, sexual dysfunction and infertility. Therefore, hypogonadism has a significant psychological impact and is associated with complications as infertility, osteoporosis and short stature which lead to additional morbidity and medical care requirements. Early diagnosis and treatment of hypogonadism has a particular importance for future fertility in female BTM in order to maintain the normal genital tract and to avoid complications as short stature and osteoporosis which will interfere with normal evolution of a future pregnancy.

Therefore, the aim of the present chapter was to perform a review of the available data regarding the prevalence, physiopathology, consequences and treatment of hypogonadism in female patients with BTM. We searched in PubMed and Google Scholar using the following key words: beta thalassemia, fertility, infertility, hypogonadism, pregnancy, short stature and osteoporosis. Only articles written in English were selected.

2. The prevalence of hypogonadism in BTM patients

Hypogonadism is the most frequent endocrine complication in BTM patients, the prevalence being as high as 50–100% in various populations [1–4]. The high variability in the proportion of patients affected by hypogonadism could be explained by ethnic factors, different availability of therapeutic agents, economic status and genetic susceptibility. For example, in the study by Gulati et al. [4], 10 of 11 BTM young patients (90%) from a developing country were presented with early form of hypogonadism. In turn, a study on 382 BTM patients treated with desferrioxamine at the **Thalassemia** Centre in Dubai showed a significantly lower prevalence of hypogonadism of only 25% [5]. However, most studies reported a prevalence of hypogonadism of approximately 50% [6].

In spite of significant progresses on the therapeutic regimens of thalassemia major aiming to correct most of the factors considered responsible for BTM complications, hypogonadism continues to be frequently found in these patients. Moreover, it seems that the prevalence was not significantly reduced along with hematologic treatment improvement [7]. Gamberini et al.

followed a cohort of 273 patients with BTM from diagnosis for 30 years in Ferrara Centre and observed that, although the incidence of the other endocrine complications (hypothyroidism, hypoparathyroidism and diabetes mellitus) has decreased over time, the proportion of patients with primary and secondary amenorrhoea was similar in the three cohorts according to the year of birth [7]. Moreover, the hypogonadism prevalence did not decrease along with the decrease in serum ferritin over time, suggesting irreversible iron-induced lesions or an increased susceptibility of pituitary cells to damages produced by excessive iron.

3. Etiopathogenesis of hypogonadism in BTM patients

3.1. Etiology of hypogonadism in BTM

Hypogonadism is defined as low levels of sex hormones as a consequence of decreased production by the gonads. There are two types of hypogonadism: hypogonadotropic hypogonadism (or secondary gonadal insufficiency) due to a pituitary defect in gonadotropins production and hypergonadotropic hypogonadism (or primary gonadal insufficiency) due to a defect in gonadal steroidogenesis. In both forms of hypogonadism, the clinical picture is the same with amenorrhoea, regression of secondary sexual characteristics (breast, uterus and vaginal atrophy) and hot flushes. In hypogonadal patients, the gametogenesis is also affected, the consequence being anovulation and infertility. In BTM, most patients with hypogonadism are presented with low gonadotropins levels indicating the presence of hypogonadotropic hypogonadism [2]. Although these data support the existence of a pituitary defect, the coexistence of an ovarian lesion cannot be excluded. However, the success of ovulation induction with gonadotropins in hypogonadic female BTM patients shows that, if ovarian lesions coexist, these are mild or reversible with a proper iron chelation as part of preconceptional care.

Iron overload is considered as the main factor involved in pathogenesis of hypogonadism in BTM patients. This hypothesis is supported by several types of observations. For instance, most studies reported an association of high serum ferritin levels with the presence of hypogonadism [5, 8] or with a faster evolution to hypogonadism [8]. Serum ferritin level is considered an accurate marker of tissue iron deposits, being correlated with T2* magnetic resonance imaging of the heart, liver [8] and pituitary [9]. Moreover, magnetic resonance imaging showed that pituitary iron and volume loss predict the presence of hypogonadism [10].

Whether the hypogonadism is reversible with a better chelation therapy is incompletely clarified. In some of the studies, the recent serum ferritin was poorly correlated to the presence of hypogonadism, suggesting that a previous deleterious effect of iron overload could not be reversed by decreasing glandular iron [6]. However, the study by Chatterjee and Katz [11] found that BTM patients with less severe iron overload and organ damage in the hypothalamic-pituitary axis could present with potentially reversible hypogonadotropic hypogonadism. In turn, patients with severe iron overloaded had an irreversible form of hypogonadism [11].

It was also suggested that the anterior pituitary has a high susceptibility to iron accumulation that could be explained by the increased number of transferrin receptors in this tissue [12].

Other factors were observed to be associated with the presence of hypogonadism, being possible contributors to its etiology. For instance, the study by Belhoul et al. demonstrated that splenectomy is related to the presence of hypogonadism independently of ferritin levels [5], the mechanism behind this association being unknown. It was also reported that the development of hypogonadotropic hypogonadism is associated with the severity of the underlying genetic defect in beta-globin synthesis gene [13, 14]. The most probable explanation is that the patients with severe defects in hemoglobin synthesis require higher quantities of transfused blood, followed by a more severe iron overload. Liver dysfunction, diabetes, hypothyroidism [15] and lower hemoglobin levels [16] were also reported as possible contributors to hypogonadism.

3.2. Mechanisms involved in hypogonadism occurrence in BTM

In patients with BTM, the excessive iron deposition in tissues, following chronic life-saving transfusion regimens, generates an increase in non-transferrin bound iron. This form of iron has high tissue toxicity due to the excessive production of the reactive oxygen species (ROS) [17]. Because ROS have a pro-oxidant activity, the excessive production of ROS disrupts the oxidants/antioxidants balance of the cells generating chronic oxidative stress [18]. Moreover, the antioxidant mechanisms in BTM patients could be decreased, contributing to oxidative stress. The antioxidative defense is performed by enzymic (superoxide dismutase, catalase, and glutathione peroxidase) and nonenzymic systems (scavenging molecules endogenously produced like GSH, ubiquinols and uric acid or derived from the diet, such as vitamins C and E). In patients with BTM, the level of vitamin E, glutathione peroxidase and superoxide dismutase [19], total antioxidant capacity [20], ascorbate, vitamin A, beta-carotene and lycopene [20] were found to be significantly lower in comparison to controls [19, 21, 22], supporting the existence of a decreased antioxidant defense. Microelements essential for the function of the antioxidative enzymes were also reported to be decreased in BTM patients [20]. Moreover, HCV infection and hepatic dysfunction, frequently found in BTM patients, might be involved in the depletion of antioxidant mechanisms [23, 24]. Growth hormone deficiency, another common complication in BTM patients, is a condition recognized for its association with increased oxidative stress [25], being a possible contributor to redox imbalance in BTM (**Figure 1**).

As a consequence of chronic oxidative stress, the fatty acids in membranes of cells suffer a peroxidation process, resulting cytotoxic products will impair cell function, protein synthesis and DNA structure [26].

Although no direct evidence exists linking oxidative stress with gonadal dysfunction in BTM patients, oxidative stress is a reasonable putative factor based on data demonstrating their involvement in female fertility in general population. For example, in the study by Appasamy et al. was showed that, in patients performing assisted reproduction, total antioxidant capacity

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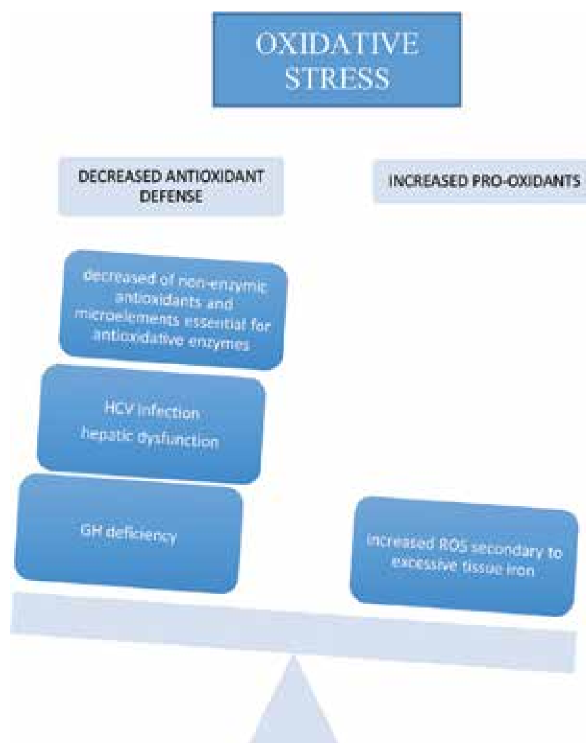

Figure 1. The mechanisms hypothesized to be involved in the appearance of oxidative stress in beta thalassemia major.

was positively associated with follicular fluid estradiol levels [27], indirectly supporting that oxidative stress might have a negative impact on follicular development. It was also suggested that oxidative stress contribute to the decrease of fertility related to age [18] and that non-heme iron deposits in ovarian stromal tissue is associated to oxidative stress-induced ovarian aging [28]. Moreover, low level of antioxidants was found to be related to anovulation in women [29].

The indirect evidence that oxidative stress is involved in iron overload-related complications is provided by interventional studies showing the supplementation of antioxidants as vitamin C and E in children with BTM is followed by improved liver function [30]. However, similar evidence regarding the improvement of female fertility in BTM following antioxidants administration does not exist.

The link between oxidative stress and iron overload in BTM is suggested by reports showing that iron chelation agents desferrioxamine (DFO) and deferasirox are effective in decreasing iron overload along with oxidative stress [31]. Similarly, the study by Kuppusamy et al. found that chelating treatment is associated with lower oxidative stress in BTM patients [32].

Other mechanisms were also proposed to be involved in hypogonadism occurrence in BTM. For example, some authors suggested that adipose tissue dysfunction due to iron toxicity could contribute to hypogonadotropic hypogonadism occurrence through a perturbed leptin

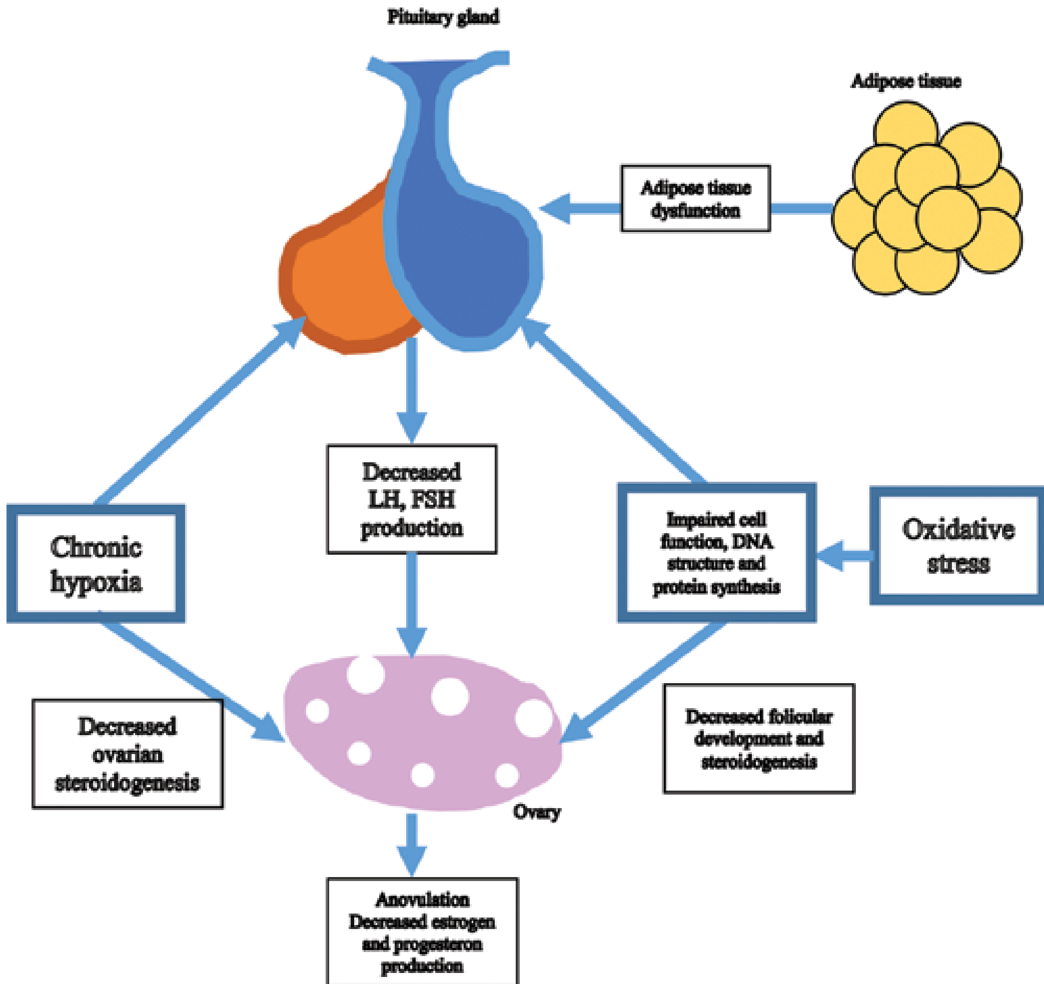

Figure 2. The etiopathogenesis of hypogonadism in beta thalassemia major.

production [33, 34]. Liver dysfunction, diabetes and hypothyroidism were found to be associated to hypogonadism [15], probably contributing to its occurrence by perturbed hormone metabolism [35].

Chronic hypoxia due to anemia could be another mechanism involved in pituitary-gonadal dysfunction in BTM. In support of this hypothesis are the studies showing decreased gonadotropin secretion few days after arriving at moderate altitude [36]. Moreover, it was observed that the fertility rate is lower in high altitude population in comparison to low altitude, probably as a consequence of a different reproductive hormones profile [37]. Animal studies show that exposure to high altitude hypoxia affects the development and function of corpus luteum [38]. Moreover, in men hypoxemia due to obstructive pulmonary disease was associated with hypogonadism and low testosterone levels [39], probably by downregulation of

androgen biosynthesizing genes in the testis as demonstrated by studies in rats [40]. However, similar effects of hypoxemia on ovary were not demonstrated (**Figure 2**).

4. Consequences of hypogonadism

4.1. Osteoporosis

Osteoporosis in BTM patients is one of the most frequent complications, multiple factors contributing to their appearance, being a major cause of morbidity in these patients as well. Hypogonadism is a widely recognized risk factor for secondary osteoporosis. However, in patients with BTM, the contribution of hypogonadism to osteoporosis pathogenesis was reported variable in different populations. The study by Anapliotou et al. showed that patients with hypogonadism had the lowest bone mineral density (BMD) and the sex steroids serum level was the only parameter related to BMD measurements [41]. Moreover, the patients who received continuous sex steroid replacement therapy had a better increase of their BMD in comparison to those with intermittent treatment [41].

Although genetic factors play a role in other types of osteoporosis, the study by Origa et al. found no association of osteoporosis with specific polymorphisms in candidate genes (vitamin D receptor, estrogen receptor, calcitonin receptor and collagen type 1 alpha 1) [42]. Instead, in female patients, osteoporosis was strongly associated with primary amenorrhea [42].

Similar findings are reported by Tzoulis et al. who find no relationship of low bone mass with vitamin D status, the only significant association observed in the multivariate analysis being between hypogonadism and low BMD at the lumbar spine [43].

However, the study by Chatterjee et al. reported that, in comparison to premature ovarian failure patients, those with BTM increased, but did not normalized their BMD following hormone replacement therapy. The authors concluded that other factors contribute to osteoporosis in patients with BTM [44]. Skordis et al. summarized the current knowledge about osteoporosis in BTM, highlighting the fact that GH-IGF 1 axis dysfunction, hypoparathyroidism, hypothyroidism, diabetes and vitamin D deficiency are important contributors as well [45]. Therefore, osteoporosis in BTM should be considered a multifactorial condition, hypogonadism being probably an important contributor to its appearance. All the contributors to osteoporosis should be addressed in order to reduce the incidence and to optimize the effect of treatment.

4.2. Infertility

Although spontaneous pregnancy can be obtained by the female patients with BTM, most of them are infertile. However, in most of the studies, pregnancies were obtained after ovulation induction with gonadotrophins or assisted reproduction. Skordis et al. reported spontaneous pregnancies among 34 patients with regular menstrual cycles and induced pregnancies in patients with primary or secondary amenorrhoea [46]. In the study by Origa et al., pregnancy was achieved with gonadotropins stimulation in 33 of 46 women with BTM [47]. Bajoria et al.

reported their experience with 11 BTM patients undergoing assisted reproductive technology (ART). They found that 60% of infertile female patients with BTM present with hypogonadotropic hypogonadism and respond favorably to gonadotropins administration [48], suggesting that central hypogonadism is the main cause of infertility in these patients. Moreover, in patients with regular menstrual cycles, fertility is usually preserved [46]. All these data suggest that pituitary dysfunction is the main cause of infertility and that the ovaries are usually intact in BTM female patients. In the light of these data, although few other factors were suggested as contributors to infertility, they do not seem to play a major role. Decreased endometrial receptivity due to iron deposition [49] is one of the factors possibly involved in infertility, although the evidences are limited. Only one study evaluated this aspect and demonstrated the presence of hemosiderin deposits in the endometrial epithelium of three patients with BTM [49]. The desferrioxamine administration resulted in reduction or disappearance of hemosiderin depositions [49].

The integrity of the ovarian tissue in patients with BTM is a matter of debate. Singer et al. [50] found that the ovarian reserve is preserved in most thalassemic patients younger than 30–35 years old. In the same study, the circulating levels of anti-Müllerian hormone (AMH), an accurate marker of ovarian reserve, were correlated with non-transferrin-bound iron, suggesting the involvement of labile iron in the regulation of ovarian reserve [50]. In turn, the study by Chang et al. demonstrated that the serum levels of AMH are lower in women with transfusion-dependent β thalassemia than in age-matched normal controls, suggesting a reduced ovarian reserve in the former [51]. Moreover, the serum AMH level in BTM patients was significantly inversely related to the ferritin level. Therefore, both studies suggest that iron overload can affect directly the ovaries, although the extent of the damage could vary depending on the severity of iron excess.

Although the presence of oxidative stress in BTM is widely recognized, its deleterious effect on oocyte maturation, fertilization, embryo development and implantation described in general population [52, 53] is not clearly linked to fertility in BTM patients. The success of ovulation induction with gonadotropins in hypogonadal patients suggests deleterious effect of iron depositions and oxidative stress on ovaries is not severe enough to impair the response to treatment. However, the hypothesis that preconceptional care of patients undergoing fertility treatment, including intensive chelation therapy prior to pregnancy, could reverse mild ovarian hemosiderosis and ovarian oxidative stress-related injuries should not be neglected. Moreover, patients referred for infertility treatment are selected in order to lower the risk of complications during pregnancy and those with severe iron-related complications are excluded. Therefore, the population of women treated for infertility is not completely relevant for the general population of female BTM patients and definitive conclusions regarding involvement of ovarian iron deposits in infertility should not be drawn.

4.3. Quality of life

The quality of life in patients with BTM was widely reported to be decreased due to affected physical, emotional and social functioning. Hypogonadism seems to be an early and significant contributor to decreased quality of life in BTM as suggested by a recent study which

evaluated the quality of life in adolescent BTM patients [54]. The authors observed that BTM adolescents had poor perception of their general health and scored significantly lower in all the subscales compared with the controls [54]. The high prevalence of short stature and pubertal delay was associated with lowest scores for physical and psychological domains [54], suggesting the important contribution of these endocrine complications to decreased quality of life of BTM patients. The siblings of BTM patients also scored significantly less in environment domain, probably reflecting the impact of BTM within families [54].

4.4. Short stature

Short stature is frequently found in patients with BTM, being variably reported in different populations, with prevalence as high as 30% in female patients [55]. Skordis et al. reviewed the growth disturbances in BTM patients [45]. They concluded that the etiology is multifactorial, causative factors including hemosiderosis, hypoxia secondary to chronic anemia, chronic liver disease, nutritional deficiencies, intensive iron chelating therapy, emotional factors, endocrinopathies (hypogonadism, delayed puberty, hypothyroidism, disturbed calcium homeostasis and bone disease) and dysregulation of the GH-IGF-1 axis [45]. The authors also describe three phases of growth in BTM children: a first phase in which growth disturbance is due to hypoxia, anemia, ineffective erythropoiesis and nutritional factors; a second phase during late childhood during which growth retardation is mainly due to GH-IGF-1 axis dysfunction probably secondary to iron overload; a third phase that starts after the age of 10–11 years when delayed or arrested puberty contribute significantly to growth failure [45].

A multicenter international study performed by Thalassaemia International Federation (TIF) in 2004 including 29 Centers and 3817 patients reported that short stature is present in 31.1% of males and 30.5% of females. However, the prevalence of growth hormone deficiency was only 7.9% in males and 8.8% in females, suggesting that other causes contributes significantly to height deficit [55]. In turn, delayed puberty was the most common endocrine complication (40.5%) [55].

Delvecchio et al. reviewed 123 papers and concluded that disproportionate short stature is frequent and aggravates at puberty because of the lack of growth spurt. Later on, partial height recovery may occur. Long-term treatment with recombinant human growth hormone seems ineffective to improve final height [56]. Therefore, short stature is associated with early forms of hypogonadism rather than with growth hormone deficiency, supporting of involvement of the former in the etiology of height deficit.

5. Treatment of hypogonadism

5.1. Hormone replacement treatment

The aim of the treatment of hypogonadism in female BTM patients is to maintain secondary sexual characteristics, to increase the quality of life and to counteract the negative impact on bone health. However, due to the complexity of conditions accompanying BTM, some

particularity should be taken into account when treating hypogonadic BTM patients. The sequential estrogen-progestogen regimens are the cornerstones of treatment in women with hypogonadism. These can be administrated as combined oral contraceptives (COC) or hormone replacement treatment (HRT). Depending on the preparation used the doses of estrogens vary, being higher in COC than in HRT, and the type of progestogen differs. The routes of hormones administration might be also different, the estrogens being administrated orally, vaginally or transdermally in most of the preparation. Progestogens can be administrated as oral tables, transdermal patch or intrauterine devices. Natural progesterone is available for oral, vaginal and transdermal routes. The major concern regarding the use of estro-progestative in BTM patients is the risk of thromboembolic events which is a well-known complication in COC and HRT users [57]. BTM is a condition characterized by an increased risk for thromboembolism [58, 59], mainly due to thrombocytosis secondary to splenectomy and to decreased production of anticoagulants due to chronic liver dysfunction. Therefore, the risk of thrombosis associated to COC or HRT administration can be even higher in BTM patients, although no study addressed this aspect. It was suggested that BTM patients with cardiomyopathy, diabetes, liver function anomalies and hypothyroidism [59], splenectomy and inadequate transfusion regimen [60] could have an increased risk of thromboembolic events, representing potential categories of patients in which COC administration should be avoided. However, among COC those containing newer progestin compounds (desogestrel, gestodene, drospirenone and cyproterone acetate) were reported to be more thrombogenic in comparison to older ones [61–63] and this aspect should be taken into account in BTM patients. Moreover, estrogens administered transdermally seem to have no prothrombotic effect [63], probably because the high estrogens concentrations in portal system generated by the oral administration of estrogens are avoided. In turn, vaginal ring has a similar venous thromboembolism (VTE) risk to third and fourth COC generation [63]. All these factors should be weighted when the decision to administer estro-progestative preparation for hypogonadism in a female BTM patient should be taken. Although a combined regimen with transdermal estrogen and natural progesterone may seem advantageous in BTM patients due to an assumed decreased risk of thrombosis, no data proving that hypothesis are published. Moreover, strict recommendations (COC versus HRT) in other categories of patients requiring hormonal replacement like those with premature ovarian failure are not available. Meanwhile, a recently published paper in International Network of Clinicians for Endocrinopathies in Thalassemia and Adolescent Medicine (ICET-A) [64] recommend that the clinician should take into consideration the United States Medical Eligibility Criteria (US MEC) for Contraceptive Use [65]. According to these criteria, no restriction exists for women with BTM, chronic hepatitis or with non-complicated insulin-dependent or non-insulin-dependent diabetes. However, in women with past VTE and known thrombogenic mutations (e.g., factor V Leiden; prothrombin mutation; and protein S, protein C and antithrombin deficiencies) and in women with diabetes associated with nephropathy, retinopathy or neuropathy, the risk is considered unacceptable if the contraceptive method is used [64, 65]. In patients with cholelitiasis COC administration represents a contraindication only in the presence of symptoms requiring medical treatment [65].

Some specific recommendations are made by ICET-A for patients with BTM [64]. They recommend that the physician should take into account the risk and benefits of estro-progestative administration in each particular patient and thoroughly discuss with the patients before

starting the treatment. Splenectomized patients should receive antiagregant or anticoagulant therapy during estro-progestative administration. In acute liver disease, estro-progestative is contraindicated, but their administration should be reinitiated after the acute episode has passed. They also consider the transdermal estradiol in association with micronized progesterone 'the most physiologic regimen with the best safety profile', being an option in women with risk factors for venous thromboembolism [64].

In order to reduce the risk of treatment, it is recommended that the serum estradiol level maintained approximately 100 pg/mL [66]. This level could be obtained with 25–50 μg 17β estradiol administered transdermally [67].

In patients with delayed puberty, the treatment should be started with low doses of transdermal, oral or parenteral estrogens. No particular regimen was reported for BTM patients and data from other populations can be used. The doses should be gradually increased (with 25–100% every 6 months) over 2–3 years to mimic normal puberty and progesterone should be added when vaginal bleeding occur or after 2 years of estrogen administration [68]. The initial proposed estrogens doses are: transdermal estradiol 3–7 μg/day, oral ethinyl estradiol 2 μg/day, micronized oral 17β estradiol 0.25 mg/day and depot estradiol 0.2 mg/month [68]. The adult doses that should be reached are: transdermal estradiol 25–100 μg/day, oral ethinyl estradiol 10–20 μg/day, micronized oral 17β estradiol 1–4 mg/day and depot estradiol 2 mg/month [68].

5.2. Infertility treatment

As a consequence of improved care of BTM patients and increased quality of life and life expectancy, an increasing number of women with BTM desire pregnancy. The existing reports show that in BTM women with normal gonadal function, the pregnancy can be obtained spontaneously, while in hypogonadal patients fertility is usually retrievable [47, 48]. Patients with BTM contemplating pregnancy should be addressed to a BTM-specialized preconception care department. During evaluation, the fertility potential and the woman's fitness for pregnancy should be assessed and the risks of pregnancy should be discussed. The iron overload consequences and especially liver and heart involvement is the main target of preconception evaluation, ensuring that associated conditions are stabilized and the potential of decompensation during pregnancy is minimized. The associated medications should be reviewed in order to exclude those with teratogenic risk and genetic screening of the partner should be provided in order to reduce the risk of a hemoglobinopathy in the baby.

Although the hypogonadotropic hypogonadism is the cause of infertility in most of the patients, a full evaluation is necessary in all the patients in order to identify associated causes (tubal obstruction and male factor). The first line of treatment in infertile BTM female patients with hypogonadism without other causes of infertility is ovulation induction with gonadotropins. Although some studies raised the question whether ovaries are injured by iron overload, most of the patients respond to this treatment [47]. There are no specific stimulation protocols for BTM patients, standard regimens being usually effective. In case of repeated failure of ovulation induction with gonadotropins or in couples with oligo/azoospermia or tubal infertility, in vitro fertilization is indicated. Growth hormone deficiency is not infrequent among adult BTM patients and can be involved in fertility treatment success in selected cases as

suggested by Surbek et al. [69] in their study. Although in patients with BTM data are limited, numerous experimental studies suggest that growth hormone acts on oocytes competence and ovarian steroidogenesis. Moreover, in patients with poor response to assisted reproduction growth hormone administration can improve the outcome of infertility treatment. In the light of these data, future studies should clarify the utility of growth hormone administration in infertile BTM female patients.

In conclusion, hypogonadism is still a frequent complication in BTM female patients having a significant impact on their health and quality of life. Early diagnosis and treatment is essential in order to prevent complications, although a careful evaluation of the risks and benefits is necessary in every patient. Fertility is usually retrievable with treatment. However, the evaluation of fitness for pregnancy and preconceptional improvement of health are of paramount importance for optimal results.

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Chapter 6

Hepatitis C Virus in Thalassemia

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Additional information is available at the end of the chapter

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Abstract

Prevalence of hepatitis C virus (HCV) infection is relatively low in children. However, seroprevalence rates of 10–20% have been reported among children who received repeated transfusion. The development and the severity of liver fibrosis are strongly related to the extent of the liver iron overload and to the presence of chronic hepatitis C (CHC). In CHC, liver iron overload has been suggested as a negative prognostic factor exacerbating inflammation with subsequent progression of liver fibrosis and decrease in antiviral therapy effectiveness. CHC may be suspected based on medical history or accidentally discovered abnormal liver functions. Hepatitis C is diagnosed by positive serology for viral antibodies and confirmed by polymerase chain reaction (PCR) to detect virus RNA. The treatment of HCV infection in children was difficult due to the limitations of pegylated interferon-α and ribavirin. In 2017, FDA approved the first direct-acting antiviral agents (DAAs) for children including ledipasvir/sofosbuvir in the adult dose, 90/400 mg, to treat HCV in children and adolescents aged 12 years and older or weighing at least 35 kg. Similarly, giving half the adult fixed-dose of ledipasvir/sofosbuvir, 45/200 mg, to children aged 6–11 years is still under clinical trials with promising results.

Keywords: chronic hepatitis C, thalassemia, direct-acting antiviral agents

1. Introduction

The prevalence of hepatitis C virus (HCV) infection is relatively high in thalassemic children. The development and the severity of liver fibrosis in those children are strongly related to the extent of the liver iron overload and to the presence of chronic hepatitis C. Chronic

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hepatitis C (CHC) in most infected patients lead to the development of liver fibrosis, cirrhosis, portal hypertension and hepatocellular carcinoma. Therefore, all persons with positive anti-HCV antibody tests must undergo additional testing for the presence of the HCV itself using polymerase chain reaction (PCR). Combined pegylated interferon-α and ribavirin with its limitations and serious complications remained the standard therapy for pediatric HCV infections until early 2017. Fortunately, the recent development of the direct-acting antiviral agents (DAAs) and the FDA approval of ledipasvir/sofosbuvir in the adult dose, to treat HCV in children and adolescents aged 12 years and older or weighing at least 35 kilograms has revolutionized the treatment of HCV infection.

2. Background

The prevalence of HCV is increasing and estimates of the future burden of CHC predict at least a threefold rise in chronic liver disease and cirrhosis by the year 2020 [1].

3. Epidemiology of hepatitis C

About 3% of the worldwide population is infected by HCV with a significant racial difference. Males are more commonly affected than females due to frequent exposure to intravenous drug and alcohol abuse. Females are frequently infected from blood product transfusions [2]. The most affected regions are Eastern Mediterranean and European Regions [3]. Egypt has possibly the highest HCV prevalence in the world where 10–20% of the general population is infected [3]. The prevalence of HCV in North Egypt and South Egypt is 15.8 and 6.7%, respectively [4].

The prevalence of HCV infection is relatively low in children. Asymptomatic HCV is detectable in 2.02% of Egyptian children [5]. However, seroprevalence rates of 10–20% have been reported among children who received blood products for conditions such as thalassemia or hemophilia or those with a variety of other potential exposures such as malignancy, hemodialysis, extracorporeal membrane oxygen, or surgery for different reasons [6, 7]. A study in Mid-Delta, Egypt, reports 40% of patients with thalassemia are infected with HCV [8].

4. Hepatitis C virus (HCV) and its genotypes

Hepatitis C virus is small, single-stranded, enveloped RNA virus of the flaviviridae family [9]. The only natural host is man. Currently, there are six HCV genotypes and many subtypes (1a, 1b, 1c, etc.). All HCV genotypes have a common ancestor virus. However, HCV genotypes 1, 2 and 4 are more prevalent in Central and Western Africa, genotype 5 in South Africa and genotypes 3 and 6 in China, South-East Asia and the Indian subcontinent [10]. Genotype 4 is widespread in East Africa and Egypt [11]. Approximately 90% of Egyptian HCV isolates belong to a single subtype, 4a, which responds less successfully to interferon therapy than other subtypes [12].

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5. Modes of HCV transmission

The main mode of transmission in children has changed over time. Before 1990, when HCV had not been identified, transfusion of blood and blood products and organ transplantation were the usual mode of transmission. One of the major routes of transmission is the reuse or inadequate sterilization of medical equipment, especially syringes and needles in healthcare settings. Currently, perinatal transmission accounts for most of cases [13].

Vertical transmission is almost always confined to women with detectable HCV RNA. The rate of vertical transmission is approximately 5%. Mothers with higher titers of HCV RNA and those who are HIV positive are more liable to pass the infection to their babies with transmission rates varying from 2 to 12%, according to maternal infectivity. Hepatitis C antibody is passively transmitted, and so all infants will have HCV antibody for up to 13–18 months. Measurement of HCV RNA is necessary to detect active infection, but it is unreliable before 3 months of age. Breastfeeding is safe in mothers with low titers of HCV RNA [14]. In children with beta thalassemia, the blood transfusion is not the only way of transmission but community and environmental factors may play a role [8].

6. Iron overload and hepatitis C virus in patients with thalassemia

Multicenter cross-sectional studies have reported that the development and the severity of liver fibrosis are strongly related to the extent of the liver iron overload and to the presence of chronic HCV infection [15]. HCV infection is the main risk factor for liver fibrosis in transfusion-dependent thalassemia. Excess liver iron is now clearly recognized as a cofactor for the development of advanced fibrosis in patients with HCV infection [16]. Despite its clinical relevance, thalassemia-associated liver damage has been insufficiently characterized [17].

Most of HCV-RNA negative patients with low iron load did not develop liver fibrosis, while HCV-RNA positive patients infected with genotype 1 or 4 and iron overload more frequently developed advanced fibrosis. Adequate chelation therapy usually prevents the development of liver fibrosis in patients free of HCV infection and reduces the risk of developing severe fibrosis in thalassemics with chronic hepatitis C (CHC) [15].

7. Relationship between chronic hepatitis C and liver iron concentration in thalassemia patients

Hepatitis C virus infection is responsible for 80–90% of post transfusion cases of hepatitis in patients who have received blood transfusions (more than 75% of HCV infection become chronic and up to 20–30% progress to cirrhosis) [18]. The main causes of liver fibrosis in transfusion-dependent thalassemia major are HCV infection and hepatic iron overload [16].

Chronic hepatitis in most infected patients lead to the development of liver fibrosis, cirrhosis and hepatocellular carcinoma. Patients with HCV have mildly to moderately increased hepatic iron concentration and occasionally have severe hepatic iron overload [13]. The increased iron stores may be due to release from damaged hepatocytes, to the virus itself or to genetic determinants so that the iron can influence the progression of chronic hepatitis C [19].

In patients with CHC, the degree of liver fibrosis and hepatic iron concentration were correlated. However, this correlation cannot be translated as a causal relation. It is not clear whether this correlation merely reflects the presence of more active disease or iron exacerbates chronic HCV-induced damage through activation of hepatic stellate cells (HSC) and regeneration of hepatocytes [20].

8. Pathogenesis and clinical consequences of iron overload in chronic hepatitis C

Iron overload induces oxidative stress leading to cell membrane damage, DNA instability and mutagenesis [21]. Due to these effects, iron can be considered a proinflammatory, profibrogenic factor and a potential carcinogen [22].

Increased hepatic iron potentiates progression toward liver fibrosis and contributes to poor response to interferon therapy [18]. There is evidence that excess iron is known to promote oxidative stress and cause direct liver damage and accelerate progression to both micronodular cirrhosis and hepatocellular carcinoma [23]. Based on the above-mentioned observations, iron overload has been suggested as a negative prognostic factor of chronic hepatitis C with influence on the increased aminotransferase activity, exacerbation of inflammation, progression of liver fibrosis and decrease in antiviral therapy effectiveness [24]. Iron accumulation in the liver induces oxidative stress which accelerates lipid peroxidation, resulting in destruction of organelle membrane and cell death via hepatocyte necrosis or/and apoptosis [25]. Also, oxidative stress products induce a focal inflammatory reaction which in turn stimulates hepatic macrophages and release of profibrogenic cytokines [26]. These mechanisms trigger the activation of hepatic stellate cells (HSC) which are major sources of collagen and other extracellular matrix elements that gradually accumulate in perisinusoidal spaces of liver parenchyma [27]. Longer hepatocyte exposure to excess iron is associated with greater risk of progressive fibrosis and development of liver cirrhosis with irreversible nodular reconstruction of the organ, replacement of the functional liver parenchyma with the connective tissue and loss of function [28]. Due to these effects, there is evidence that moderate to severe liver iron concentration and chronic HCV infection in thalassemia patients have a potentiating effect on hepatic fibrogenesis [29].

9. Symptoms and signs of hepatitis C

Acute HCV infection becomes chronic in 70% of patients, which represents a high rate of chronicity for a viral infection. Most patients with CHC and compensated hepatic synthetic

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functions are usually asymptomatic. They may have nonspecific complaints like fatigue or malaise. However, patients with decompensated cirrhosis post HCV infection have the same symptoms observed in other patients with decompensated liver diseases.

Manifestations in patients with decompensated liver disease occur merely due to synthetic dysfunction and portal hypertension. These include mental status changes (hepatic encephalopathy), ankle edema and abdominal distention (ascites), and hematemesis or melena (variceal bleeding). Sometimes, extrahepatic manifestations of HCV are the first symptoms observed; most commonly, it involves the joints, muscle and skin [30].

10. Diagnosis of hepatitis C

As most patients of hepatitis C experience no or non-specific symptoms during acute phase, they usually do not seek medical advice and diagnosis is rarely made at this phase of the disease. The clinical diagnosis of hepatitis C in chronic phase is also challenging as the patient may spend decades with compensated liver functions lacking any specific symptoms until advanced liver disease develops [31].

Chronic hepatitis C may be suspected clinically on the basis of the medical history. A history of unsafe injections as IV drug abuse or inhaled substance usage, such as cocaine, piercings or tattoos, sharing toothbrushes or personal sharp objects (e.g. nail scissors or cutters) with HCV patient raise the suspicion of HCV infection. Unexplained easy fatigability, with abnormal liver enzymes or liver function tests found during routine blood testing may be the clue. Also, hepatitis C may be diagnosed as a result of targeted screening; such as screening of blood donors or HCV contact tracing [32].

Diagnosis of HCV can never be made on clinical bases only. Serological blood test is the first step in diagnosis. The test is used to detect antibodies to HCV. Anti-HCV antibodies can be detected in 80% of patients within 15 weeks after exposure, in >90% within 5 months, and in >97% by 6 months after exposure and may persist for life even in treated patients. This makes the test more valuable to diagnose infection but not to test cure of HCV chronic infection. HCV antibody tests have a strong positive predictive value for exposure to HCV but may miss patients in early phase who have not yet developed antibodies, or those who have an insufficient level of antibodies to detect [31, 32].

Immunocompromised individuals infected with HCV may never develop antibodies to the virus. So, using HCV antibody test is of no value in screening immuncompromised patients for HCV, and they should be screened using a test to detect the virus itself if HCV infection is suspected [33].

Anti-HCV antibodies indicate exposure to the virus but cannot determine the presence of ongoing infection. So, all persons with positive serology must undergo additional testing to confirm the presence of current infection. Molecular nucleic acid testing methods, such as polymerase chain reaction (PCR) are used to test the presence of virus RNA in the blood [34]. HCV RNA PCR is a quantitative test that has the capacity to detect not only the presence but also the amount of virus copies present in the blood which is also known as “viral load”. HCV

viral load neither indicate disease severity nor prognosis. It was a predictor of response to interferon-based therapy but not important now with the current therapy [33].

Genotype testing was generally recommended before therapy as it was used to determine the required length and potential response to interferon-based therapy. This concept was changed with the introduction of direct-acting antiviral drugs [35].

11. Treatment of hepatitis C

11.1. Standard PEG interferon/ribavirin (PEG-IFN/RBV) combination therapy

The treatment of HCV infection in children was difficult as few options were available. The standard therapy for children aged 3 years and older was combined pegylated interferon (PEG-IFN) α-2a or 2b and ribavirin, and the duration of therapy was dependent on HCV genotype. This treatment regimen was developed first in adults [36, 37]. Combined pegylated interferon-α and ribavirin with its limitations and serious complications remained the standard therapy for pediatric HCV infections till 2017 [38].

11.2. New direct-acting antiviral agents

In recent years, many direct-acting antiviral agents (DAAs) are under development for treatment of CHC. DAAs reduce the amount of HCV in the body by blocking viral replication inhibiting directly one of the several steps of the HCV lifecycle preventing the virus from multiplying, and in most cases, they cure HCV. DAAs are classified into several categories based on their molecular target [39, 40]. The recent development of DAAs has shown promising results in clinical trials for use in children and adults and has dramatically increased the rates of sustained virological response (SVR) while improving side effect profiles as compared to interferon-based treatments [41]. Prior to 2017, new DAAs available for adults have still not been approved for treatment in children [37, 38, 41, 42].

Recently (on April 7, 2017), the US Food and Drug Administration (FDA) approved the first DAAs for children that included sofosbuvir (Sovaldi) and sofosbuvir/ledipasvir (Harvoni) to treat HCV in children and adolescents aged 12 years and older or weighing at least 35 kg. These DAAs (Harvoni and Sovaldi) were previously approved to treat HCV in adults. These approvals will help change the landscape for HCV treatment by addressing an unmet need in children and adolescents [43]. The adult fixed-dose of ledipasvir/sofosbuvir, 90/400 mg, resulted in similar plasma exposure of ledipasvir, sofosbuvir and GS-331007 (the inactive metabolite of sofosbuvir) in adolescents as in adults, thus the adult dose was used for this age group [44]. Similarly, giving half the adult fixed-dose of ledipasvir/ sofosbuvir, 45/200 mg, to children ages 6–11 years resulted in comparable plasma exposure of ledipasvir, sofobuvir and GS-331007 as in adults, without any severe adverse events or laboratory abnormalities [45].

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Section 5

Other Hemolytic Anemia

Chapter 7

Sickle Cell Disease: A Genetic Disorder of Beta-Globin

Karen Cordovil

Additional information is available at the end of the chapter

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Abstract

Sickle cell disease (SCD) is a structural and monogenetic genetic disorder due to a mutation that occurs in the globin β-chain, resulting in the formation of hemoglobin S (Hb S), a protein composed of two normal, and two β-type mutant chains. Estimates indicate that the prevalence among live births is 4.4% in the world. The difficulty in circulating the sickle cell, its interaction with endothelial cells, leukocytes, platelets, endothelial dysfunction, and the abnormal expression of adhesion molecules permeate the beginning of the blood vessel occlusion process as well as pathophysiological aspects of SCD. Among the secondary complications are the stroke, pulmonary hypertension, leg ulcer, renal disorders, and all complications associated with vascular dysfunction. Clinical and biochemical markers of disease severity can be used to predict risk, prevent complications, and increase the expectation and quality of life of the SCD population. The entire scenario generated by Hb S has implications for the health and social inclusion of patients, so the treatment of the person with SCD needs an approach focused on the prevention of these complications in an individualized way.

Keywords: sickle cell disease (SCD), hemoglobin, genetic disturber, nucleation, molecular interaction

1. Introduction

According to global estimates, approximately 5% of the population has some type of hemoglobin variant, and more than 300,000 babies are born each year with hemoglobinopathies, with sickle cell disease (SCD) being the most prevalent type [1–2]. It is estimated that the prevalence of live births with the disease is 4.4% in the world, where rates remain high on the main continents of Africa, Southeast Asia, and the Americas [2].

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In 2013, perform a first evidence analysis focusing on sickle hemoglobin using a 2010 dataset combined with demographic data and modern geostatistical modeling techniques that explain spatial heterogeneities and precision measurements of global statistics about sickle cell disease neonates (**Figure 1**) [3]. In 2010, the births of infants with sickle cell anemia (SCA-Hb SS) accounted for 2.4% of the world's most severe cases of the disease [3]. However, worrying estimates indicate that the number of newborns with SCA will increase from approximately 305,000 in 2010 to 404,000 in 2050 [4, 5].

The African continent, which has 3.6 million new cases of sickle cell trait (HbAS) and 238,000 SCA, remains the largest cradle of SCD genetic inheritance [3]. Nigeria, and the Democratic Republic of Congo would urgently need to plan policies for prevention and management of SCA, so that implementations carried out in 2015 could save many lives by 2050 (**Figure 2**) [4, 5].

In Southeast Asia where a hemoglobin variant Hb E is more prevalent, a heterozygosity with Hb S has increased mainly due to immigration and interracial relationships [6–8]. Nevertheless, according to data between the years 1990 and 2013, an annual mortality rate SCD HbSE per 100,000 inhabitants decreased by 63.9%, keeping them in the media of 2.8% per year [9]. It is estimated that the prevalence of live births with the SCD is 1.1% in the American continent [2]. In the United States, it is estimated that 113,000 hospitalizations are in the occurrence of the disease and the cost of hospitalization for SCD reaches 488 million dollars per year [10].

In Brazil, the estimated incidence of SCD is 1 case per 2700 live births: Bahia, Rio de Janeiro, and Minas Gerais being the main states with the highest prevalence [11–13]. According to data from the Ministry of Health of Brazil, child and perinatal care lethality rates can reach 80% and between 20% and 50%, respectively, of uncared children who cannot reach 5 years of life [14]. Among the adults followed in the high prevalence states, such as Bahia and Rio de Janeiro, the median age of death due to SCD is still low, 26.5 years and 31.5 years, respectively [15]. Nevertheless, in the last 13 years, the Brazilian government implemented several public health policies focused on the detection of new cases by neonatal screening and on improving the quality of treatment provided to these patients, implying an increase in life expectancy, with individuals reaching the fourth, fifth, and up to the sixth decade of life [16–19].

Figure 1. Distributions HbS data points. Red points indicate surveys showing the presence of HbS and blue points indicate surveys showing an absence of HbS. Source: Adaptation of Piels et al. [3].

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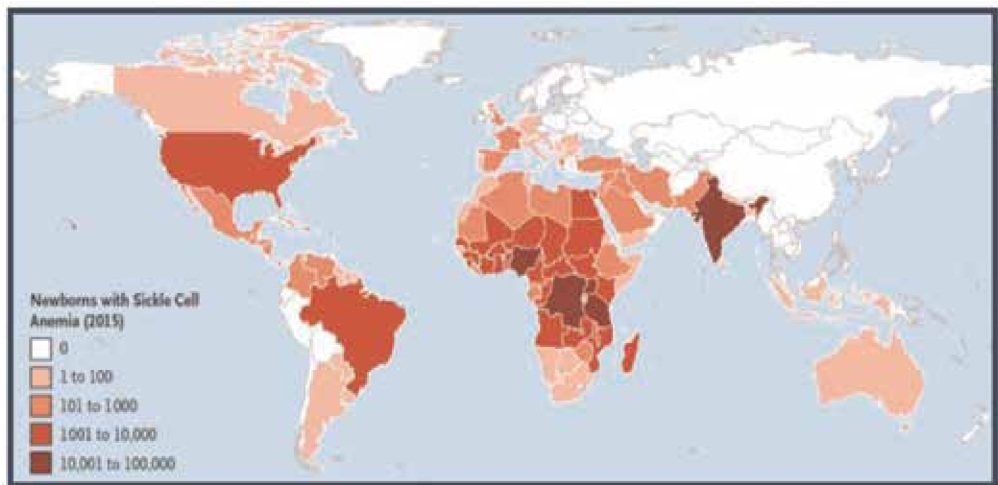

Figure 2. Numbers of Newborns with Sickle Cell Anemia (SCA) in 2015. Source: Adaptation of Piels et al., 2017.

The pathological presentment of SCD begins with the process of formation of Hb S polymers triggers dehydration and increased cell stiffness, giving rise to the vaso-occlusion event [20, 21]. This phenomenon leads to the appearance of several pathophysiological events such as tissue ischemia, anemia, inflammation, and hemolysis [20–24].

Hemolysis consists of the early destruction of the erythrocytes by membrane rupture, being a common event in the pathophysiological process of SCD [25–27]. During hemolysis, vasodilation, transcriptional activation of endothelin and vascular adhesion molecule are reduced, whereas nitric oxide is exposed directly to free Hb S, causing its degradation [28, 29]. Chronic hemolysis in SCD causes vascular imbalance, reflecting directly on hemoglobin concentration, reticulocyte count, bilirubin levels, lactic dehydrogenase (LDH), and nitric oxide bioavailability [28, 30, 31]. The reduction of the supply of oxygen to the tissues and organs causes the appearance of several complications secondary to disease [5].

Nevertheless, genetic, age, gender, hematological, and environmental factors afford to interfere on the characteristics of SCD and also impact on the quality and life expectancy of patients, mainly reducing their social insertion [32–35].

2. The hemoglobin: origins and function

Hemoglobin is one of the most abundant proteins in animals, performing important functions such as oxygen transport, started when hemoglobin binds to oxygen that arrives from the airways in the lungs and is taken to organs and tissues that need it to maintain life through red blood cells [36–38]. The genomic structure of genes encoding hemoglobin subunits, characterized by three exons and two introns, are highly similar among vertebrate animal strains [39].

Despite this, the function of some proteins belonging to the contemporary hemoglobin family in vertebrates is to store oxygen in tissues such as myoglobin, a protein formed by a globin chain, gives the red color to the muscular tissues and has structural and genomes similar to globins that form hemoglobin [37, 40–43].

Composed of four polypeptide subunits, two alpha chains and two beta chains ($\alpha 1\beta 1$; $\alpha 2\beta 2$), respectively, each of the four globin groups has a porphyrin ring (Heme group) containing the iron element in its constitution (**Figure 3**) [38, 44].

Hemoglobin is considered an allosteric molecule because it regulates its functionality very well, especially in situations of change in the environment where it is present, in the increase or decrease of the concentration of a certain ligand [45, 46]. A classic example of this can be highlighted in how oxygen binds cooperatively in the heme cluster [47, 48].

Previously, researchers admitted that the base of hemoglobin allosterism was based on the Monod Wyman-Changeux (MWC) two-state allosteric model, which corresponded to oxyhemoglobin (bound) and deoxyhemoglobin (unlinked) forms [44, 46, 49]. It is currently believed that hemoglobin can adopt several allosteric conformations in dynamic equilibrium, also implying different functionalities (**Figure 4**) [44, 48].

Over time hemoglobin has been consistently an object of scientific research given its relevance to biology [50–52]. One of the most important aspects is related to the study of its origin and its relation with oxygen, a very reactive metal, but necessary for mammalian metabolism [53–55].

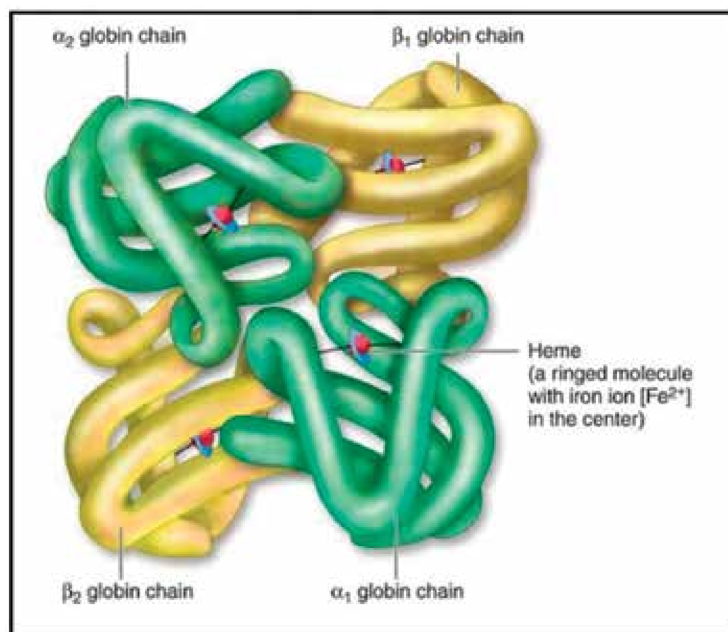

Figure 3. Structure quaternary of hemoglobin. Source: Antranik website: Available in http://antranik.org/blood-components-hemoglobin-typerh-factor-agglutination.

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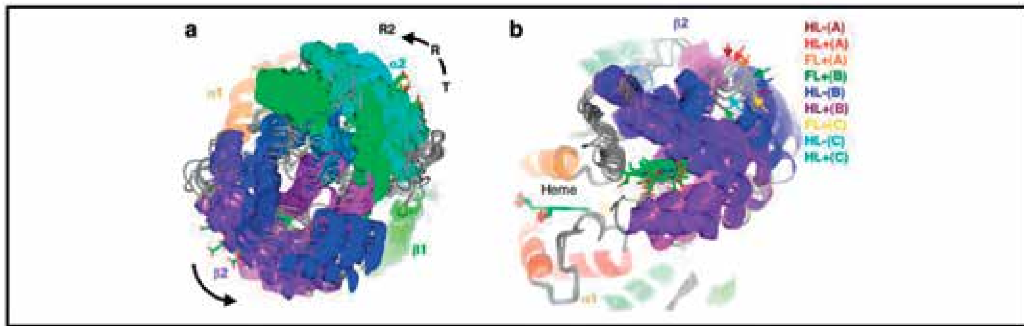

Figure 4. Presentation and comparison of nine quaternary structures of hemoglobin. In (a) diagram showing the orientation of $\alpha_2\beta_2$ dimers relative to $\alpha_1\beta_1$. In (b) the presentation of the β2 subunit with the same nine conformations represented in nine colors and at different angles. Source: Adapted from Shibayama et al. [44].

From the evolutionary point of view, about 4 billion years ago, the gaseous layer that enveloped the Earth was composed only of nitrogen, methane, water effluvia, and ammonia [37]. Probably many organisms that emerged in the early days used these gases for their own subsistence [56]. It is believed that iron and magnesium were involved in many of these actions in the metabolism of these extremely primitive organisms [57, 58].

In order to increase the efficiency of life-generating energy systems, somehow still not so enlightened and despite being toxic, oxygen has been incorporated by organisms [37, 50]. It is believed that initially this large protein complex that now bears oxygen-dependent organisms, organs, and tissues was very primitive, probably composed only of a metal that was able to bind and carry oxygen [37].

In the process of evolution, at one point, it was necessary that this structure is wrapped within a porphyrin ring and then embedded in enovelled protein [52]. During evolution, this ring-shaped structure has accompanied generations of organisms of animal origin (Heme group) and plant (Clorofila group) [37, 59].

The Heme group not only binds to globin molecules to form hemoglobin but can bind other molecules with a certain function to give rise to oxygenases proteins, cytochromes, and even fungal ligninases [37]. Chlorophyll, the green-coloured substance in plants, is basically an organic molecule characterized by a porphyrin ring that contains magnesium, and its function is to absorb electromagnetic energy through sunlight, which will be used in photosynthesis [58, 60, 61].

Studies to identify the origin of hemoglobin compare their respective coding genes with several parent organisms in order to detect the changes that have been made throughout evolutionary history and time [37]. But the change identified in hemoglobins was more in the form of how they are genetically regulated than in their structural basis from which they were strongly conserved [58]. In general, studies indicate that hemoglobin appeared about 500 million years ago (**Figure 5**), prior to the time that eukaryotic cells diverged from eubacterial cells [37].

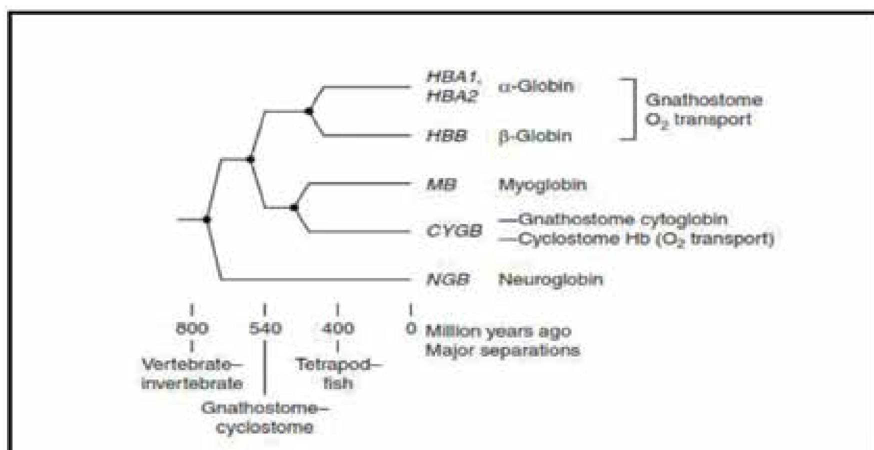

Figure 5. Phylogenetic tree model of globin genes in vertebrate animals. Source: Adapted from Hardison [58].

3. Pathophysiology of Hb S: a mutation, an amino acid, a disease

Multipotent hematopoietic stem cells have the potential to be targeted to a number of special differentiation pathways that originate several blood cell lines in mammals [62–64]. One of the pathways, erythropoiesis, is responsible for the production of red blood cells, discoid and anucleated cells that carry oxygen (O2) and carbon dioxide (CO2) through an intracellular metalloprotein called hemoglobin throughout the body [39, 65].

As seen previously, hemoglobin is a heterotetramer composed of two α-globin and β-globin subunits linked by a non-covalent bond [2, 39]. Each globin subunit has a heme group containing the bivalent iron ion [64, 66].

Different globin genes are activated or deactivated both in embryonic, fetal and adult life in order to meet different oxygen demands and facilitate the placental transfer of oxygen from the mother to the embryo (**Figures 6** and **7**) [64, 66, 67].

In humans, throughout embryonic life to adulthood, various types of hemoglobin can be expressed and this process is regulated in a complex manner, involving several molecular mediators in order to stimulate hemoglobin production (**Figure 6**) [2, 66, 68]. The globin genes α and β, arranged on chromosomes 16 and 11, respectively, control the production of globins through the expression of the subunits from the α globin locus: ζ (embryonic) and α-globin (adult) genes; and locus β globin: ε (embryonic), γG and γA (fetal), and δ and β-globin (adult) (**Figure 7**) [64, 66].

However, due to spontaneous mutations, variant hemoglobins may arise and be structurally different [68, 69]. These mutations can, for example, trigger a change in the amino acid sequence,

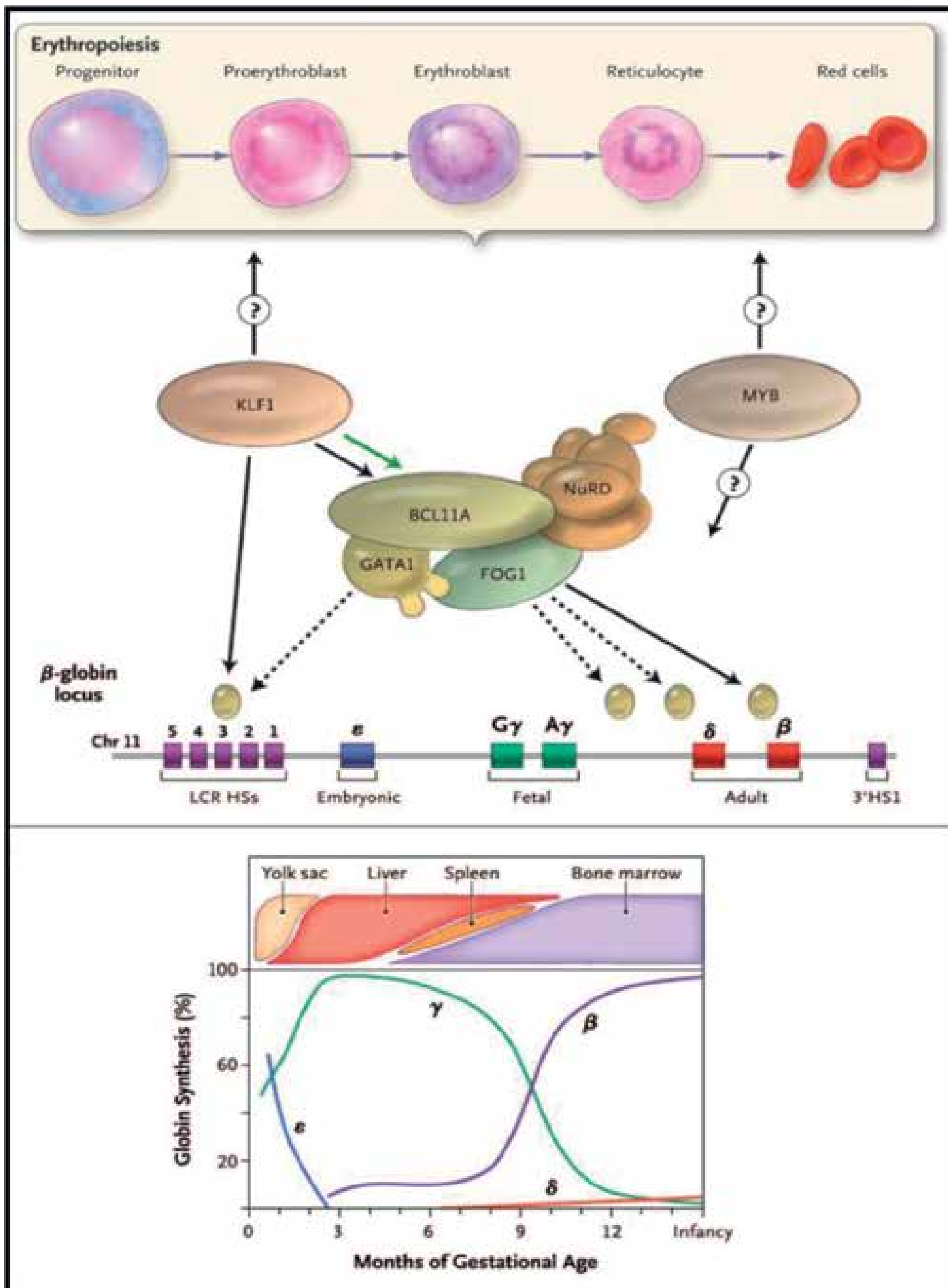

Figure 6. Representation of the red cell maturation process, molecular regulation of hemoglobin (embryonic, fetal, and adult) with focus on β globin and globin synthesis. Source: For more details, look up the Sankaran article reference of the year 2011 [68].

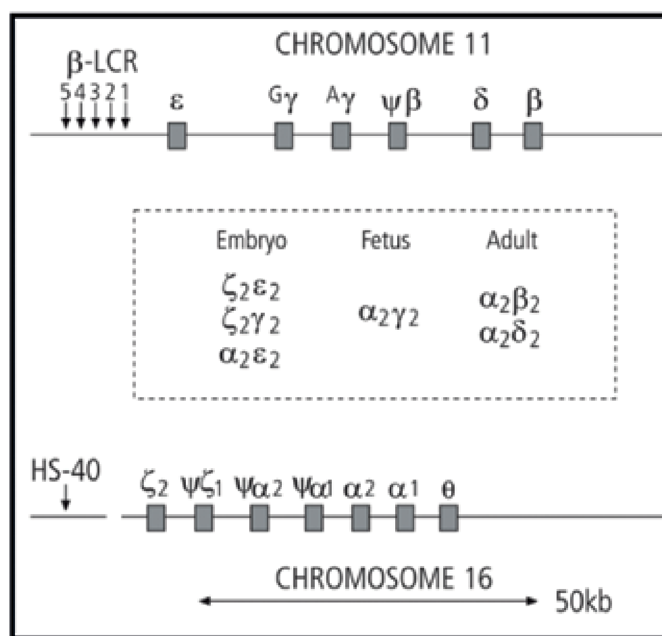

Figure 7. Variation of hemoglobin types in the embryonic, fetal, and adult period. Source: Adapted from Weatherall and Clegg [113].

leading to the decrease or suppression of the production of a globin chain, as observed in β Thalassemia [70, 71]. Such genetic changes often lead to the onset of diseases, which are called hemoglobinopathies [2, 8, 72].

A mutation in the gene of the sixth codon of exon 1 in the DNA of chromosome 11, which synthesizes the β globin, leads to the replaced adenine nitrogen base (from the GAG codon) by thymine (GTG), resulting in the substitution of glutamic acid for valine in position 6 of the N-terminal end in the Beta (β) chain of globin [73–76] .The pathophysiology of sickle cell disease (SCD), a monogenetic disorder that gives rise to the formation of hemoglobin S (Hb S), a protein composed of two normal α-chains and two mutant chains of the β-type (α2A β2S) (**Figure 8**).

Three levels direct the scientific knowledge related to the pathophysiological changes present in SCD: molecular and cellular, tissue and organism [77–80]. At the molecular level, the exchange of amino acids with different isoelectric points, glutamic acid (IP = 5.97) per valine (IP = 2.77), causes an imbalance because of the loss of negative charges of Hb S in relation to Hb A (**Figure 9**) [81, 82]. These changes in the physical structure of hemoglobin will imply impairments in its functionality, mainly related to oxygen loading [83–85].

In certain periods or situations where hypoxia occurs (absence or decrease of oxygen tension in the body), oxygenated mutant hemoglobin (oxy-HbS) loses oxygen, adopting deoxygenated conformation (deoxy-Hb S) [81, 86, 87].

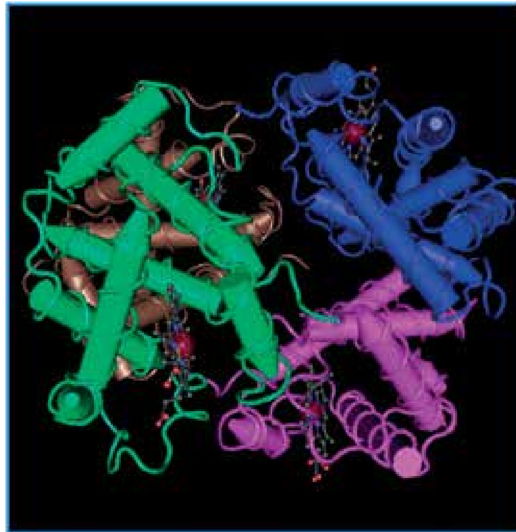

Figure 8. Crystalline structure of deoxy hemoglobin S (deoxy-Hb S). Source: For more information, see details in the study by Harrington et al., 2017.

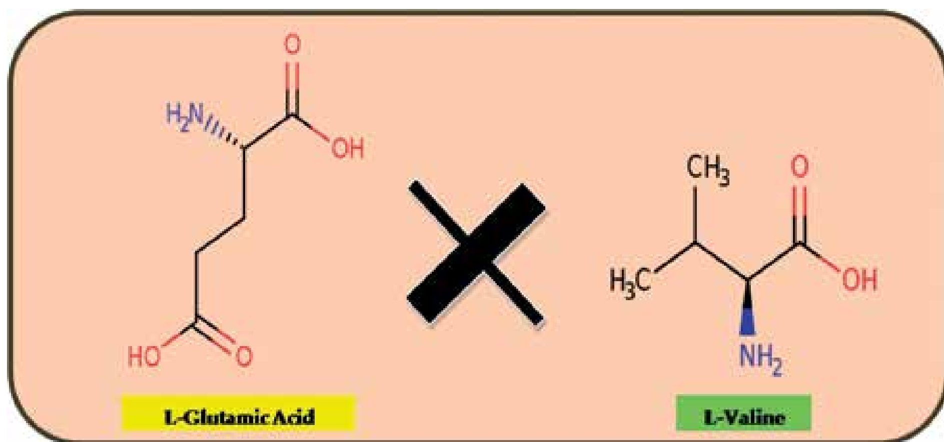

Figure 9. Representation of the mutated amino acid structures present in HbS. Glutamic acid has an acted structure and with more negative charges. Valine is an amino acid with hydrophobic characteristics that tend to have the most neutral charge. Source: Wishart et al., 2013.

In its own structure, the formation of hydrogen bonds between the amino acids valine of position n1 of the globin beta S (normal position) and the mutant valine of the same globin begins [82, 83, 84]. Hydrogen bridges promote intermolecular approximations and contacts between the amino acids of hemoglobins (GLU121→ GLY16, ASP73→ THR4, etc.) that favor the formation of Hb S polymers [84, 85]. However, it is through the hydrophobic interactions between valine (βVAL6) and the hydrophobic concavity formed mainly by leucine (βLEU88) and phenylalanine (βFEN85) that the formation of Hb S polymers occurs [81, 83, 88].

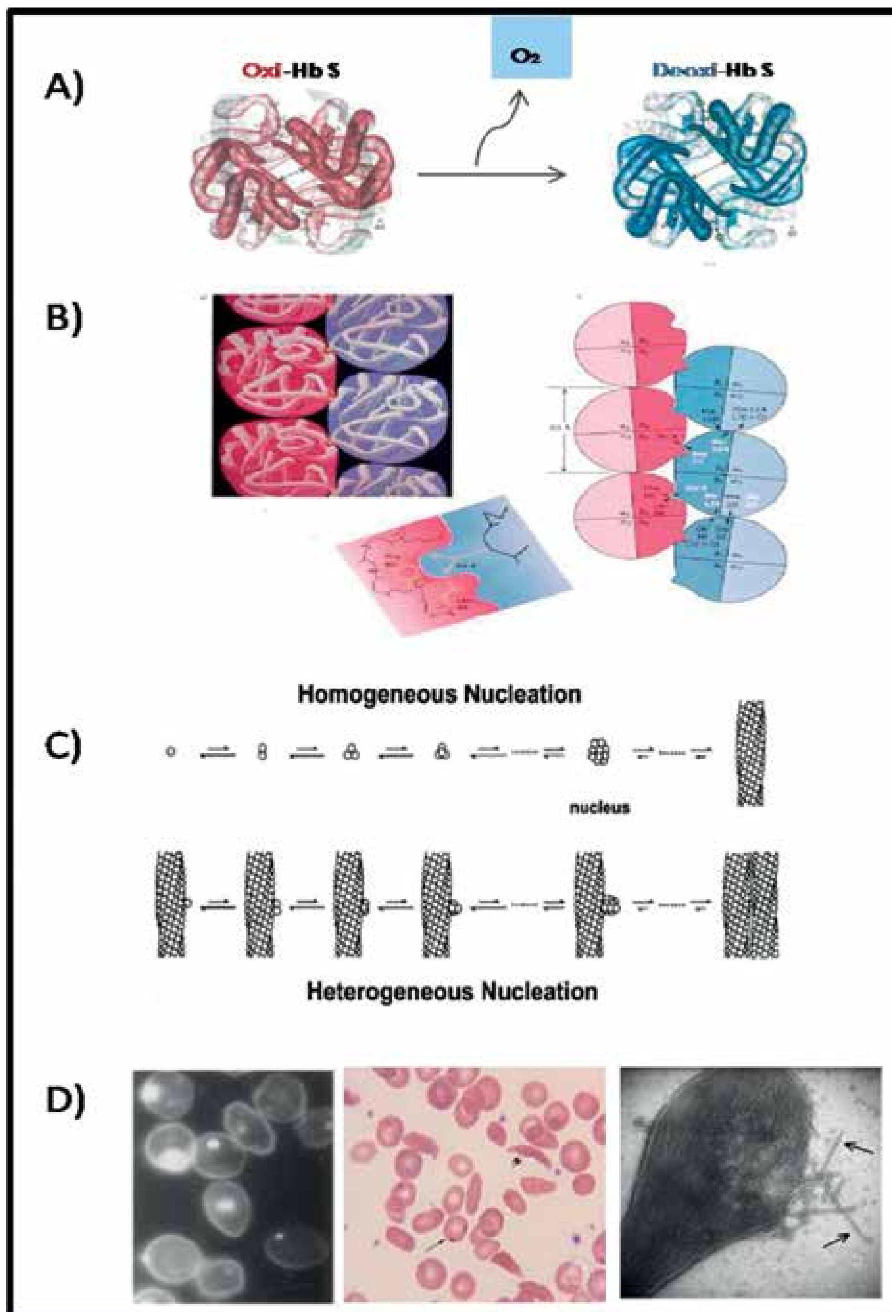

Figure 10. Summary of the pathophysiology of SCD. (A) Representation of the structural differences in the conformation of HbS when it is in oxygenated and deoxygenated form. (B) HbS polymerization process with details of the main amino acids involved in the mechanism. (C) Formation of the deoxy-HbS fibers through the phenomenon of homogeneous and heterogeneous nucleation. (D) Microscopic findings of sickle cell. Left cells of the blood with the formation of Heinz bodies (fluorescence method). In the center, smear blade containing scythe-shaped cells. On the right is a lysed sickle cell showing several deoxy-HbS fibers. Source: See details at Howard Hughes Medical Institute, 2018; Galkin et al. [88]; Rooter, 2005; Liu et al., 1996.

Polymerization in SCD is a process triggered by a phenomenon known as nucleation in which a number of molecules come together within an embryo of the new phase that resembles a first transition phase similar to a gas-solid transformation [88, 89]. The nucleation progressively progresses through the initial fiber growth and its branching, due to the secondary nucleation of new fibers on top of the existing ones, as if it were a double nucleation [77, 88, 90, 91].

Polymerization of HbS is a primary event in the pathophysiology of SCD, generally favored by several factors such as insufficient oxygen saturation, loss of potassium and water, reductions in blood pH, increased the concentration of 2, 3-diphosphoglycerate [81, 82, 86]. In the formation of HbS fibers, they are capable of generating 14 members of T-shaped conformation fibers when hemoglobin is in the deoxygenated state [87, 88]. Among these aligned fibers hydrophobic contacts occur, which are initiated between the valine of the HbS molecule and alanine, phenylalanine and leucine of adjacent Hb S molecules [88]. In the case of a high degree of polymerization, the deoxy-HbS presents a behavior characteristic of a polymer gel [88, 90].

After polymerization progresses through enveloped fibers, which will alter the structure of the red cell, mainly through the formation of more elongated fibers and mechanisms of precipitation in the cell wall with the formation of Heinz bodies, triggering the appearance of sickle-shaped red blood cells, rather than discoid and malleable (**Figure 10**) [81, 82, 87].

The affinity of oxygen for hemoglobin, Hb S concentration, dehydration, the minimum concentration of gelation, acidosis and elevated temperature are determinant events, which directly influence the falcization process [92].

Sickle cells have a rigid, adherent and fragile structure, which compromises their circulation in the bloodstream [86, 87]. Cell damage and deformation of erythrocytes occur as a result of polymerization of deoxy-HbS and high concentrations of unpolymerized oxy-HbS, as well as influenced by cellular levels of HbF, water content, pH, temperature and mechanical stresses that will result in membrane injury [84].

The difficulty of circulating the sickle cell, its interaction with endothelial cells, leukocytes, platelets, endothelial dysfunction and the abnormal expression of adhesion molecules permeate the beginning of the process of occlusion of the blood vessels, generating tissue hypoxia, hemolysis, increased oxidative stress and other pro-inflammatory phenomena [80, 87, 91, 93].

4. Clinical consequences of the presence of Hb S

SCD is a chronic hemolytic anemia characterized by clinical events involving recurrent vaso-occlusion, and its main clinical manifestations are anemia, pain, and multiple organ failures [18, 80, 87]. To understand the clinical aspects of SCD, we must go a bit further into the pathophysiological and molecular aspects of this genetic disorder.

As we saw earlier, the presence of a genetic alteration in the nitrogen base in the gene that encodes the β globin production triggers the formation of HbS, modifies the structure of the erythrocytes (**Figure 11**), and implies a series of pathophysiological complications for individuals

Figure 11. Microscopic finding showing structural differences observed in normal form (oxy-HbS) and sickle cell (Deoxy-HbS), responsible for the pathological aspects in individuals with SCD. Source: Site of Howard Hughes Medical Institute, 2018.

with SCD. Many of the following events do not occur in isolation and are directly involved in the pathogenesis of SCD.

The sickle cell has many difficulties in permeating the blood vessels. Due to the speed of the bloodstream, many end up clinging to each other thus harming the passage. Sickle cell occlusion mechanism is started. Spleen cells are pounded, violently pushed, lysed, and intravascular hemolysis causes the red blood cells to release a series of biocomponents, mainly hemoglobin and arginase that will interact with nitric oxide (NO) produced in the endothelium, reducing its bioavailability and arginine and its main precursor [84, 94, 95].

The vessel occlusion plus constant hemolysis initiates tissue hypoxia. At the same time, early oxidation of NO increases oxidative stress implying endothelial dysfunction, with imbalances in the mechanisms of vessel dilatation and constriction [84, 85].

At a time when local occlusion ends, and blood perfusion returns, more free radicals are produced, and they further increase lesions to the endothelium, which becomes more adherent, especially to red blood cells and leukocytes, making the vascular wall again exposed to a new occlusion [84, 95, 96].

Among the main adhesion pathways that progress the sickle cell and endothelial cell interactions are the soluble adhesion proteins (thrombospondin, fibrinogen, fibronectin, and von Willebrand factor), integrins ($\alpha 4\beta 1$, $\alpha V\beta 3$) and their membrane-bound receptors and sulfated glycolipids), immunoglobulins VCAM-1 and ICAM-4, endothelial selectin, as well as leukocyte activation by epinephrine through β-AR stimulation [85, 96].

Recurrent hemolysis eventually becomes chronic, and the inflammatory state is established. Thus, the organism needs to increase the production of red blood cells by the bone marrow,

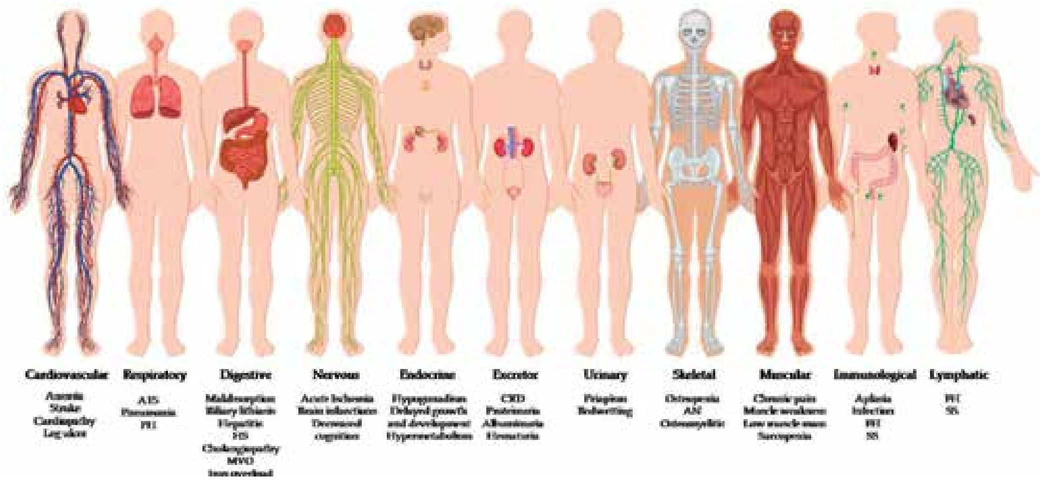

Figure 12. Major complications secondary to SCD. Source: The illustration has been adapted from the Toda Matéria website. For more details, refer to the articles Ballas et al. [78]; Saraf et al. [104]; Saraf et al., [105]; Gordbach et al., 2012; Piels et al., 2017; Di nuzzo & Fonseca [76]; Machado, 2007; Gumiero et al., 2007; Brunetta et al., 2010; Paladino, 2007; Hyacinth et al. [99]; Marques et al., 2012; Cançado, 2007; Lobo et al., 2010; Borsato et al., 2000; Saad eTraina, 2007, Marques et al., 2012; Caridade et al., 2007.

resulting in high cardiovascular work, with increased cardiac output in order to facilitate the rapid delivery of blood with a higher content of oxygen to the organs, avoiding hypoxia and tissue death [97]. More precisely, a compensatory mechanism is established that increases heart rate, leading to increased myocardial energy demand with the effect between myocardial energy requirements and total body [98, 99, 100].

The hypermetabolism present in these patients has an impact on body composition and has been related to increased energy expenditure, increased protein turnover, increased oxidative stress, higher reticulocyte levels, and reduced body mass [97, 99, 101, 102].

Progressive degeneration of the organs results from infarctions in the affected areas, leading to several secondary complications that directly compromise patients' lives and survival [18, 80, 103].

Patients with SCD are more likely to have episodes of vascular accident, pulmonary hypertension, proteinuria and chronic kidney disease, all complications associated with vascular dysfunction caused by the disease [78, 94, 104, 105].

Vasodilation is reduced in patients with SCD and may have other consequences, such as the appearance of leg ulcers [94, 106, 107]. These lower limb ulcer lesions represent 8 to 10% of the cases and have a higher incidence in people with SCA males and in the age group between 10 and 50 years [99, 107, 108, 109].

Ulcerations may appear after trauma, insect bites, excessive dryness of the skin or spontaneously generally in the ankle or malleolar region (middle or lateral portion), where there are less subcutaneous tissue and blood flow as a consequence of tissue hypoxia, endothelial dysfunction, and vaso-occlusion [107, 108, 110].

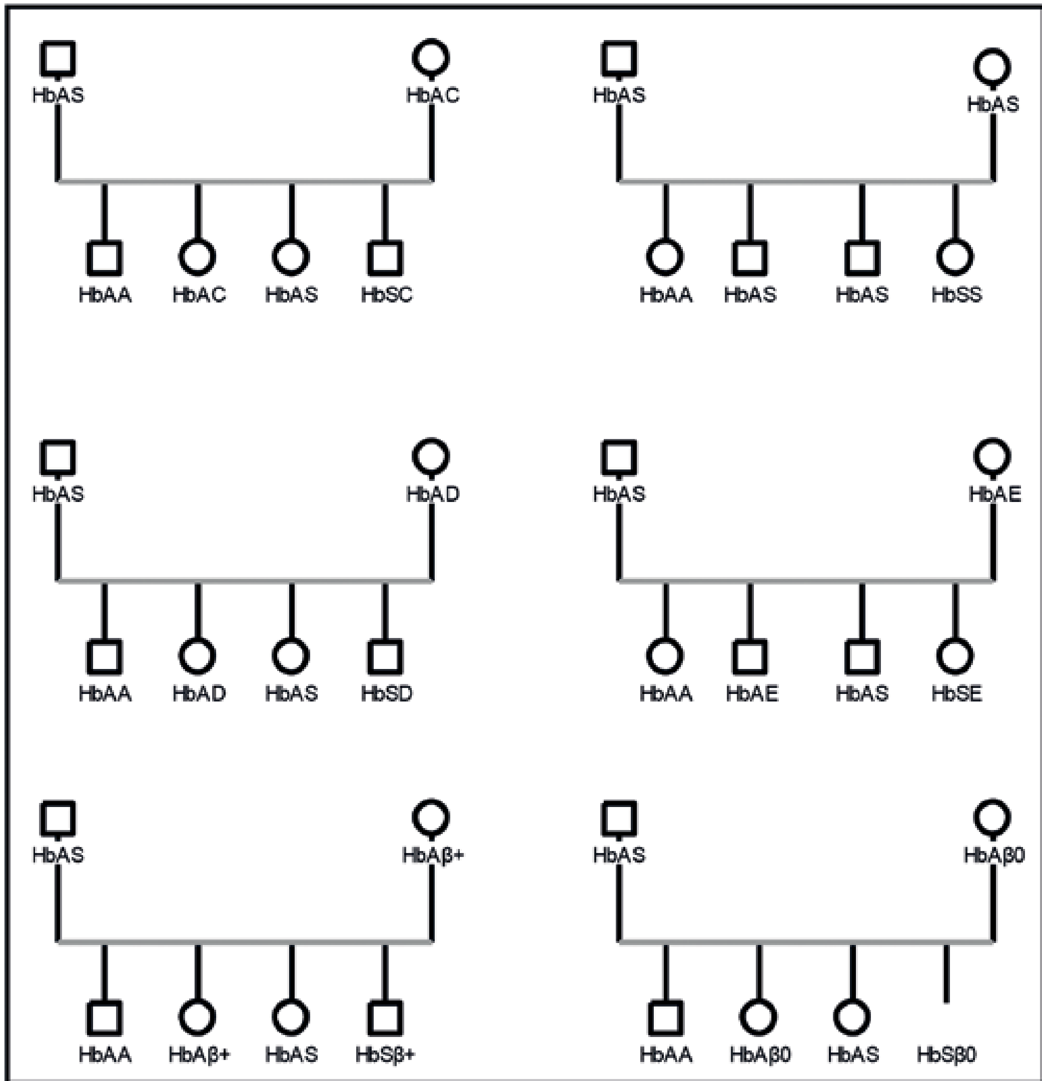

Figure 13. Genograms showing the most prevalent SCD genotypes in the world and the likelihood of homozygous or heterozygosis independent of sex when the parents have some type of hemoglobin variant that can generate SCD. The GENOPRO® software version 2016 was used to make the genograms.

Infections in these patients are also a major cause of concern both in childhood and in adulthood [76, 78, 111, 112]. In general, this and other complications (**Figure 12**) bring many misfortunes to individuals with SCD and basically compromise the quality of life of these patients. Despite all the consequences of HbS formation, the degree of severity of the disease depends on numerous factors, and the first one is the genotype.

SCD can be subdivided into distinct genotypes, six of which are more frequent in the world, SCA (Hb SS), heterozygotes (Hb SC, Hb SE, Hb SD), sickle thalassemia (Hb Sβ+ and Hb Sβ0),

and sickle cell trait (Hb AS) [8, 84, 104, 113, 114]. Individuals with Hb SS genotypes, heterozygoses and associations with thalassemia are generally symptomatic, and at each gestation, there is a 25% chance that the child will be born with SCD from parents carrying some S gene or other variant hemoglobin (**Figure 13**) [84, 104, 115]. In general, the HbAS genotype is considered to be asymptomatic in that it hardly develops any clinical picture, but it represents a type of hemoglobinopathy, since the recessive gene is likely to be inherited for the next generation [115–118].

Other indicators of disease severity are bilirubin, PCV, erythropoiesis rate, leukocytes, LDH, fetal hemoglobin, creatinine, proteinuria, reticulocytes, HSV, phenotypes, days of hospitalization per year, severe vaso-occlusive crisis per year, number of transfusions per year, hip disease, leg ulcer, hepatobiliary complications, neurological events, renal disorders and body mass index [84, 119–123].

5. Treatment of SCD: general aspects

Treatment, in general, is differentiated by pathophysiological changes during life and will also depend on the type of genotype, which is accompanied by a hematologist. The use of folic acid supplements is included in order to contain hemolysis and to accelerate the production of red blood cells.

Also used are: (A) antibiotics, especially in children under 5 years, since generalized infections can lead to death within a few hours due to splenic sequestration; (B) analgesics, codeine, morphine, and anti-inflammatories in the presence of acute or chronic pain crises; venous hydration in the vessel occlusion; (C) transfusion or blood exchange; (D) periodic and special immunizations; and (E) treatment of the sequelae or chronic consequences caused by the disease [18, 124, 125].

The use of hydroxyurea medication over the years as a treatment that greatly increased the quality of life of patients. However, not all individuals are eligible or adapted to their use [77, 126]. Alternative treatments, transplantation, and gene therapy are welcome measures for clinical treatment; however, some of these are still under discussion and require technical and scientific clarification for their implementation.

Clinical and biochemical markers of disease severity should be used to predict risk, prevent complications, and increase the expectation and quality of life of the population with SCD [77, 87, 127]. Often patients with SCD report the development of vaso-occlusive symptoms after emotional/psychological stress, temperature changes, and physical exertion [95]. Therefore, patients undergoing treatment and their caregivers are encouraged to practice self-care, with measures that can prevent acute events, improve prognosis, and allow a better quality of life [128].

In general, people with SCD due to chronic hemolysis and inflammatory state have higher energy expenditure to develop daily activities and tendency to anorexia [109, 129, 130]. Pain crises generate a decrease in food consumption, which has a direct impact on caloric and nutrient intake. Probably, the pain crises associated with the constant hospitalizations contribute to the

lower food consumption that consequently compromises the nutritional status [127, 129, 130]. Thus, this population calls for nutritional monitoring for the intervention of the problems related to food and nutrition. In general, it is important the presence of a multi-professional team, centered in the assistance and matrix support to the hematologist doctor and the patients assisted with SCD.

6. Conclusion

Scientific research and technical work around the world have been done to better understand the pathophysiological and clinical aspects of SCD. It is a severe hemolytic disease that causes great morbidity and mortality, especially in underdeveloped countries. The entire scenario generated by HbS has implications for the health and social inclusion of patients, so the treatment of the person with SCD needs an approach focused on the prevention of these complications in an individualized way.

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Conflict of interest

The author does not present conflicts of interest.

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Chapter 8

Direct Anti-Globulin Test and Clinical Diagnosis

Takeshi Sugimoto

Additional information is available at the end of the chapter

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Abstract

This chapter focuses on anti-red blood cells antibody and anti-globulin test. The relationship between warm or cold antibody and hemolysis is explained. Direct anti-globulin test (DAT) is a useful clinical examination tool on the diagnosis of autoimmune hemolytic anemia (AIHA); however, false positive or negative results are sometimes detected. This chapter shows the disposition about the surroundings of the IgG antibody in DAT examination. In addition, this chapter contains pointing issue on the diagnosis of AIHA. Some pitfalls about diagnostic AIHA are presented from our experienced cases. To diagnose the background diseases such as lymphoproliferative disorders or autoimmune diseases under the analysis of secondary AIHA is important.

Keywords: direct anti-globulin test, autoimmune hemolytic anemia, secondary AIHA, complement activity, autoimmune diseases, lymphoproliferative disorders

1. Introduction

Anti-globulin test is one of the standard examinations in clinical laboratory. This test is predominantly used in hematology area, transfusion medicine, or organ transplantation area. The positive status of anti-globulin test expresses the existence of antibody against the protein related to the blood type; however, the clinical application of this test is various. The potentiality of anti-globulin test is introduced in this chapter.

2. Anti-red blood cells antibody

Anti-red blood cells antibody is the antibody against the protein related to the blood type on the surface of red blood cells. Anti-red blood cells antibody is composed of autoantibody

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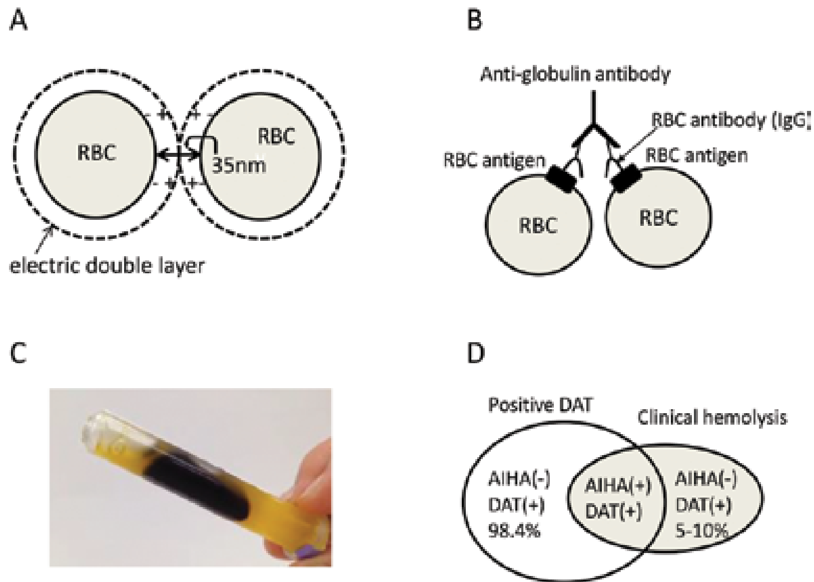

Figure 1. (A) The distance between RBCs is 35 nm. The surroundings of each RBCs obtain electric double layer. (B) The principle of coombs test, IgG-type antibodies bound to RBCs are united by anti-globulin antibody (Coombs antibody) with bridge-bindings. (C) Macroscopic aggregation in cold agglutinin disease (CAD). Aggregation part is floating on the plasma. (D) Schematic model about the relationship between direct anti-globulin test (DAT) and hemolysis. Only few percentage cases among positive DAT reach to hemolysis. In clinical AIHA, 5–10% of cases show negative of DAT. The figure is modified from original manuscript Ref. [1].

produced against self-red blood cells, and alloantibody produced against red blood cells of other persons. Antibodies classified according to thermal condition, warm antibody reacts to red blood cells (RBCs) mostly at around 37°C, and cold antibody reacts mostly at low temperature around 4°C. The main immunoglobulin antibody classes related to reaction to RBCs are IgG, IgA and IgM, in each of which light (L) chain has two types, that is, k and λ. Generally, IgM antibody is able to agglutinate by itself by reacting to RBCs (complete antibody). However, IgG antibody is not able to agglutinate alone (incomplete antibody) and requires activation of complement component to develop the agglutination. The capacity of binding of IgG antibody to complement is different among the IgG subclass, in which IgG3 followed by IgG1 is strong. In this context, high affinity of IgG antibody binding to complement brings about strong agglutination and hemolysis with activation of complement. **Figure 1B** shows a schematic model of the reaction with RBCs and anti-globulin.

3. Anti-globulin test

Anti-globulin test is a tool detecting RBCs which IgG antibody or complement bind to, and this test was developed by Coombs in 1945. There are two methods for anti-globulin test: direct anti-globulin test (DAT) and indirect anti-globulin test (IAT). DAT determines the existence of

IgG antibody or complement binding to RBCs antigen and IAT determines the existence of any IgG antibody reacting with RBCs antigen in the serum (or blood plasma). In the DAT method, anti-globulin antibody reagent (Coombs reagent) makes crosslink between IgG binding RBCs together, resulting in RBCs agglutination (**Figure 1A** and **B**). There are two kinds of anti-globulin antibody reagent: one is multispecific Coombs reagent reacting to any of IgG antibody, C3b or C3d component, and the other is mono-specific Coombs reagent reacting to only one component of them. When DAT shows positive result using multispecific Coombs reagent, the type of antibody will be determined by DAT using mono-specific Coombs reagent. Because the universal multispecific Coombs reagent is the cocktail reagent against IgG, C3b and C3d component, the antibody of IgA or IgM type is unable to be detected.

In clinical practice, DAT is useful for investigating AIHA, neonatal hemolytic disease, drug-induced immune hemolytic anemia (DIHA), and hemolytic transfusion reactions. DAT is also used for identifying the membranous protein sensitizing RBCs. IAT method is useful for irregular antibody examination, cross-match test for transfusion, mother's serum (blood plasma) examination in neonatal hemolytic disease, and identification of antibody eluted samples from antibody binding RBCs.

4. Autoimmune hemolytic anemia

4.1. Warm antibody

Warm antibody exerts to bind to RBCs mostly at around 37°C. Complement component will deposit on the surface of RBCs in activating warm antibody. The main detected protein by DAT is IgG antibody and complement component. The sensitized RBCs by warm antibody are recognized and received phagocytosis by hepatic and splenic macrophage, leading to cause extravascular hemolysis. Macrophage recognizes IgG antibody via IgG-Fc receptor (FcγR), in the subclass of IgG antibody IgG3 and IgG1 have strong affinity to macrophage. Therefore, IgG3 followed by IgG1 antibody are related stronger to causing hemolysis than IgG2 and IgG4 antibody. It is another mechanism of phagocytosis that macrophage recognizes and perform phagocytosis via C3b receptor protein deposited on the surface of RBCs. Warm antibody causes intravascular hemolysis, where IgM antibody reaction or antibody-dependent cell mediated cytotoxicity (ADCC) is involved. In clinical laboratory, warm antibody usually shows pan-hemagglutination character and tends to agglutinate all irregular antibody test panels. The main auto-antigens corresponding to warm antibody are Rh, band 3 and glycophorin A (GPA) protein [2, 3]. Blood-type specific warm auto-antibody (anti-E, -c, -e, -Kell, -Jk, -Ii, etc.) is detected in some of AIHA cases.

4.2. Cold antibody

Cold antibody exerts to bind to RBCs at close to 0°C. The activation of complement components is possible to be proceeded on above 12°C. If the cold antibody binds to RBCs on the thermal range of complement activation, then intravascular hemolysis will occur. It is known

that there are two types of cold antibodies, namely cold agglutinin and Donath-Landsteiner antibody (DL antibody), each of which develops cold agglutinin disease (CAD) and paroxysmal cold hemoglobinuria (PCH), respectively. Each disease is divided into primary (idiopathic) and secondary type.

The immunoglobulin class in cold agglutinin (cold antibody) is mostly IgM type, and this single IgM antibody can activate C1 component at low temperature. If the cold agglutinin binds to erythrocyte in below body temperature, blood circulatory status is disturbed by intravascular hemolysis and hemaggutination. Circulatory disturbance emerges as pain and acrocyanosis in the extremities. As blood flows back to the central trunks from extremities, cold agglutinin leaves from RBCs. However, complement remains to be bound to RBCs, and complement cascade activates sequentially developing into intravascular hemolysis. On the other hand, RBCs escaped from hemolysis is going to receive hemophagocytosis in the reticuloendothelial system in the liver via remaining complement components C3b and C3d on RBCs, developing into extravascular hemolysis. Reflecting the abovementioned reason, DAT shows positive for complement component and negative for IgM antibody in CAD. Cold agglutinin usually has the specificity for Ii blood-type antigen. In clinical laboratory about CAD, RBCs have characteristics of self-aggregation on blood smear sample and macroscopic aggregation on sample tubes which disappear in warm condition (**Figure 1C**). Low titer CAD is known to be a subtype of the CAD, in which clinical manifestation accompanied by RBCs agglutination is not due to the quantity of cold agglutinin but to the activating thermal range of it. Mixed type AIHA is other subtype related to cold agglutinin.

PCH occupies in 1.7–10% of the hemolytic anemia. PCH comes from pediatric infectious diseases (measles, varicella, mumps, influenza, etc.) and adult idiopathic type in recent years. When patient exposes to cold circumstances, intravascular hemolysis appears attackingly several minutes to hours later. Patient raises hemoglobinuria, fever, abdominal and limb’s pain. PCH is caused by DL antibody, which has the character of polyclonal IgG-type antibody and of strong hemolysin in low titer level. DL antibody reacts with C1 component in cold condition and called the biphasic hemolysin. DL antibody has the specificity for P blood-type antigen. During and immediately after the attacks of PCH, DAT with complement components shows positive. IAT test under cold condition shows positive with IgG antibody. The titer of serum complement becomes decreased due to consumption.

5. Relationship between DAT and hemolysis

DAT is a useful clinical examination tool at the diagnosis of AIHA during more than 70 years. Although RBCs autoantibody is observed at around 7% of all hospitalized patients [4], most of DAT positive cases indeed have no hemolysis [5]. The intensity of DAT reaction is generally related to the degree of hemolysis. Some AIHA cases show DAT negative status and other non-AIHA cases show DAT positive status (**Table 1**) (**Figure 1D**). Therefore, we need to consider whether the presence of RBCs autoantibody actually works for hemolysis or not and to discriminate the background disease in independent case. Also, in the diagnosis of

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False positive result
Hypergammaglobulinemia
Intravenous immunoglobulin administration
Anti-phospholipid antibody
Infections
Technical problem
False negative result
Hemolysis caused by IgA or IgM antibody
Below sensitivity level of amounting RBC-bound IgG antibody
Low-affinity IgG antibody
Technical problem

Table 1. Main causes of false positive and false negative results in direct anti-globulin test.

DAT negative AIHA, we should differentiate non-AIHA status like as mechanical hemolysis or thrombotic microangiopathy [6].

The following is the pointing condition about the occurrence of AIHA in clinical condition:

1. The amount of IgG antibody on RBCs. The situation of suspecting AIHA strongly by symptom irrespective of DAT negative status, Coombs negative AIHA should be considered. Autoimmune hemolysis can occurred in some condition even if the amount of IgG antibody on RBCs is below the detection limit of positive DAT, and 5–10% of all AIHA cases fall into this category [7]. The amount level of IgG antibody which reaches to DAT positive status is 200 or more molecules per each RBC in conventional irregular antibody test (tube test), and is 120–150 or more molecules per each RBC in column agglutination technology (CAT) [8, 9]. The newly high sensitive quantitative method for detecting RBCs-bound IgG antibody developed recently. Flow cytometoric analysis (FCM), immuroradiometric assay (IRA) and enzyme-linked immunosorbent assay (ELISA) are useful methods for measuring low amount of RBCs-bound IgG antibody [10, 11].

2. The subclass of IgG antibody. As mentioned above, the cause of hemolysis depends on the affinity to IgG antibody and Fc gamma receptor (FcγR). IgG3 antibody having high affinity to FcγR mediates hemolysis with fewer amounts. Regarding this, RBCs bound with many complement components tend to become hemolysis regardless of the small amount of bounding IgG antibody [12].

3. Non-IgG antibody bound to RBCs. Conventional DAT becomes negative if the hemolysis occurred by IgA or IgM class antibody because universal Coombs reagent does not contain these classes of antibody [13]. DAT with the FCM method by using IgA or IgM antibody is able to detect autoantibody bound to RBCs.

4. Hyper-IgG status. A part of cases accompanied with hyper IgG status as a consequence of hyper-gammaglobulinemia or intravenous immunoglobulin administration show DAT or

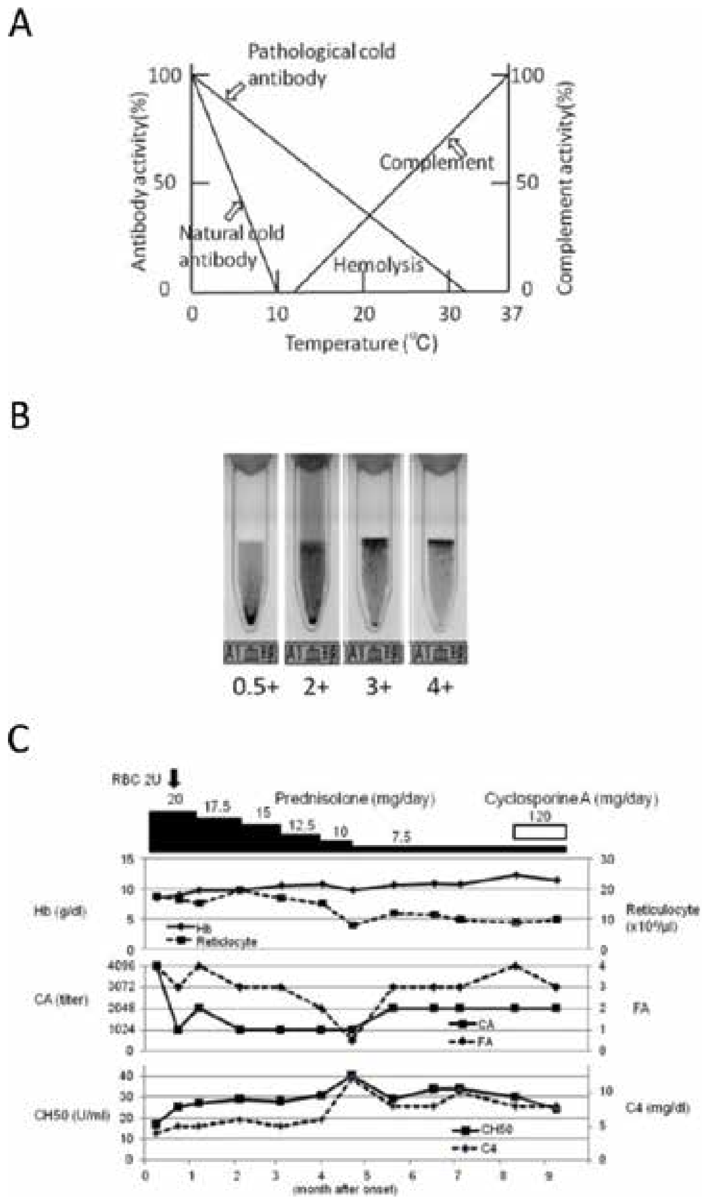

Figure 2. (A) Schematic model about the relationship between antibody activity and complement activity in IgG antibody-mediated hemolysis. Each component has optimal activating temperature. Cold hemolysis requires adequate temperature in which both factor activate. (B) Evaluation of false positive result in the column agglutination technology (CAT) in cold agglutinin disease (CAD) (Ref. [16]). The agglutination in micro-bead matrix reaction shown as the sets of small dots is evaluated as false positive in CAT machinery, and the grade of false positive reaction was expressed by semi-quantitative scale (0.5+ ~ 4+). (C) Clinical course in the CAD case (modified from Ref. [16]). The degree of this false positive (FA) reaction in CAT is more correlated inversely than cold agglutinin (CA) to complement titer (CH50, C4).

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IAT positive status [14, 15]. The reason of this is explained that plasma IgG antibody united to RBCs with nonspecific fashion. However, autoantibody for RBCs is produced by autoimmune mechanism in some of hyper-gammaglobulinemia cases. To analyze autoantibody for RBCs, examiner should pay attention to the existence of alloantibody hiding from autoantibody. If IAT is positive, free autoantibody for RBCs will be absorbed from serum (plasma) by using patient's own RBCs, and eluted autoantibody will be analyzed.

5. Thermal range of operating IgG antibody. Complement activity is essential to develop hemolytic reaction. RBCs autoantibody has optimal thermal range for bindings, and hemolysis requires adequate temperature in which complement activates and autoantibody binds to RBCs (**Figure 2A**). To take an example of CAD, not the quantity of cold agglutinin but the operating thermal range of cold agglutinin is important to emerge disease. We experienced a CAD case having blood type A (Rh+), in which the agglutination in micro-bead matrix area was interpreted to false positive. This agglutination in CAT is caused by cold agglutinin (IgM autoantibody) induced in CAD (**Figure 2B**). The degree of this false positive reaction in CAT seems to be correlated inversely to complement titer (**Figure 2C**). The range of temperature on activation of cold agglutinin is $22 \pm 10°C$ [17]. The temperature of operating CAT in the presenting case is between 18 and 32°C, where both cold agglutinin and complement component can activate, bring about the agglutination reaction in CAT test tube [16].

6. The pitfalls in the diagnosis of AIHA

The pitfalls about AIHA diagnosis are described as follows:

1. In some cases, blood-type specific autoantibody is capable to be produced without receiving either previous transfusion or the history of pregnancy. As mentioned earlier, a part of AIHA cases have blood-type specific autoantibody, like as Rh type. Immunological disorders related to inappropriate gammaglobulin production seem to be a high risk group for occurrence. In this situation, discrimination between autoantibody and alloantibody for RBCs should be required. We experienced a 14-year-old female's Evans syndrome case with hereditary IgA deficiency. As she had no medical history of receiving transfusion or pregnancy, her hemolysis may be caused by JKa autoantibody [18].

2. Try to clarify AIHA or ineffective erythropoiesis. Hemolytic anemia is defined as the condition composed of anemia, increase of reticulocyte, rising serum indirect bilirubin and lactate dehydrogenase (LD) level and decrease of the serum haptoglobin level. AIHA is one of the hemolytic anemia occurred by autoimmune reaction. On the other hand, ineffective erythropoiesis such as megaloblastic anemia, sideroblastic anemia and myelodysplastic syndromes show similar laboratory data as hemolysis. However, the status of ineffective erythropoiesis is not the same status as that of AIHA, and most of erythroblasts are dying in the intramedullary

area by apoptosis in ineffective erythropoiesis. We experienced an 80-year-old female who has pernicious anemia accompanied with DAT positive status. Anemia was suspected of involving autoimmune mechanism by laboratory data expecting hemolysis; however, anemia was normalized with only vitamin B12 administration. As shown with this case, pernicious anemia cases with positive DAT status do not always be concerned with autoimmune mechanism (**Figure 3**) [19]. It is sometimes difficult to clarify AIHA and ineffective erythropoiesis at the onset of anemia.

3. Consider the background disease in secondary AIHA. Secondary AIHA is caused by infection, allogeneic blood transfusion, pregnancy, autoimmune disease and malignancy. We need to diagnose any AIHA cases carefully whether autoimmune disease or malignancy is exist (**Table 2**) [20]. In terms of autoimmune diseases, collagen diseases including systemic lupus erythematosus (SLE) or rheumatoid arthritis may sometimes complicate AIHA. Especially for SLE, the current SLE diagnostic criteria contain the existence of AIHA or DAT

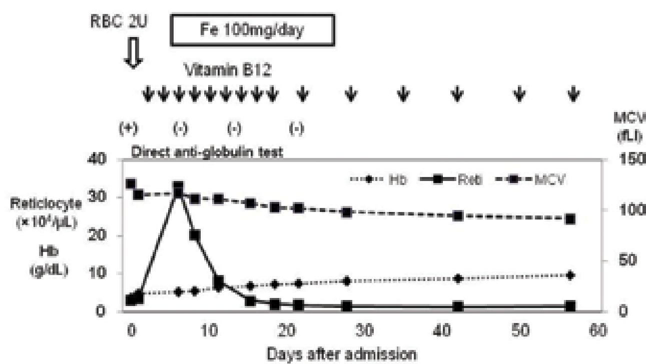

Figure 3. Clinical course in the pernicious anemia case (modified from Ref. [19]). Autoimmune hemolytic anemia (AIHA) was suspected due to the hemolytic aspect and DAT positive status in initial presentation of this case. However, treatment with vitamin B12 administration improved anemia and the disappearance of hemolysis or DAT positive status turned out to be the diagnosis of pernicious anemia which was not involvement of true AIHA conclusively.

Hematological malignancy	
Chronic lymphocytic leukemia (CLL)	4.3–9%
Non-Hodgkin lymphoma excepting CLL/SLL category	0.23–2.6%
Angioimmunoblastic T-cell lymphoma	17.8%
Hodgkin lymphoma	0.19–1.7%
Autoimmune disease	
Systemic lupus erythematosus	6.6–7.5%
Anti-phospholipid syndrome	9.7%
Ulcerative colitis	1.4–1.7%

Modified from Ref. [20] with refined WHO classification.

Table 2. Causes of secondary AIHA.

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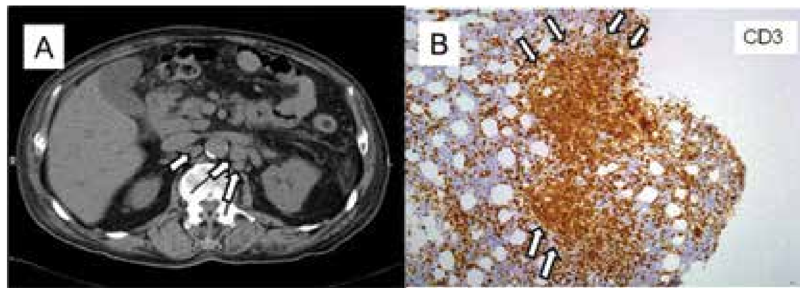

Figure 4. (A) Abdominal lymph nodes were swollen in CT scan (arrow) in an AITL case. (B) Bone marrow biopsy specimen (CD3 immunostaining (100×)). AITL cells infiltrate to bone marrow, which were shown as clear cells (arrow).

positive status [21], which is the reflection of high frequency of AIHA in SLE. AIHA is also complicated with inflammatory bowel diseases such as ulcerative colitis or Crohn's disease. Searching patient's medical history or following patient during clinical course is required for the diagnosis of background disease because these autoimmune diseases and AIHA are not always manifested at the same time. Anti-phospholipid antibody syndrome and idiopathic thrombocytopenic purpura are another responsible cause of DAT positive status; therefore, the possibility of existence of these diseases is also considered in DAT positive status.

4. About malignancy as a cause of secondary AIHA, lymphoproliferative disorders are predominantly observed than solid tumors. As shown in **Table 2**, angioimmunogenic T cell lymphoma (AITL) and chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL/SLL) seem to be the main cause. AITL is known to have symptoms of autoimmune diseases including AIHA. We show an AIHA case in which background disease is AITL (**Figure 4A**), and show another case involved in bone marrow (**Figure 4B**) [22]. In these presenting cases, computed tomography (CT) revealed abdominal lymphadenopathy. DAT positive status turned to be negative when AITL was in good control. In solid tumors, squamous cell carcinoma and adenocarcinoma are seen as a paraneoplastic syndrome [23]. To diagnose malignancy as the secondary disease of AIHA, especially for AITL and CLL/SLL, image diagnosing including CT scan or bone marrow examination is required.

7. Conclusions

The potentiality of anti-globulin test and pointing issue under the diagnosis of AIHA is explained. We need to make much of diagnosing the background disease such as lymphoproliferative disorders or autoimmune diseases when diagnosing AIHA.

Conflict of interest

The authors have no conflict of interest.

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Thalassemia is a very common disease first described by pediatrician Thomas Benton Cooley in 1925 who described it in a patient of Italian origin. At that time, it was designated as Cooley's anemia. George Hoyt Whipple, a Nobel prize winner, and W. L. Bradford, a professor of pediatrics at the University of Rochester, coined the term thalassemia in 1936, which in Greek means anemia of the sea (Thalassa means "sea", and *emia* means "blood"), due to the fact that it is very common in the area of the Mediterranean Sea. This name is actually misleading because it can occur everywhere in the world. Thalassemia is not a single disease; it is rather a group of hereditary disorders of the production of globulin chain of the hemoglobin. Throughout the world, thalassemia affects approximately 4.4 of every 10,000 live births. It represents a major social and emotional impact on the patient and his family and a major burden on health services where the prevalence is high.

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