2022

Beta thalassemia: pathogenesis, progression, and treatment

BOSTON UNIVERSITY

SCHOOL OF MEDICINE

Thesis

BETA THALASSEMIA:

PATHOGENESIS, PROGRESSION, AND TREATMENT

by

MICHAEL KITIASHVILI

B.S., New York University, 2020

Submitted in partial fulfillment of the

requirements for the degree of

Master of Science

2022

Approved by

First Reader

David Atkinson, Ph.D.
Professor of Physiology & Biophysics

Second Reader

Aaron William Young, Ph.D.
Assistant Professor of Physiology & Biophysics

BETA THALASSEMIA:

PATHOGENESIS, PROGRESSION, AND TREATMENT

MICHAEL KITIASHVILI

Abstract

β-thalassemia is an autosomal recessive blood disease caused by mutations in β-globin genes that either reduce or altogether abolish β-globin chain synthesis. Normally, two β-globin chains would combine with two α-globin chains and a heme group to form hemoglobin. Because α-globin chain synthesis is unaffected in β-thalassemia patients, the unpaired α-globin chains accumulate and precipitate. The reduced formation of hemoglobin and accumulation of unpaired α-globin chains are the two fundamental molecular pathologies. In the most serious cases of the disease, the resulting complications develop before two years of age. Most often, these include severe anemia, pallor, jaundice, abdominal enlargement, and distinct craniofacial features. If left untreated, the disease is fatal before the age of three in the most serious cases. Each year, more than 40,000 births, mostly in Southeast Asia, Middle East, or Africa, are affected with β-thalassemia. With increased migration, however, β-thalassemia is becoming more common in Europe and North America. Currently, the most widespread treatment for the disease is transfusions and iron chelation therapy, and the only cure is hematopoietic stem cell transplantation. In recent years, however, multiple treatments and potential cures such as fetal hemoglobin inducers and gene therapy have shown promise. By analyzing the cost-efficiency, viability, and therapeutic benefits of current and future treatments, it can be seen that a combination of fetal hemoglobin inducers, transfusions, and iron

chelation therapy will have the greatest impact for the vast majority of β-thalassemia patients.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

BCL11A	B-cell lymphoma/leukemia 11A protein
CHF	congestive heart failure
DFO	desferrioxamine
DFP	deferiprone
EMH	extramedullary hematopoiesis
EPO	erythropoietin
GATA1	GATA-binding protein 1
Hb	hemoglobin
HCT	hematocrit
HIF-2	hypoxia-inducible factor 2
HLA	human leukocyte antigens
HPFH	hereditary persistence of fetal hemoglobin
HSCT	hematopoietic stem cell transplantation
IE	ineffective erythropoiesis
IVS	intervening sequences
KLF1	Kruppel like factor 1
LCR	locus control region
LRF	lymphoma/leukemia-related factor
MCH	mean corpuscular hemoglobin
MCV	mean corpuscular volume
NF-Y	nuclear transcription factor Y

NTBI	non-transferrin bound iron
RBC	red blood cells
ROS	reactive oxygen species
SA	self-assembly domain of Ldb1
TI	thalassemia intermedia
TM	thalassemia major
UTR	untranslated region
ZF	zinc finger

Introduction

β-thalassemia is an autosomal recessive blood disorder characterized by reduced levels of hemoglobin. There are more than 350 mutations associated with the disorder, and an estimated 80-90 million people worldwide (1.5% of global population) are carriers (Origa, 2017). All the β-thalassemia mutations either reduce or completely abolish β-globin chain synthesis. In adults, two β-globin chains normally combine with two α-globin chains to form the hemoglobin protein (Origa, 2017). The reduced levels of β-globin chain synthesis lead to decreased levels of circulating hemoglobin and accumulation of unpaired α-globin chains. These underlying cellular defects manifest as anemia, hemolysis, and iron overload (Croteau et al., 2013). If the underlying anemia is inadequately controlled, bone marrow expansion and consequent skeletal abnormalities ensue. On the other hand, increased hemolysis leads to hypercoagulability and elevates the risk for thromboembolism in β-thalassemia patients (Taher et al., 2018). Although skeletal abnormalities and hypercoagulability can be quite problematic, by far the most significant pathology in β-thalassemia is iron overload. It can develop because of increased iron absorption (secondary to chronic anemia) and/or regular transfusion in patients who require them. Over time, the excess iron accumulates in the heart, liver, and endocrine glands (Rund & Rachmilewitz, 2005). Cardiomyopathy due to iron overload is by far the most common cause of death among the β-thalassemia patients. Nonetheless, hepatic abnormalities and endocrine dysfunctions (most commonly diabetes mellitus) are common as well (Musallam et al., 2021).

Currently, β-thalassemia can be treated by transfusions (the most prevalent treatment) and fetal hemoglobin induction or cured by hematopoietic stem cell transplantation. Transfusions, usually required once every two to five weeks, deliver a large quantity of iron that can accumulate over time and cause iron overload. As a result, most of the patients who receive transfusions also undergo iron chelation therapy to remove excess iron. Some β-thalassemia patients require chelation up to five times per week for several hours each day. The burdensome iron chelation therapy and transfusions greatly reduce the quality of life in β-thalassemia patients, leading to low compliance. Consequently, many β-thalassemia patients are inadequately transfused and chelated and experience complications. In addition to the reduction in quality of life, β-thalassemia management can be costly. Lifetime cost of the disease management is more than $700,000 in the UK, for example (Weidlich et al., 2016).

In recent years, a great deal of research has been devoted to finding novel treatments and cures for the disease such as erythroid maturation agents and gene therapy. Although such approaches are promising, their safety, efficacy, and viability remain to be evaluated. The first goal of this thesis is to explain the genetic basis of β-thalassemia. It will then explore the disease's pathophysiology, pathogenesis, and symptomatology. Finally, the thesis will compare the safety, efficacy, and viability of current and future treatments or cures for β-thalassemia and discuss which treatments are likely to be most impactful.

Genetics

β-globin Gene Cluster and Regulatory Elements

The β-globin gene cluster consists of approximately 70 kb of DNA on the terminal portion of the short arm of chromosome 11 (p15). The gene cluster contains ε-globin gene (*HBE*), duplicated γ-globin genes (*HBG2* and *HBG1*), δ-globin gene (*HBD*), β-globin gene (*HBB*), and 2 pseudogenes (Figure 1). Gene products of these five genes (β-like globin genes) each contain a total of 146 amino acids. The duplicated γ-globin genes, *HBG2* and *HBG1*, code for nearly identical gene products G_γ and A_γ. Although G_γ has glycine and A_γ has alanine residue at codon 137, the two globin chains are functionally indistinguishable (Gilman, 1988). In addition to these genes, there is a powerful distal enhancer, locus control region (LCR), which can be brought in close proximity with different globin genes by chromatin looping. In humans, LCR-binding transcription factors have been shown to interact and alter expression of *HBG2*, *HBG1*, and *HBB* genes at different developmental stages (Deng et al., 2014; Palstra et al., 2003).

The β-globin gene itself consists of three exons and two intervening sequences or introns (IVS1 and IVS2). The promoter region contains several crucial consensus sequences such as CACCC box and TATA box which serve as binding sites for transcription factors (Figure 2). Mutations causing β-thalassemia can arise in the promoter region, 5' and 3' untranslated regions (UTRs), IVS1 and IVS2, and any of the codons (Cao & Galanello, 2010; Thein, 2013). Depending on the severity of mutations, a β-globin allele can be classified as either β^+ or β^0. The β^+ and β^0 alleles exhibit reduced or

absent β-globin chain syntheses, respectively. β^+ alleles are usually a result of point mutations in the promoter region and 5' and 3' UTRs.

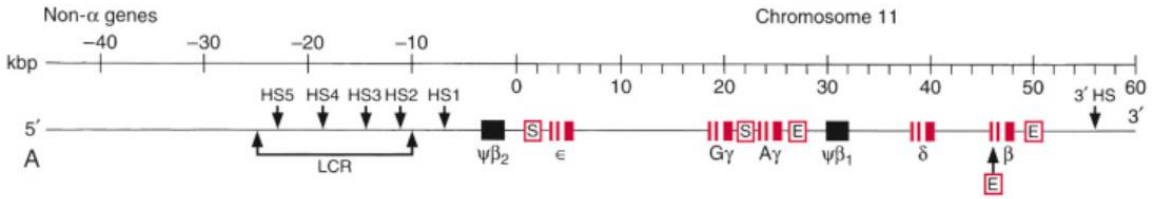

Figure 1: Human β-globin Gene Cluster. Active genes are shown in red boxes and pseudogenes are shown in black boxes. G_γ is encoded by *HBG2*, and A_γ is encoded by *HBG1*. Taken from (Hoffman et al., 2018, Chapter 33).

β^0 alleles, on the other hand, typically stem from splice junction, initiation codon, nonsense, and frameshift mutations (Origa, 2017; Thein, 2013). Splice junction mutations can arise from either abolition of an existing splice site or introduction of a new splice site.

Although more than 350 β-thalassemia-related mutations have been described worldwide, no more than five or six mutations usually account for over 90% of mutations in a given region (Jaing et al., 2021). Furthermore, the dominant form of mutation varies heavily between and within regions (Cao & Galanello, 2010). In Sardinia, for example, over 95% of β-thalassemia mutations are caused by a nonsense mutation in codon 39 (CAG to TAG), leading to absence of β-globin chain production (Pirastu et al., 1984). In Cyprus, a country with highest incidence of β-thalassemia, more than 60% of mutations are caused by a point mutation in IVS1 (Kountouris et al., 2020). Finally, a four base pair deletion in codons 41 and 42 is the dominant β-thalassemia mutation in Malaysia (George & Ann, 2010).

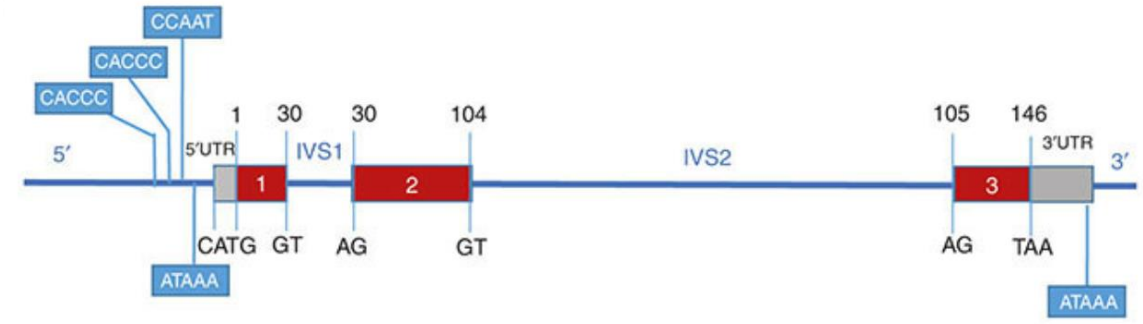

Figure 2: Human β-globin Gene. Blue boxes represent consensus sequences, red boxes represent exons, and grey boxes represent UTRs. The codons that each exon codes for are represented by the numbers flanking each exon. Taken from (Origa, 2017, fig. 3a).

Inheritance Patterns

Inheritance patterns of β-thalassemia are somewhat complicated. In almost all cases, the disease is transmitted in autosomal recessive fashion. Therefore, for the disease to be present, two copies of mutated β-globin gene must be inherited. Because the mutated alleles can be classified in two groups, there are three possible homozygous genotypes: β^0/β^0, β^0/β^+, and β^+/β^+ (Chonat & Quinn, 2017). Rarely, β-thalassemia can be inherited in autosomal dominant fashion. In such cases, mutations usually allow for β-globin mRNA and mutant protein synthesis (Thein, 1992). Rather than reduction or complete absence of β-globin chain synthesis, the molecular basis for the disease is the formation of unstable hemoglobin (Hb) tetramers or inability of β-globin chains to associate with α-globin chains (Chen et al., 2021; Croteau et al., 2013). In the latter case, unpaired α- and β-globin chains precipitate and their accumulation overwhelms proteolytic capacity of erythroid precursors, leading to the formation of inclusion bodies (Croteau et al., 2013). Lastly, β-thalassemia can arise due to mutations in transcription

factors that would otherwise regulate expression of β-globin gene. Mutations in GATA-binding protein 1 (GATA1), a master transcriptional regulator of erythropoiesis, have been associated with X-linked β-thalassemia (Freson et al., 2002). The X-linked pattern of inheritance is displayed simply because the gene that codes for GATA1 is located on the X chromosome. Additionally, mutations in Kruppel like factor 1 (KLF1) have been associated with β-thalassemia (Tamaddoni et al., 2019). KLF1 binds to CACCC boxes in the promoter region of β-globin gene (Figure 2) and directly promotes its expression (Siatecka & Bieker, 2011).

Differential Expression of Globin Genes

Depending on the stage of development, different globin genes are expressed (Figure 3). The first globin genes to be expressed are ε- and ζ-globin genes which combine to form the earliest form of embryonic Hb ($\zeta_2\varepsilon_2$). ζ-globin resembles adult α-globin while ε-globin resembles adult β-globin. For the sake of simplicity, these two globin genes and other embryonic Hb forms will be considered no further. Starting at around 6 weeks of gestation, α- and γ-globin genes are activated. High levels of α-globin synthesis are quickly attained and continue for the remainder of fetal life and all of adult life, whereas γ-globin synthesis peaks during fetal development and drops to insignificant levels by 6 months postnatally (Pace & Zein, 2006). β-globin synthesis starts at around 12 weeks of gestation, but its rate of increase in synthesis is relatively low. Once it peaks, however, β-globin synthesis remains high for the entire adult life (Pace & Zein, 2006).

Close to birth, low levels of δ-globin synthesis begin and remain relatively low for all adult life.

Therefore, at birth, about 75% of circulating Hb is HbF ($\alpha_2\gamma_2$) and 25% is HbA ($\alpha_2\beta_2$) (Felicetti et al., 1984). In adults, HbF makes up less than 0.6% and HbA almost 97% of circulating Hb (Rochette et al., 1994). It is worth pointing out that about 2.5% of circulating Hb in adults is HbA_2 ($\alpha_2\delta_2$). By 6 months of age, β-globin synthesis dominates while γ-globin synthesis is reduced to insignificant levels (Edoh et al., 2006).

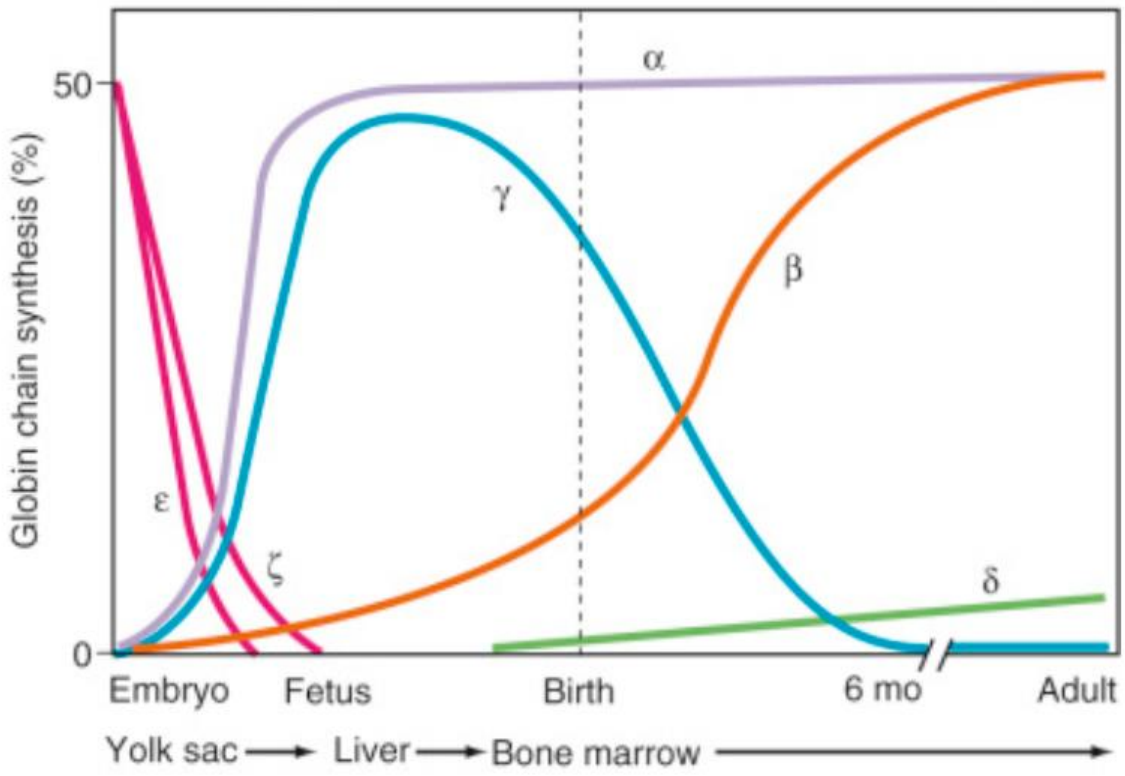

Figure 3: Differential Expression of Globin Genes. Organs in which erythropoiesis occurs are shown underneath the graph. Taken from (Hoffman et al., 2018, Chapter 33).

Transcriptional Regulation

What accounts for this drastic switch from HbF to HbA? Firstly, in a small portion of population (≈10%) affected by hereditary persistence of fetal hemoglobin (HPFH), the complete switch HbF to HbA does not occur (Thein & Menzel, 2009). In such cases, varying levels of HbF are observed into adulthood. Usually, individuals with HPFH express 0.8-30% of HbF; however, cases of HbF levels greater than 90% have been reported (Martyn et al., 2018; Sharma et al., 2020). Mutations that lead to HPFH have been mapped to two clusters (-115 cluster and -200 cluster) within γ-globin gene promoter (Figure 4). Perhaps the two most important γ-globin repressors bind these two clusters. During the fetal stage of development, however, the γ-globin gene promoter is first occupied by nuclear transcription factor Y (NF-Y), a ubiquitous CCAAT-binding protein that promotes chromatin accessibility (Oldfield et al., 2014). NF-Y recruits coactivators near the promoter region, leading to high levels of γ-globin gene transcription (N. Liu et al., 2021).

In order for the γ- to β-globin synthesis switch to occur, γ-globin synthesis must first be repressed. This action is achieved by B-cell lymphoma/leukemia 11A (BCL11A) protein which is a regulatory zinc-finger. It has become clear that BCL11A is essential for repression of γ-globin gene, but the mechanism of action has been somewhat controversial (Sankaran et al., 2008). BCL11A was shown to bind LCR regions (Figure 1) which led to the suggestion that it acted through long-range interactions, mediated by chromatin looping (Carter et al., 2002; Palstra et al., 2008; Xu et al., 2010). However, in transgenic mice incorporating individual human globin genes, removal of LCR does not

disrupt the correct sequence of globin expression. Rather, decreased levels of globin expression are observed (Bender et al., 2000). More recent studies have demonstrated that in addition to LCR, BCL11A directly binds to -115 cluster in the γ-globin gene promoter (N. Liu et al., 2018). As described earlier, mutations in this region *in vivo* are associated with HPFH.

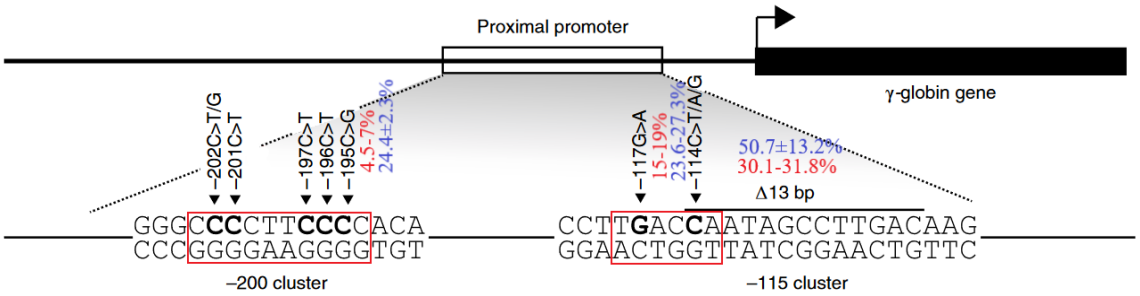

Figure 4: LRF and BCL11A Binding Sites in γ-globin Gene Promoter. LRF binds to -200 cluster and BCL11A binds to -115 cluster. Respective consensus sequences are outlined by red boxes. Black arrows indicate sites of mutations within each binding sites that lead to HPFH. Levels of HbF expression for three mutations (195C>G, 117G>A, and Δ13 bp) are shown. Red percentages reflect *in vivo,* and blue percentages reflect *in vitro* HbF expression for each of the three mutations. Results of X-ray crystallography are shown in the bottom half of the figure. Taken from (Martyn et al., 2018) and (Yang et al., 2021).

Corresponding mutation *in vitro* have been demonstrated to lead to elevated levels of HbF, establishing a causal relationship. Higher levels of HbF were observed *in vitro* than *in vivo* because individuals with HPFH tend to be heterozygous whereas the human erythroid cells were genetically engineered to be homozygous (Martyn et al., 2018). Only 24 base pairs downstream of BCL11A binding site, there is CCAAT-box which is a consensus sequence for NF-Y (Doerfler et al., 2021). The proximity of the two binding sites and the opposing effects of BCL11A and NF-Y on γ-globin expression give rise to possible binding competition at the promoter. In BCL11A knockout human erythroid

cells, targeted binding of dCas9 (mutant Cas9 with no endonuclease activity) to BCL11A binding site led to decreased NF-Y occupancy and reduced γ-globin expression (N. Liu et al., 2021). Therefore, through steric hindrance, BCL11A outcompetes NF-Y at the promoter region and leads to γ-globin repression (N. Liu et al., 2021). Once displaced from γ-globin promoter, NF-Y binds the β-globin promoter and promotes chromatin accessibility. Note that the authors did not assess γ-globin expression levels beyond five days of erythroid differentiation, so no direct conclusions about long-term repression can be drawn. Nonetheless, BCL11A has been shown to recruit corepressors that remodel nucleosomes and deacetylate histones to maintain γ-globin gene inaccessible (Xu et al., 2013).

Another transcription factor, lymphoma/leukemia-related factor (LRF), has been shown to directly bind γ-globin gene promoter at -200 cluster (Martyn et al., 2018). Similar to BCL11A, LRF is a zinc-finger protein which binds to a consensus sequence within the γ-globin gene promoter (Figure 4), recruits corepressors, and represses γ-globin expression through chromatin remodeling and histone deacetylation (Masuda et al., 2016; Sankaran et al., 2008). Despite similarities, the two repressors seem to act in independent rather than cooperative fashion: human erythroid cells with either LRF or BCL11A gene knockout express lower levels of HbF (≈70%) than LRF/BCL11A double-knockout cells (>90%), and LRF-associated corepressors do not seem to include BCL11A (Masuda et al., 2016). The fact that HbF expression levels increased from 1.79% in controls to well over 90% in double-knockout cells indicates that repression of γ-globin gene is almost entirely due to LRF and BCL11A (Masuda et al., 2016).

In addition to γ-globin gene repression, β-globin gene expression must be promoted for γ- to β-globin synthesis switch to occur. This is achieved in large part by GATA1 and its coactivators. As discussed above, mutations in GATA1 are associated with rare forms of X-linked β-thalassemia (Freson et al., 2002). GATA1 has been shown to bind to β-globin gene promoter and LCR and bring the two regions in close proximity, forming a chromatin loop (Drissen et al., 2004). Once juxtaposed with β-globin promoter, LCR increases the gene expression by enhancing the transition from transcription initiation to elongation (Sawado et al., 2003).

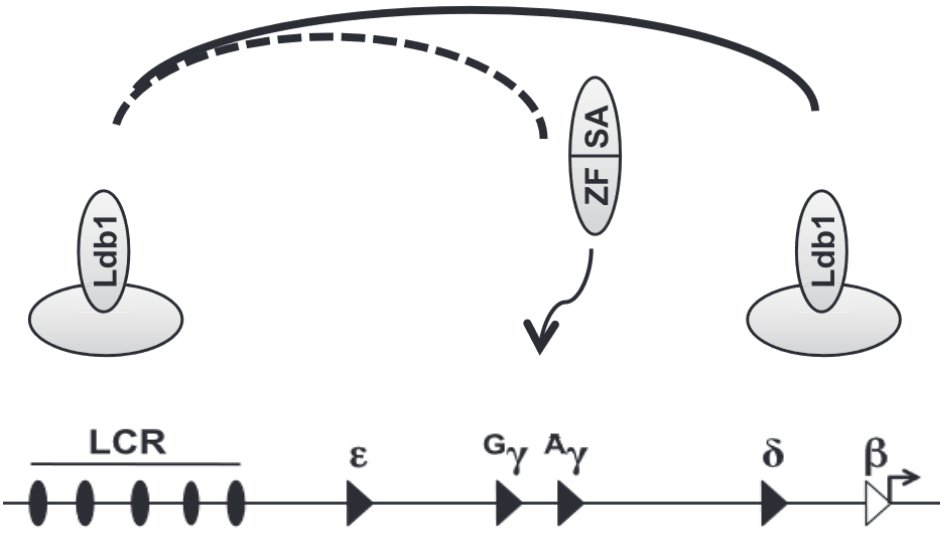

Figure 5: Reactivation of γ-globin Gene Expression. Within the β-globin gene cluster in adult human erythroid cells, Ldb1 brings β-globin gene promoter and LCR together as shown by the solid line. ZF-SA denotes the self-assembly (SA) domain of Ldb1 attached to artificial zinc finger (ZF) which binds to γ-globin gene promoter. Dashed line denotes novel interaction between γ-globin gene promoter and LCR. γ-globin synthesis was ~25% of total in controls, and total globin synthesis was defined as β-globin plus γ-globin. Modified from (Deng et al., 2014).

Interestingly, GATA1-associated chromatin loop formation was shown to be, in part, the action of one of its coactivators, Ldb1. Ldb1 is recruited at LCR and β-globin promoter

(Ldb1 does not bind the promoter directly) with the latter being entirely GATA1-dependent (Deng et al., 2012; Soler et al., 2010). When Ldb1 was artificially recruited to β-globin promoter (via zinc fingers) in GATA1 null cells, 1000-fold increase in β-globin transcription was observed (Deng et al., 2012). To demonstrate that this effect was LCR-dependent, the experiment was repeated in cells with no LCR. As expected, no β-globin transcription amplification was observed (Deng et al., 2012). Notably, γ-globin synthesis (85% of total) was induced in adult human erythroid cells by artificially recruiting one of Ldb1 domains to γ-globin gene promoters (Figure 5). The fact that γ- to β-globin synthesis switch was effectively reversed by forced recruitment of LCR at the γ-globin promoter indicates that LCR proximity is the most significant factor mediating this switch (Deng et al., 2014).

To summarize, repression of the γ-globin gene is chiefly achieved by independent actions of BCL11A and LRF. BCL11A acts to displace and sterically hinder binding of NF-Y (Figure 6B), a transcription factor that promoters chromatin accessibility (N. Liu et al., 2021; Oldfield et al., 2014). In addition, both BCL11A and LRF recruit corepressors that remodel nucleosomes and deacetylate histones (Masuda et al., 2016; Xu et al., 2013). The two repressors bind the γ-globin gene promoter no more than 100 base pairs apart (Figure 4). At the β-globin gene promoter, GATA1 and NF-Y act to promote transcription (Figure 6A). GATA1 and its coactivators bring LCR in close proximity with the promoter, enabling faster transition from transcription initiation to elongation (Deng et al., 2012; Sawado et al., 2003). As discussed above, absence of LCR does not disrupt the correct sequence of globin expression (Bender et al., 2000). Nonetheless, in

order for a globin gene to be expressed in adequate amounts, juxtaposition of LCR to its promoter is essential (Figure 6, Deng et al., 2014).

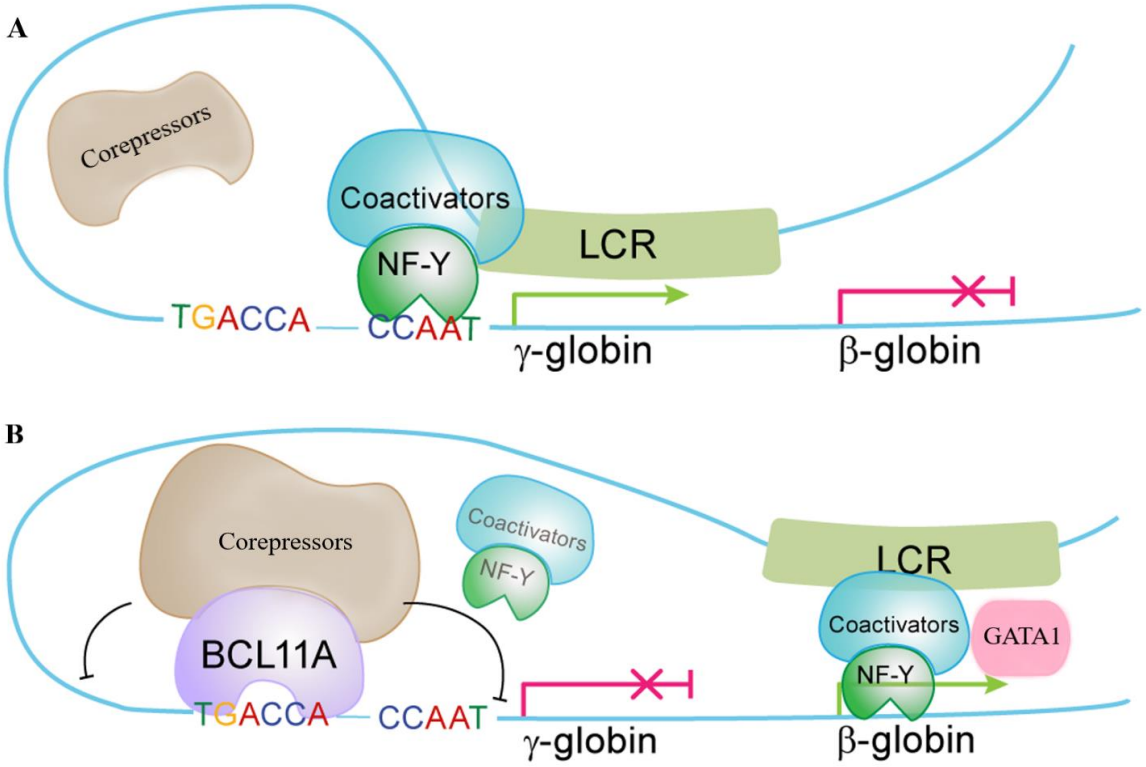

Figure 6: Hemoglobin Switching. γ-globin and β-globin gene promoters in A) fetal erythroid cells and B) adult erythroid cells. NF-Y binding site is 24 base pairs downstream of BCL11A binding site. Note that this is by no means an exhaustive list of transcription factors involved in globin synthesis regulation. Modified from (N. Liu et al., 2021).

Pathophysiology

The adult Hb protein consists of two α-globin chains and two β-globin chains held together by hydrogen bonds, hydrophobic interactions, and ionic bonds. The β-globin chain contains eight alpha helices, named A-H, and non-helical connecting regions (Devlin, 2011). The α-globin chain is very similar, but only contains seven alpha helices (D helix is absent). Each of the globin chains contains a heme molecule which consists of a porphyrin ring and a ferrous iron atom. The heme is located in a hydrophobic pocket where 18 mostly apolar amino acids provide approximately 80 interactions to stabilize it (Devlin, 2011). Both the porphyrin ring and the globin chain form covalent bonds with the iron atom. It forms four bonds with the porphyrin ring (Figure 7). In the absence of oxygen, proximal and distal histidines on a globin chain are also covalently bonded to the iron atom. In the presence of oxygen, however, oxygen replaces the distal histidine (Figure 7). Oxygen binding flattens the heme porphyrin ring system and displaces the proximal histidine, triggering allosteric changes in the quaternary arrangement of the two α- and β-subunits. This change drives the cooperative binding of oxygen which is vital to the function of hemoglobin. One of the most important features of Hb is its high solubility. In fact, Hb is more soluble than the α- and β-globin chains individually (Devlin, 2011). As a result, excess unpaired globin chains tend to precipitate. Note that since the underlying pathology in the recessively inherited β-thalassemia is either the absence or reduction of β-globin chain synthesis, the synthesized hemoglobin (however little) is normal HbF and/or HbA. Only in the very rare, dominantly inherited cases of β-

thalassemia is there the formation of abnormal Hb (Thein, 2013). Due to its rarity, the dominantly inherited form will not be discussed further.

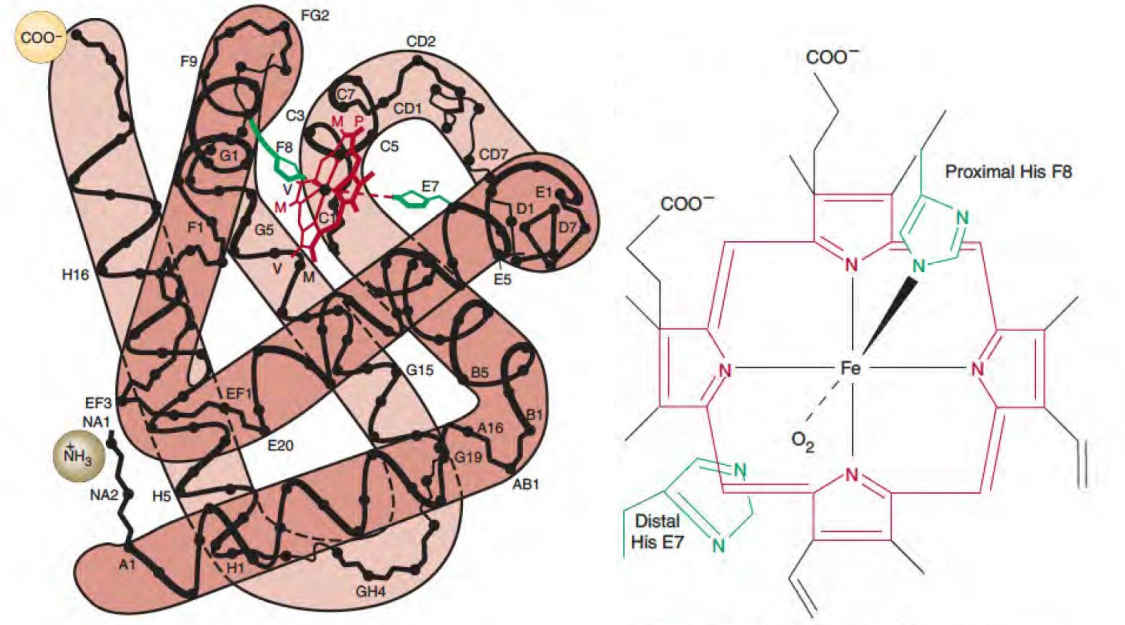

Figure 7: Molecular Structure of β-globin Chain. The molecular structure of deoxy β-globin chain is shown on the left. The porphyrin rings are displayed in red. F8 refers to the proximal histidine while E7 refers to the distal histidine. An oxygen bound heme is shown on the right. The wedge denotes a bond coming out of the page, and the dash denotes a bond going into the page. The iron atom and the four bonds to the porphyrin ring (shown in pink) are in the same plane. Modified from (Devlin, 2011).

As already discussed, there are three possible homozygous genotypes that can cause the recessive form of β-thalassemia: β^0/β^0, β^0/β^+, and β^+/β^+. Individuals with β^0/β^+ and β^+/β^+ are usually diagnosed with thalassemia intermedia (TI) and display mild to moderate anemia with mean corpuscular hemoglobin (MCH) of 16-24 pg. Individuals with β^0/β^0 genotype, on the other hand, are diagnosed with the thalassemia major (TM) which is characterized by severe anemia and MCH of 12-20 pg (Galanello & Origa, 2010). The values for hematological diagnostic criteria are summarized in Table 1.

Although this genotype-phenotype-based dichotomy generally holds true, there is some overlap between the two categories. That is, depending on the severity of β-globin chain synthesis reduction, β^0/β^+ and β^+/β^+ genotypes may manifest as the TM instead of TI (Asadov et al., 2018). The TM patients become symptomatic between ages of 6-24 months and require regular transfusions (every two to five weeks) throughout life (Origa, 2017). Note that the onset of symptoms is delayed because the γ-globin chain synthesis persists for several months after the birth (Figure 3). In contrast, the TI patients usually only require transfusions because of pregnancy, infection, or other events that increase oxidative stress (Weatherall, 2012).

Since one of the diagnostic criteria for β-thalassemia is the reduction of MCH, and the genetic basis for the disease is the reduction or absence of β-globin chain synthesis due to mutations, the pathophysiology for the disease seems straightforward; the anemia is a simple consequence of the reduced MCH and the decreased levels of circulating hemoglobin. However, a few observations indicate that this understanding is incomplete.

Table 1: Hematological Diagnostic Criteria. MCH = $\frac{[Hb]}{[RBC]}$ and MCV = $\frac{HCT \times 10}{[RBC]}$ where [Hb] = concentration of Hb in g/dL, [RBC] = concentration of RBCs in millions/μL, and HCT = hematocrit in percent. In other words, MCH is the average amount of Hb per RBC and MCV is the average volume of an RBC. Table put together from (Galanello & Origa, 2010).

	Thalassemia major	**Thalassemia intermedia**
Hb (g/dL)	<7	7-10
MCH (pg)	12-20	16-24
MCV (fL)	50-70	50-80

Firstly, the TM patients would be expected to have elevated hematocrit (HCT) to compensate for decreased MCH, but their HCT is actually depressed (Table 2). Secondly, the untransfused TI patients can show very high levels of HbF (Musallam et al., 2012). Similar results are found in the TM patients (Galanello & Origa, 2010). Finally, the inheritance of α-thalassemia in the TI patients leads to a milder rather than more severe thalassemia phenotype (Nienhuis & Nathan, 2012). Furthermore, the thalassemia carriers (normally asymptomatic) that inherit additional copies of α-globin gene express mild TI phenotype (Karim et al., 2016).

The last observation indicates that the degree of imbalance between α- and β-globin synthesis determines the degree of thalassemia severity. In fact, increase in the α-globin/β-globin mRNA ratio was shown to correlate with a more severe thalassemia phenotype (Ranjbaran et al., 2014).

Table 2: Hematological Characteristics of the TM patients. These results were obtained from 54 adult Bangladeshi TM patients and 54 adult controls. At the cellular level, the reduced MCV manifests as microcytosis and the reduced MCH as hypochromia. P-values were calculated with ANOVA followed by independent t-test. Modified from (Karim et al., 2016).

Parameters (Unit)	Thalassemia major (Mean ± SD)	Control (Mean ± SD)	P-value
Hb (g/dL)	7.2 ± 1.5	13 ± 1.4	P< 0.001
HCT (%)	21.5 ± 5.3	38 ± 6.2	P< 0.001
MCV (fL)	70 ± 9.5	80 ± 11	P< 0.05
MCH (pg)	23.8 ± 3.8	28 ± 5	P< 0.05
Ferritin (µg/L)	1249 ± 59.2	45 ± 17	P< 0.05
Fe (µg/dL)	123 ± 40.5	110 ± 32	P>0.05

In the TI patients, α-globin chain synthesis is three to four times that of β-globin, and any greater globin imbalance manifests as the TM (Nienhuis & Nathan, 2012). At the cellular level, this can be explained by the α-globin chain aggregations. Since the unpaired α-globin chains are unstable, they tend to aggregate and form inclusions which have been shown to colocalize with membrane proteins (e.g. spectrin) at the sites of membrane defects (Aljurf et al., 1996). The inclusions cause disintegration of heme and the release of iron species which generate reactive oxygen species or ROS (Taher et al., 2018). In addition, the unpaired α-globin chains autoxidize at a higher rate (compared to Hb), thereby producing additional ROS (Nagababu et al., 2008). Higher levels of ROS damage cytoskeletal proteins and further compromise membrane integrity (Ribeil et al., 2013). The heightened oxidative stress also causes increased peripheral hemolysis, shortening the lifespan of mature erythrocytes by more than half (Singer et al., 2004).

The α-globin chain inclusions and increased generation of ROS both lead to erythroid precursor death (Voskou et al., 2015). In particular, higher number of erythroblasts at polychromatic stage of erythropoiesis undergo apoptosis (Figure 8, Mathias et al., 2000). This stage of development is normally marked by a great increase in α- and β-globin chain syntheses (Ribeil et al., 2013). Since the β-thalassemia patients have defective β-globin genes, the globin chain imbalance worsens at this stage. Consequent oxidative stress and membrane damage trigger apoptosis. Similarly, the extent of globin chain imbalance and apoptosis can either be attenuated or exacerbated by the inheritance of fewer or additional α-globin genes, respectively (Karim et al., 2016; Nienhuis & Nathan, 2012). Finally, the TM patients show lower HCT because the vast

majority of erythroid precursors never reach maturity (Figure 8). This process is aptly called ineffective erythropoiesis (IE). The IE is so severe in the TM patients that despite erythropoietin-induced erythroid hyperplasia (secondary to anemia) and bone marrow expansion of up to 25 to 30 times the normal, HCT remains depressed (Galanello & Origa, 2010).

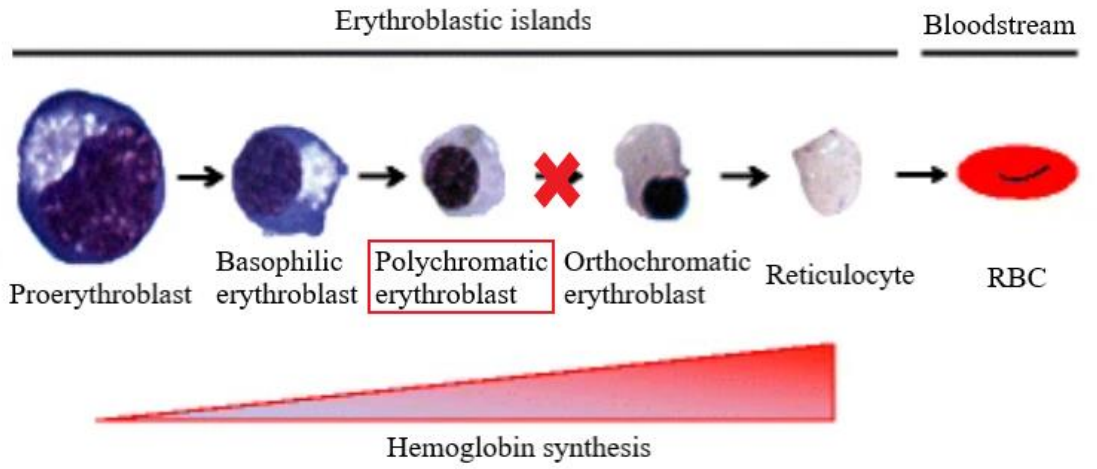

Figure 8: Overview of Erythropoiesis. As erythropoiesis progresses, the hemoglobin synthesis gradually increases while non-hemoglobin protein synthesis declines. Erythroid precursors in the β-thalassemia patients fail to differentiate beyond polychromatic erythroblast stage. Note that reticulocytes are formed in erythroblastic islands but mature in bloodstream by losing internal organelles. Modified from (Zivot et al., 2018).

However, this observation raises the question: if the erythroid precursors fail to mature, why do the TM patients have elevated levels of HbF? Recall that HbF synthesis is insignificant by six months of age, and by two years of age, the circulating levels of HbF are less than 1% (Figure 3, Rochette et al., 1994). Nevertheless, rather than it being uniformly distributed, HbF synthesis is limited to a small group of cells called F-cells. It is worth noting that the F-cells resemble adult, not fetal, erythrocytes in every respect other than the HbF synthesis. Although highly variable, these cells usually make up no

more than 5% of adult erythrocytes and 14-28% of total Hb in each cell is HbF (Khandros & Blobel, 2021; Rochette et al., 1994). Since some of the unpaired α-globin chains pair with γ-globin chains to form HbF, the globin chain imbalance is less severe in the F-cells. As a result, they are able to fully mature and enter circulation (Ribeil et al., 2013). Thus, the TM patients have elevated levels of HbF for two reasons. There is a great increase in the number of F-cells due to the erythroid hyperplasia and they have a selective survival advantage which enables them to fully mature and enter the circulation.

In summary, the anemia in β-thalassemia patients is brought about by the IE and the reduced MCH. The former is caused by the inclusions of excess unpaired α-globin chains and the oxidative stress, whereas the latter is a simple reflection of the reduced or absent β-globin chain synthesis. Another factor that contributes to the anemia is the more than two-fold reduction in the lifespan of mature erythrocytes due to the increased oxidative stress (Singer et al., 2004). The inclusions and oxidative stress damage the erythroid precursor cells' membranes and lead to apoptosis, especially at the polychromatic erythroblast stage (Aljurf et al., 1996; Mathias et al., 2000). In the TM, only the F-cell erythroid precursors differentiate beyond the polychromatic erythroblast stage (due to lower globin chain imbalance) and enter the circulation (Khandros & Blobel, 2021; Ribeil et al., 2013). The same cell population is also less susceptible to apoptosis in the TI. Moreover, the number of F-cells is greatly increased because of erythropoietin-induced erythroid hyperplasia (Galanello & Origa, 2010). As a result, the TI and TM patients show highly elevated levels of HbF (Musallam et al., 2012). Lastly, the coinheritance of α-thalassemia in the TI patients attenuates the β-thalassemia

phenotype by reducing the globin chain imbalance (Karim et al., 2016; Nienhuis & Nathan, 2012).

Pathogenesis and Symptomatology

TM patients most often present with severe microcytic anemia, hepatosplenomegaly, and mild jaundice before the age of two (Rund & Rachmilewitz, 2005). Similarly, the TI patients present with milder symptoms but at a later stage in life. The diagnosis of β-thalassemia can be made by a hematologic exam, peripheral blood smear, and genetic analysis (Galanello & Origa, 2010). As shown in Table 2, the adult TM patients have very low Hb levels and decreased MCV and MCH. In fact, the Hb levels tend to be even lower (~5g/dL) at the time of diagnosis, and HbF comprises 92-95% of the circulating Hb (Galanello & Origa, 2010; Susanah et al., 2021).

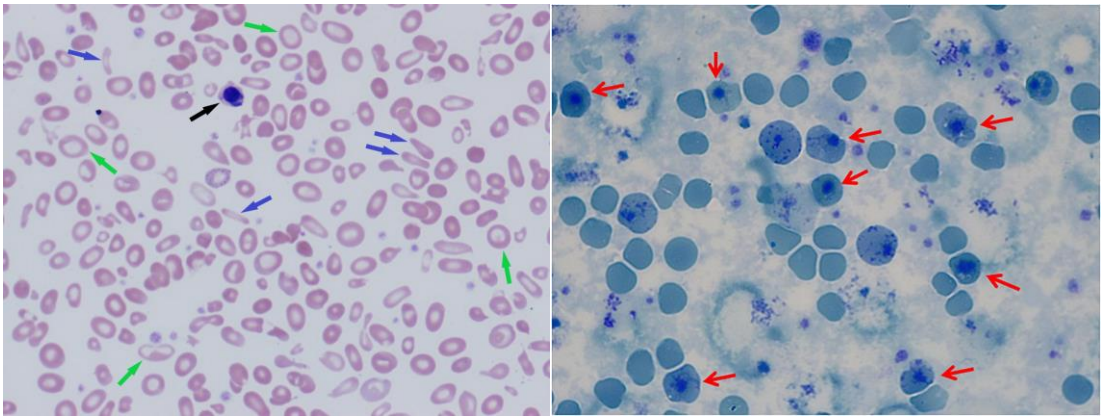

Figure 9: Histopathological Features of Erythrocytes. The two peripheral blood smears are from the TM patients. The smear on the left is stained with hematoxylin-eosin stain, and the one on the right is stained with brilliant cresyl blue stain. The arrows point to the erythrocytes that display typical thalassemia features: black – nucleated erythrocyte, blue – poikilocytosis, green – hypochromia, and red – inclusion bodies. Modified from (Needs et al., 2022) and (Rizzuto et al., 2021).

The erythrocytes in peripheral blood smear usually display microcytosis, poikilocytosis, hypochromia, and inclusions (Brancaleoni et al., 2016). Note that the highly elevated

levels of HbF are one the distinguishing features of the TM and TI (Brancaleoni et al., 2016). The histopathological features of the TM erythrocytes are shown in Figure 9.

Bone Expansion and Extramedullary Hematopoiesis

Secondary to the anemia, the β-thalassemia patients show the erythroid bone marrow expansion which often leads to the growth impairment, osteoporosis, and fragility fractures. Moreover, the patients often experience significant endocrine dysfunction and micronutrient deficiencies, further compromising their bone health (Piga, 2017). On face, the bone marrow expansion manifests as larger cheekbones, protruding maxilla, and depressed nasal bridge, some of the most readily identifiable features of the TM (Karakas et al., 2016). The craniofacial features of a TM patient are shown in Figure 10.

Despite its considerable extent, the bone marrow expansion is unable to curb the anemia, especially in the TM patients. Consequently, extramedullary hematopoiesis (EMH) ensues. Although the EMH can occur in just about any location, the most common sites are paravertebral regions and the skull (Ben Ammar et al., 2020; Merchant et al., 2018). The less common sites of EMH include the liver, spleen, and lymph nodes (Subahi et al., 2022). Unexpectedly, the prevalence of EMH is 20% in the TI but only ~1% in the TM (Subahi et al., 2022). However, this is a simple reflection of the fact that the severe anemia in the TM requires aggressive treatment (which prevents the EMH), whereas the TI patients may live with the chronic mild or moderate anemia (Subahi et al.,

2022). While the EMH is harmless at first, the continual expansion of erythroid soft tissue in certain locations can become symptomatic.

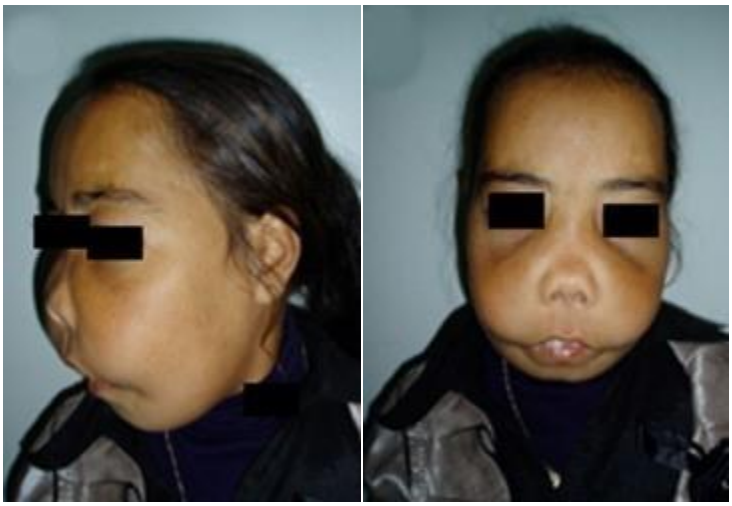

Figure 10: Craniofacial Features. This is a typical example of protruding maxilla, larger cheek bones, and depressed nasal bridge due to bone marrow expansion in the TM patients. Modified from (Bouguila et al., 2015).

Most often, such soft tissues masses compress the spinal cord and result in neurological symptoms, necessitating immediate treatment (Sohawon et al., 2012). Most often, such complications are managed by blood transfusions, but radiotherapy and surgical decompression may be required in some instances (Sohawon et al., 2012). Nonetheless, other organs such as the brain can be compressed, and the splenomegaly can cause pain in the left upper quadrant (Ben Ammar et al., 2020; Merchant et al., 2018). These complications usually occur later on in life; however, if the underlying anemia is poorly managed, they can occur within the first few years after birth (Merchant et al., 2018).

Hypercoagulability

The increased oxidative stress in β-thalassemia promotes the expression of erythrocyte senescence antigens such as phosphatidylserine, leading to the erythrocyte aggregation and premature removal from the circulation (Tavazzi et al., 2001). Also, the increased levels of phosphatidylserine (and other negatively charged phospholipids) seem to promote thrombin generation (Taher et al., 2018). Furthermore, the cell adhesion molecules that promote the platelet adhesion and activation, including von Willebrand factor and E- and P-selectins, are upregulated on the endothelial cells (Taher et al., 2018). Simultaneously, the concentrations of circulating antithrombin, protein C, and protein S are significantly depressed (Sultan, 2018). All three are glycoproteins, primarily synthesized in the liver, that act to degrade multiple factors in the coagulation cascade (Dahlbäck & Villoutreix, 2005; Hsu & Moosavi, 2022). When combined, these factors result in a chronic state of hypercoagulability.

Similar to the EMH, the hypercoagulability is more common in the TI than TM patients (Taher et al., 2018). Over time the hypercoagulability can lead to thromboembolisms. In one study, almost a third of the TI patients developed at least one thromboembolic event over a ten year period, compared to only 2% in the TM patients (Cappellini et al., 2000). Interestingly, none of the patients had a positive family history for thrombosis or were exposed to any transient risk factors such as prolonged immobilization and pregnancy (Cappellini et al., 2000). In this study, all except one patient who developed a thromboembolic event were splenectomized. The TI patients are

also at increased risk for stroke and the prevalence of silent cerebral infarct may be as high as 80% (Hashemieh & Jafari, 2022).

Iron Overload

Another effect of the anemia and consequent hypoxia in β-thalassemia is the upregulation of erythropoietin or EPO (Chaisiripoomkere et al., 1999). The oxygen levels are sensed by the α subunit of hypoxia-inducible factor 2 (HIF-2α) which is degraded under normoxia (Haase, 2013). Under hypoxia HIF-2α persists, heterodimerizes with HIF-2β, and elevates EPO expression by binding to the hypoxia response element and recruiting transcriptional cofactors (Haase, 2013). More importantly, the number of EPO-producing cells increases exponentially as the severity of anemia increases (Koury et al., 1989). In addition to promoting the erythroid hyperplasia, EPO downregulates the synthesis of hepcidin, a hepatocyte-synthesized peptide hormone that inhibits iron release into the circulation (Ganz & Nemeth, 2012). Rather than acting directly on hepatocyte EPO receptors, EPO downregulates hepcidin synthesis by stimulating the release of hepcidin inhibitors from the erythroblasts (Gammella et al., 2015). Hepcidin normally binds to ferroportin, an iron exporter found on the plasma membranes of enterocytes, macrophages, and hepatocytes, and induces its internalization and degradation (Nemeth et al., 2004). With decreased hepcidin concentrations, transferrin (an iron-carrying glycoprotein) saturation rises. Unsurprisingly, increasing transferrin saturation stimulates hepcidin synthesis and release (Ganz & Nemeth, 2012).

Due to the opposing effects of EPO and transferrin saturation on hepcidin, it is difficult to predict its concentrations in the β-thalassemia. If left untreated, it appears that the effects of EPO dominate (Tanno et al., 2007). That is, hepcidin concentrations remain low despite rising iron levels. This understanding is substantiated by the fact that the TI patients, who seldom require transfusions, have significantly depressed levels of hepcidin despite high transferrin saturation (Origa et al., 2007). In the TI, therefore, the increased intestinal absorption and iron release from the intracellular stores (due to low hepcidin concentrations) is the fundamental cause of the iron overload.

In the TM patients, who require regular transfusions, the hepcidin concentrations have been found to be normal, elevated, and depressed (Jawad et al., 2018; Kaddah et al., 2017; Origa et al., 2007). Note that the transfusions in the TM patients are followed by iron chelation therapy to prevent the iron overload. Nevertheless, these results are not necessarily contradictory. The transfusions would increase the hepcidin levels by ameliorating the anemia, thereby decreasing EPO concentrations, and delivering additional iron to the circulation. However, if compliance to the transfusions is poor, the TM patients would be expected to have decreased levels of hepcidin due to the anemia. Similarly, poor compliance to the iron chelation therapy would elevate both the iron and hepcidin levels. In fact, among the TM patients, more frequent transfusions and inadequate chelation were associated with significantly increased hepcidin concentrations (Kaddah et al., 2017). Thus, in the TM, the iron overload can be caused by inadequate iron chelation and/or by low hepcidin concentrations (Nemeth, 2010).

The iron overload is by far the most significant pathology in β-thalassemia. The excess iron can deposit in endocrine glands and result in hypopituitarism, hypothyroidism, hypoparathyroidism, and diabetes mellitus (Rund & Rachmilewitz, 2005). Although endocrine deficiencies and all that they entail can be quite significant, the most consequential site of iron deposition is the heart. As the transferrin saturation increases, the iron increasingly resides as non-transferrin bound iron (NTBI). The NTBI can enter cardiomyocytes through the calcium channels, divalent metal transporters, and transferrin-receptor-mediated endocytosis (Oudit et al., 2006). Nevertheless, it appears that the high-capacity L-type calcium channels (which also conduct ferrous iron) play the most important role. This idea is supported by the fact that iron uptake from transferrin was shown to be insignificant, and iron overload downregulates transferrin receptors in the cardiomyocytes (Y. Liu et al., 2003). Moreover, transferrin receptors and divalent metal transporters are expressed at very low levels in cardiomyocytes (Murphy & Oudit, 2010). Note that the basis for the excess iron deposition in the endocrine glands is very similar. In the pancreatic β-cells, for example, the calcium influx that ultimately leads to the insulin granule exocytosis is mostly brought about by the L-type calcium channels (Rorsman & Ashcroft, 2018). At high levels of NTBI, the calcium influx would be accompanied by the iron influx. The progressive destruction of the β-cell due to the iron overload could result in diabetes mellitus. In fact, diabetic ketoacidosis and diabetes mellitus are a very common reasons for hospitalization in TM patients (Karimi et al., 2011).

Once inside the cardiomyocyte, the highly reactive iron ions generate the ROS which have several deleterious effects. Firstly, the oxidative damage to the mitochondrial membranes leads to the inhibition of oxidative phosphorylation and ATP depletion (Zhang et al., 2019). Secondly, the iron ions compete with the calcium ions and disrupt the excitation-contraction coupling in the cardiomyocytes. Finally, the ROS damage the plasma membrane proteins (e.g. sodium-calcium exchangers and potassium channels) and the sarcoplasmic reticulum proteins (e.g. sarcoplasmic reticulum calcium ATPases), hindering the cardiomyocyte repolarization and relaxation (Murphy & Oudit, 2010). The disrupted excitation-contraction coupling, and insufficient relaxation cause the cardiac arrhythmias and diastolic dysfunction, respectively. The heart compensates by ventricular dilation, but the increasing oxidative stress and ATP depletion eventually lead to the systolic dysfunction and congestive heart failure or CHF (Gujja et al., 2010).

In β-thalassemia patients, the circulating iron levels and its deposition in the heart were shown to be directly correlated with the increased mortality and higher risk of developing CHF (Taher et al., 2021). In fact, before the advent of iron chelation therapy in the late 1960s, two thirds of the TM patients developed serious cardiac complications such as CHF and arrhythmias and four fifths had some cardiac involvement (Engle, 1964). The average age of onset was 16 years and death by the mid-twenties was the norm (Engle, 1964). In the more recent studies, the cardiomyopathy accounted for ~70% of death is the TM patients (Bazrgar et al., 2011; Borgna-Pignatti et al., 1998; Ladis et al., 2005). Although the cardiomyopathy has continued to be the major cause of death, the lifespan of the TM patients has been greatly extended: the mean survival age was

estimated to be 50.07 years (Ansari-Moghaddam et al., 2018). Almost certainly, this increase in the life expectancy was brought about by the iron chelation therapy. A similar study found that patients with poor compliance to the iron chelation therapy accounted for 90% of CHF cases and developed cardiomyopathy between the ages of 11-20 years (Karimi et al., 2011).

Another organ that is particularly affected by the iron overload is the liver. Normally, the liver takes up the extra circulating iron through transferrin-receptor-mediated endocytosis and acts as a storage site for it (Anderson & Shah, 2013). Unlike in the cardiomyocytes, the iron overload leads to the upregulation of transferrin receptors in the hepatocytes (Oudit et al., 2006). The excess iron uptake in the liver results in the elevated oxidative stress on the hepatocytes and fibrosis and eventual cirrhosis ensue (Philippe et al., 2007). In the TI, the liver diseases contribute significantly to the mortality even though cardiomyopathy is the most prevalent (Musallam, Vitrano, et al., 2021). The causes of death in the TM and TI are summarized in Table 3.

To summarize, the underlying anemia in the β-thalassemia patients leads to the bone expansion and EMH. The bone expansion causes osteoporosis, growth impairment, and fragility fractures which are further worsened by the concomitant endocrine dysfunctions (Piga, 2017). The EMH generally tends to be harmless, but erythroid soft tissue masses can compress spinal cord, brain, or other internal organs (Ben Ammar et al., 2020; Merchant et al., 2018). In addition, the increased oxidative stress causes erythrocyte senescence, aggregation, and removal from the circulation. The endothelial

cells show upregulated levels of the cell adhesion molecules that promote the platelet adhesion and activation (Taher et al., 2018).

Table 3: Causes of death in TM and TI patients. The mortality due to thromboembolisms, anemia, and endocrine dysfunctions is summarized as the other β-thalassemia-related deaths. Similarly, the mortality due to unknown or β-thalassemia-unrelated causes is summarized as other β-thalassemia unrelated deaths. n = 240 for the TM group and n = 113 for the TI group. Table put together from (Musallam, Vitrano, et al., 2021) and (Borgna-Pignatti et al., 1998).

Cause	Thalassemia major (%)	Thalassemia intermedia (%)
Cardiomyopathy	71.0	36.3
Liver disease	6.0	20.4
Infection	12.0	11.5
Cancer	3.0	12.4
Other (β-thal. related)	5.0	15.0
Other (β-thal. unrelated)	3.0	4.4

At the same time, the circulating levels of proteins that inhibit the coagulation cascade are decreased (Hsu & Moosavi, 2022; Sultan, 2018). The resulting chronic state of hypercoagulability increases the risk of thromboembolism in the β-thalassemia patients (Hashemieh & Jafari, 2022). Most importantly, the anemia (in TI) or blood transfusions (in TM) lead to the iron overload. The iron deposition in the liver is mediated by transferrin-receptor-mediated endocytosis, whereas the iron deposition in the heart and the endocrine organs is mediated by the L-type calcium channels (Anderson & Shah, 2013; Murphy & Oudit, 2010; Rorsman & Ashcroft, 2018). The excess iron inside the cells disrupts the cellular processes and eventually results in the cell death. As a result,

the overwhelming majority of the TM patients (77.0%) and the majority of the TI patients (56.7%) die from either the cardiomyopathy or liver diseases (Table 3).

Clinically, the TM-affected infants present with progressive pallor, jaundice, abdominal enlargement, and failure to thrive before the age of two. Over the next few years, growth retardation, poor musculature, skeletal changes, and hepatosplenomegaly will develop if the anemia is inadequately controlled (Galanello & Origa, 2010). By 11 to 15 years of age, splenomegaly becomes symptomatic and requires surgical intervention. In fact, splenectomy is the most common reason for hospitalization among the TM patients (Karimi et al., 2011). In addition, the endocrine dysfunctions lead to delayed puberty and late development of secondary sexual characteristics (Rund & Rachmilewitz, 2005). By twenty years of age, the iron accumulation in the heart likely causes diastolic dysfunction and arrhythmias (Murphy & Oudit, 2010). The heart compensates by ventricular dilation for some time, but systolic dysfunction and CHF eventually develop (Gujja et al., 2010). Also, the iron accumulation in the liver can compromise hepatic function and lead to liver fibrosis and cirrhosis (Philippe et al., 2007). Without the appropriate treatment, death by the mid-twenties is the norm for the TM patients.

Current Treatments

Regular Transfusions and Iron Chelation Therapy

The TM patients are commonly started on a regular transfusion regimen to alleviate the anemia and consequent pathogenesis. For the regular transfusions to be initiated, a patient must meet the following criteria: 1) two measurements of very low Hb concentration (<7 g/dL) more than two weeks apart, 2) significant clinical manifestations such as poor growth/failure to thrive, significant EMH, and bone abnormalities, and/or 3) confirmed diagnosis of the TM (Cappellini et al., 2021). Ideally, the transfusions are initiated based on a combination of clinical and laboratory findings since several factors can modify the severity of the disease. The goal of the transfusions is to maintain pretransfusion Hb concentrations above 9 g/dL which typically corresponds to 13-15 g/dL of Hb posttransfusion (Cappellini et al., 2021). The normal Hb concentrations are 11-16 g/dL with males on the higher and females on the lower end of this range. The transfusions are required every two to four weeks and 8-15 mL of packed erythrocytes/kg are usually transfused (Rachmilewitz & Giardina, 2011). The transfusion volumes in mL can be estimated by the following equation:

weight (kg) × increment in Hb (g/dL) × 3/HCT (Davies et al., 2007).

For the sake of illustration, let us suppose that a 70 kg man requires 2 g/dL of Hb to reach the satisfactory posttransfusion Hb concentration and the HCT of the transfused blood is 60%. Then, 70 × 2 × 3/0.6 mL = 700 mL of blood must be transfused. If we assume that 1 mL of erythrocytes contains ~1 mg of iron and 12 transfusion are required per year, then this man is receiving 700 mL × 0.6 × 1 mg/mL × 12 = 5,040 mg of iron per

year (Mast & Murphy, 2017). Since the total body iron is 3.5-5,000 mg, the iron overload would soon develop. In fact, the iron chelation therapy is usually initiated after 10-12 transfusions (Cao & Galanello, 2010).

The first iron chelator to be approved for the treatment of iron overload in the TM patients was desferrioxamine (DFO). The DFO enters the hepatocytes (and other cells) and binds the iron from labile iron pools in 1:1 ratio, forming a stable chelator-iron complex. This complex can either be secreted in bile and subsequently exerted in the feces, or it can recirculate and be excreted in the urine (Cappellini et al., 2021). Because the labile iron is being constantly generated, the iron chelation therapy works the best if the chelator is constantly present. The iron-free DFO has the half-life of about 30 minutes, and it is too large a molecule to be absorbed directly from the gut. As a result, the DFO must be delivered either subcutaneously or intravenously at least five days a week for 8-12 hours per administration (Cappellini et al., 2021). Since the DFO, oral iron chelators with longer half-lives such as deferasirox and deferiprone (DFP) have been developed. Note that two molecules of deferasirox and three of DFP are required to form the chelator-iron complexes. The three iron chelators can be used to treat the iron overload individually or in combination with each other (Rachmilewitz & Giardina, 2011). In fact, the severe cases of iron overload are treated with a combination of an oral iron chelator and the DFO (Galanello et al., 2010). With adequate transfusions and iron chelation therapy, the cardiac, hepatic, and endocrine complications of the TM can either be prevented or ameliorated to a certain extent (Galanello et al., 2010).

Nevertheless, the iron chelation therapy can have some significant side effects. In some patients, the treatment with the DFO can result in visual and auditory symptoms which rarely progress into vision and hearing loss (Cohen et al., 1990). In addition, the DFP can cause neutropenia, a basis for discontinuation. Other side effects include gastrointestinal disturbances, decreased renal function, and retarded bone growth (Cappellini et al., 2021).

The regular blood transfusion can also lead to some significant complications, the most prevalent being transfusion-transmitted infections. Such infections are much more prevalent in the developing countries. A study from India found that the prevalence of viral diseases in the blood donor population was ~2% (Shyamala, 2014). If we assume no screening, after only 250 transfusions a patient would have $1 - (1 - 0.02)^{1 \times 250} = 0.99$ or 99% chance of being virally infected. Some TM patients may require as many as 600 transfusions throughout their lives, and this back-of-the-envelope calculation assumes one blood source per transfusion. The same study found that 45% of the TM patients suffered from the transfusion-transmitted infections due to insufficient serological testing. Another study from India reported similar results (N. Shah et al., 2010). In addition to the viral infections, transfusion-related bacterial, fungal, and parasitic infections may also occur. The second most common complication of regular transfusions is the development of antibodies against specific erythrocyte antigens or alloimmunization. The alloantibodies shorten the lifespan of donor erythrocytes, necessitating more frequent transfusions and accelerating the rate of iron accumulation. Although highly variable, it is estimated that 10-20% of the TM patients develop alloantibodies (F. T. Shah et al., 2019).

Hematopoietic Stem Cell Transplantation

Fundamentally, the hematopoietic stem cell transplantation (HSCT) involves 1) collection of the donor stem cells, 2) myeloablation and immunosuppression of the recipient, and 3) the transplantation of the collected stem cells. The donor selection, based on human leukocyte antigens (HLA), is especially important because immunological complications may result in transplant failure or death (Rachmilewitz & Giardina, 2011). To date, the most prevalent sources of stem cells have been HLA matched donors (siblings, related, or unrelated to the recipient). The transplantation with HLA-matched sibling donor has been shown to be very effective with overall survival of 91% and thalassemia-free survival of 83% (Baronciani et al., 2016). In fact, the donor in the very first HSCT in a TM patient was an HLA-matched sibling (Thomas et al., 1982). Moreover, the transplantation with related or unrelated HLA-matched donors has been shown to be just as effective (Cappellini et al., 2021). There are also several risk factors (of the recipient) such as age, hepatomegaly, portal fibrosis, and inadequate iron chelation that influence the outcome of the HSCT (Rachmilewitz & Giardina, 2011). Finally, the severity of myeloablation and immunosuppression can be modulated to optimize the clinical outcomes.

While these results are certainly encouraging, 50-60% of the TM patients will not have an HLA-matched donor (Oevermann et al., 2019). For such patients, haploidentical or mismatched unrelated donors present a possibility. Early on, haplo-HSCTs showed high rates of graft rejection and transplant related mortality of 30% (Oevermann et al., 2019). However, with the better immunosuppression and T-cell depletion strategies, over

90% overall survival and very low incidence of the graft versus host diseases have been reported in the mismatched unrelated donor HSCTs (Oostenbrink et al., 2021). Nonetheless, a significant side effect of the enhanced immunosuppression is the delayed immune recovery. Although this study found it to be inconsequential, the reactivated latent viruses can be life-threatening due to the inadequate immune function (Oostenbrink et al., 2021). In addition, following the HSCT, the patients are usually required to take immunosuppressants which can further expose them to infections.

Splenectomy

One of the most widespread treatments for the splenomegaly in the β-thalassemia used to be splenectomy. For the patients born in the 1960s, the probability of undergoing the surgical procedure within the first 10 years of life was 57% (Piga et al., 2011). The goal is to increase the circulating levels of hemoglobin by decreasing the rate of erythrocyte removal. This leads to decreased transfusion requirements and less severe iron overload (Rachmilewitz & Giardina, 2011). More recently, the rates of splenectomy among the β-thalassemia patients have declined for a few reasons. Firstly, the absence of a spleen leads to increased susceptibility and mortality due to infections, especially in the young thalassemic patients (Vento et al., 2006). Secondly, splenectomy has been associated with higher incidence of thromboembolic events (Cappellini et al., 2021). As already discussed, the erythrocytes in the TM express higher levels of senescence antigens such as phosphatidylserine which seem to promote thrombin generation. Allowing these erythrocytes to remain in the circulation for longer exacerbates the

already present hypercoagulability. In addition, the spleen also functions to remove platelets from the circulation. As a result, splenectomized patients have elevated levels of platelets (Piga et al., 2011). Lastly, with the improved anemia control and consequent reduction in the IE and EMH, the prevalence of splenomegaly has declined (Cappellini et al., 2021). For the patients born in the 1990s, only about 7% have received splenectomy before the age of 10 (Piga et al., 2011).

Fetal Hemoglobin Inducers

Perhaps the two most extensively studied HbF inducers are hydroxyurea and thalidomide which can be used as monotherapies or in combination. In a recent prospective study from Bangladesh, 51 TM patients were treated with thalidomide. Of the participants, 40 (78.4%) showed significant increases in their mean Hb levels: 18 (35.3%) were able to forego transfusions and 22 (43.1%) showed significant increase in the time between transfusions (Begum et al., 2020). In addition, 19 participants (37.3%) showed only mild side effects, and 32 participants (62.7%) reported no side effects at all (Begum et al., 2020).

A more recent retrospective study, analyzing outcomes in 133 patients treated with hydroxyurea and thalidomide, showed even more promising results. Of the participants, 76 (57.2%) achieved a complete response and 19 (14.3%) achieved a partial response (Bhurani et al., 2021). The authors defined the complete response as the maintenance of at least 9 g/dL of Hb without transfusions and the partial response as at least 50% reduction in the transfusion burden. Once again, the participants mostly

reported mild side effects such as increased sedation and constipation. Nonetheless, a few cases of more severe side effects were observed. Seven cases of thrombocytopenia, four of thrombocytosis, and one of deep vein thrombosis were observed. Moreover, four participants developed peripheral neuropathy, and four more participants experienced syncope (Bhurani et al., 2021). It is plausible that hydroxyurea or some interaction between thalidomide and hydroxyurea accounts for the development of more severe side effects since this study used lower dosage per kg of bodyweight of thalidomide than the prospective study. Whatever the case may be, dosage titration as well as close monitoring are required to prevent adverse outcomes (Bhurani et al., 2021).

Interestingly, the efficacy of HbF induction by hydroxyurea and thalidomide differs depending on the patient population. The patients from India and Bangladesh, for example, respond better than patients from Italy. Although it is unclear exactly what accounts for these differences, they have been associated with polymorphisms in the gene that codes for G_{γ} globin chain (Figure 1, Musallam et al., 2021).

Future Treatments

Gene Therapy

Similar to the HSCT, gene therapy involves 1) collection of the donor stem cells, 2) myeloablation and immunosuppression of the recipient, and 3) the transplantation of the collected stem cells. In the case of gene therapy, however, the stem cells are collected from the recipient and are genetically modified before the transplantation. The genetic modifications fall into two general categories: gene insertion and gene editing. Since β-thalassemia is fundamentally caused by non-functional or poorly-functional β-globin genes, gene insertion aims to introduce a functional copy of the globin gene in the collected stem cells (Locatelli et al., 2022). On the other hand, gene editing aims to cure the disease by modifying genes other than the β-globin genes (Musallam, Bou-Fakhredin, et al., 2021).

In a recent phase 3 clinical study, 22 TM patients were treated with the gene insertion approach. The collected autologous stem cells were transduced with a self-inactivating lentiviral vector encoding a functional β-globin gene (Locatelli et al., 2022). These stem cells were then returned to the patients who had undergone myeloablation. The authors reported transfusion independence in 20 of the 22 participants (91%), and the transfusion independence was defined as the weighted average Hb levels of more than 9 g/dL without transfusions for at least 12 months (Locatelli et al., 2022). The two participants who did not achieve transfusion independence showed reduced transfusion requirements. Importantly, the side effects associated with the treatment such as

infections in the presence of neutropenia were consistent with myeloablation (Locatelli et al., 2022).

In another clinical study, 7 TM patients were treated with the gene editing approach. In this case, *BCL11A* erythroid-specific enhancer was targeted in the collected stem cells using CRISPR-Cas9 nuclease system to reduce the levels of BCL11A (Frangoul et al., 2021). Recall that BCL11A (along with LRF) binds the γ-globin gene promoter and represses its transcription by steric hindrance and recruitment of corepressors (Figure 4). Like the previous study, the genetically edited stem cells were returned to the patients who had undergone myeloablation. High levels of HbF expression and transfusion independence were soon observed. In one of the participants, for example, HbF levels increased from 0.4 g/dL at the baseline to 8.4 g/dL three months after the treatment. By 18 months, the HbF levels reached 13.1 g/dL (Frangoul et al., 2021). Once again, the side effects were consistent with the myeloablation and no off-target editing was observed (Frangoul et al., 2021). Note that the same *BCL11A* erythroid-specific enhancer can also be targeted using zinc-finger nucleases, but this approach fails to induce sustained HbF synthesis, and the peak HbF synthesis levels are not as high (Walters et al., 2021). Although these results are certainly encouraging, the long-term efficacy and safety of the gene insertion and gene editing approaches has yet to be demonstrated.

Erythroid Maturation Agents

As previously discussed, the reduced HCT is one of the features of TM (Table 1). As their name implies, the erythroid maturation agents elevate the HCT by accelerating erythroid maturation and reducing the IE. In a recent phase 3 clinical trial, an erythroid maturation agent, luspatercept, showed promising results. Luspatercept is simply an extracellular domain of a receptor fused with Fc domain of immunoglobulin G. It acts as a ligand trap which disrupts a signaling pathway in the erythroid precursors, eventually leading to increased expression and availability of GATA1 and other erythroid-differentiation-promoting transcription factors (Cappellini & Taher, 2021). For this study, a total of 336 TM patients were divided into luspatercept and placebo groups in 2:1 ratio (Cappellini & Taher, 2021). Significantly more luspatercept-treated participants showed decreases in the transfusion burden of at least 33% and even 50% over a period of 64-weeks (Cappellini et al., 2020). Furthermore, at least 11% of the participants at any 8-week interval were transfusion independent (Cappellini et al., 2020). The side effects were tolerable with bone pain and headaches being the most common complaints.

The authors observed that non-β^0/β^0 participants responded better to the treatment than β^0/β^0 participants (Cappellini & Taher, 2021). Although the exact reason for this observation is unclear, it is possibly due to GATA1-mediated chromatin looping. Recall that Ldb1 is recruited at the β-globin promoter (in GATA1-dependent fashion) and LCR, and it brings the two regions in proximity (Figure 5). The juxtaposition of the β-globin gene promoter and LCR enables a faster transition from transcription initiation to elongation, thereby increasing the gene expression. The increased expression and

availability of GATA1 would promote chromatin looping; however, the consequently elevated gene expression would not be observed in the β^0/β^0 participants since both β-globin gene copies are non-functional. On the other hand, the non-β^0/β^0 participants could show slightly elevated β-globin expression because they have at least a poorly functional β-globin gene copy. If this is indeed the case, the β-globin chain imbalance would be less severe in these participants. Therefore, relatively more erythrocyte precursors would be able to fully mature and enter the circulation, accounting for the differential efficacy.

Discussion

Each year, 25,000 individuals are born with TM, and the regional distribution of the births is shown in Figure 11 (Modell & Darlison, 2008). Unfortunately, more than 22,000 newborns are never started on transfusions and die within the first three years of life. There are almost 100,000 people living with the TM worldwide, of which close to 3,000 die each year due to iron overload (Modell & Darlison, 2008). Note that all of these statistics are the minimum estimates.

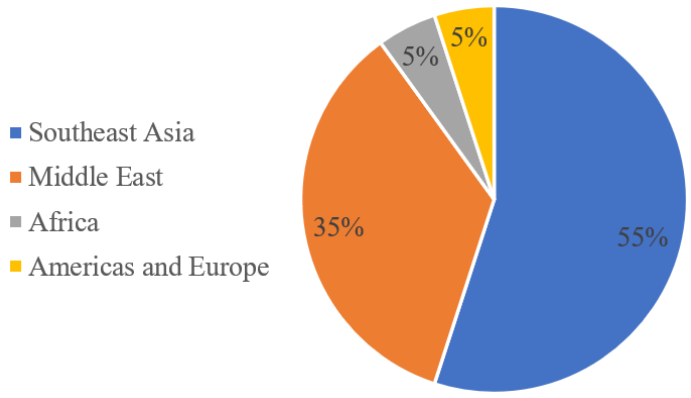

Figure 11: Regional Distribution of the TM births. The data were collected from the 2003 United Nations Demographic Yearbook. There were estimated total of 25,511 TM-affected births. Put together from (Modell & Darlison, 2008).

Although there are many effective treatments currently available and a few exciting prospects, it is unclear which of the treatments or a combination of treatments will ultimately solve the β-thalassemia challenge. The most common treatment for TM right now is blood transfusions and iron chelation therapy. Despite its widespread availability, the cost of this treatment varies greatly depending on the country; in the UK, the annual cost of transfusions and iron chelation is ~$14,500, but the corresponding amount is only ~$1,400 in Thailand (Riewpaiboon et al., 2010; Weidlich et al., 2016). In

addition to the transfusion-transmitted infections and alloimmunization, patient compliance with the cumbersome and time-consuming transfusions and iron chelation (especially in the developing countries) is a major problem. Often the patients are under transfused and under chelated, leading to chronic anemia and iron overload (Riewpaiboon et al., 2010).

So far, the only cure for TM is HSCT. From the first successful case in 1982 to 2010, 3783 TM patients have received transplants with excellent outcomes (Baronciani et al., 2016). However, the overwhelming majority of the transplantations have been carried out in European countries such as Italy and the UK. Once again, the cost of the treatment varies depending on the country. In the European countries, for example, HSCT costs ~$175,000 while it costs only ~$37,500 in China (Matthes-Martin et al., 2012; Tan et al., 2015). The most common complications of this treatment are graft rejection, chronic graft-versus-host diseases, and immunosuppression-related infections.

Regardless of its high one-time cost, HSCT has been demonstrated to be a more cost-efficient treatment than the transfusion and iron chelation therapy. When comparing the two treatments, a European study found that the lifetime cost of managing the TM could be reduced by 37% if half of the patients received transplants (Weidlich et al., 2016). A similar study from China found that HSCT reduced the lifetime cost of TM by 62% when compared to the conventional treatment (Tan et al., 2015). In addition, the successfully transplanted patients have a far better quality of life and suffer fewer complications (Weidlich et al., 2016). Nevertheless, the fundamental limitation of HSCT appears to be the need for an HLA-matched donor, and about half of the patients will

never find one (Oevermann et al., 2019). While haploidentical and mismatched unrelated donors present a possibility, the data on efficacy and long-term safety are lacking.

Gene therapy can cure β-thalassemia and solve the fundamental issues of HSCT. Because the stem cells are harvested from the patient in this treatment, there is no need to find an HLA-matching donor, and there is almost no risk of graft rejection or chronic graft-versus-host diseases. As a result, the immunosuppression following the transplantation is not required, greatly reducing the risk of infections (Thuret et al., 2022). Moreover, the gene therapy patients would enjoy the same improvements in the quality of life as HSCT patients.

Despite its promises, however, there is low likelihood that the gene therapy will contribute substantially to the solution of the β-thalassemia challenge for a few reasons. Firstly, the evidence of long-term safety and efficacy is simply lacking. No more than 130 TM patients have ever been treated with this approach and less than seven years of long-term follow-up data is available (Thuret et al., 2022). The concerns of insertional mutagenesis (and consequent oncogenesis) and the disruption of genomic DNA at non-specific targets must be addressed more comprehensively (Oikonomopoulou & Goussetis, 2021). Secondly, the gene insertion approach discussed previously, which was conditionally approved by the European Medicines Agency in 2019, was priced at $1.9 million per patient. Needless to say, none of the European countries accepted the absurd price tag, and the highest offer of $950,000 with the condition of persistent clinical efficacy by the German health system was rejected (Thuret et al., 2022). Thirdly, virtually all TM patients are born in Southeast Asia, Middle East, or Africa (Figure 11).

The three regions are comprised, almost exclusively, of poorly developed countries that lack the necessary medical expertise and infrastructure to administer the gene therapy. How could the countries in which more than 22,000 TM-affected infants die each year due to the lack of transfusions appropriately administer this sophisticated treatment? Finally, gene therapy is unlikely to achieve the necessary scale. Let us not forget that a comparable, highly effective, and cost-efficient treatment (i.e., HSCT) has existed for more than four decades. In the first three decades since its advent, no more than 4,000 HSCTs have been performed (Baronciani et al., 2016). If, over this period, we assume a preexisting and constant population of 100,000 TM patients, 3,000 TM-affected births per year surviving past infancy, and 50% of the patients ever finding an HLA-matched donor, there would be $\frac{1}{2}(100{,}000 + 3{,}000 \times 30) = 95{,}000$ patients who could have been treated with HSCT. That is, only 4.2% of the eligible TM patients received a transplant. While this statistic is underwhelming to begin with, it is a great overestimate: the developing countries would presumably address the TM-caused infant mortality before offering the gene therapy. As a result, ~25,000 (rather than 3,000) TM-affected births per year would survive past infancy, making the number of HSCT-eligible TM patients over this period 425,000. In this counterfactual scenario, not even 1% of the eligible patients would have been treated with the HSCT. Thus, the availability of HLA-matched donors clearly does not limit the number of transplantations. The fact that the gene therapy renders HLA-matched donors unnecessary is its main advantage since the immunosuppression-related complications in HSCT are manageable. Therefore, for just about every TM-affected country, the gene therapy offers no additional benefits over

transplantations. Note that the gene therapy may be cost-efficient once all the HSCT-eligible patients have received transplants, but this is far from being the case. Since HSCT failed to scale, so will the gene therapy.

Most likely, the TM patients will be treated with a combination of transfusions, iron chelation therapy, and HbF inducers (e.g., thalidomide and hydroxyurea). The erythroid maturation agents such as luspatercept may also be used; however, they are currently too expensive. Hydroxyurea, on the other hand, has been demonstrated to be a cost-effective treatment, and thalidomide is used in low doses to treat the TM (Ravangard et al., 2018; S. Shah et al., 2020). The reduction in transfusion burden due to HbF induction has multiple benefits. Less frequent transfusions greatly reduce the risk of transfusion-transmitted infections and alloimmunization. Also, the extent of iron overload will be lessened which will lead to better cardiac, hepatic, and endocrine functions in the TM patients. Furthermore, less frequent transfusions and chelation will undoubtedly improve the quality of life and compliance. A small portion of the TM patients, predominantly residing in the well-developed countries, will be cured with either the HSCT or gene therapy. For the vast majority of the TM patients, reduction in the transfusion burden and iron overload by HbF induction is the most pragmatic, cost-efficient solution to achieve better health outcomes.

Bibliography

1. Aljurf, M., Ma, L., Angelucci, E., Lucarelli, G., Snyder, L. M., Kiefer, C. R., Yuan, J., & Schrier, S. L. (1996). Abnormal Assembly of Membrane Proteins in Erythroid Progenitors of Patients With β-Thalassemia Major. *Blood*, *87*(5), 2049–2056. https://doi.org/10.1182/blood.V87.5.2049.2049

2. Anderson, E. R., & Shah, Y. M. (2013). Iron homeostasis in the liver. *Comprehensive Physiology*, *3*(1), 315–330. https://doi.org/10.1002/cphy.c120016

3. Ansari-Moghaddam, A., Adineh, H. A., Zareban, I., Mohammadi, M., & Maghsoodlu, M. (2018). The survival rate of patients with beta-thalassemia major and intermedia and its trends in recent years in Iran. *Epidemiology and Health*, *40*, e2018048. https://doi.org/10.4178/epih.e2018048

4. Asadov, C., Alimirzoeva, Z., Mammadova, T., Aliyeva, G., Gafarova, S., & Mammadov, J. (2018). β-Thalassemia intermedia: A comprehensive overview and novel approaches. *International Journal of Hematology*, *108*(1), 5–21. https://doi.org/10.1007/s12185-018-2411-9

5. Baronciani, D., Angelucci, E., Potschger, U., Gaziev, J., Yesilipek, A., Zecca, M., Orofino, M. G., Giardini, C., Al-Ahmari, A., Marktel, S., de la Fuente, J., Ghavamzadeh, A., Hussein, A. A., Targhetta, C., Pilo, F., Locatelli, F., Dini, G., Bader, P., & Peters, C. (2016). Hemopoietic stem cell transplantation in thalassemia: A report from the European Society for Blood and Bone Marrow Transplantation Hemoglobinopathy Registry, 2000–2010. *Bone Marrow Transplantation*, *51*(4), 536–541. https://doi.org/10.1038/bmt.2015.293

6. Bazrgar, M., Peiravian, F., Abedpour, F., & Karimi, M. (2011). Causes for Hospitalization and Death in Iranian Patients with β-Thalassemia Major. *Pediatric Hematology and Oncology*, *28*(2), 134–139. https://doi.org/10.3109/08880018.2010.503336

7. Begum, M., Moslem, M. H. M., Begum, N. N. F., & Rahman, M. Z. (2020). Outcome of Treatment with Thalidomide in Transfusion Dependent Thalassemia Patients: A Prospective Study in a Thalassemia Center, Dhaka, Bangladesh. *American Journal of Pediatrics*, *6*(3), 168. https://doi.org/10.11648/j.ajp.20200603.11

8. Ben Ammar, L., Ferjani, H., Maatallah, K., Bouallegue, S., Riahi, H., Kaffel, D., & Hamdi, W. (2020). Spinal cord compression by extramedullary hematopoiesis in beta-thalassemia major. *Clinical Case Reports*, *8*(8), 1433–1436. https://doi.org/10.1002/ccr3.2924

9. Bender, M. A., Bulger, M., Close, J., & Groudine, M. (2000). β-globin Gene Switching and DNase I Sensitivity of the Endogenous β-globin Locus in Mice Do Not Require the Locus Control Region. *Molecular Cell*, *5*(2), 387–393. https://doi.org/10.1016/S1097-2765(00)80433-5

10. Bhurani, D., Kapoor, J., Yadav, N., Khushoo, V., Agrawal, N., Ahmed, R., Arora, J. S., & Mehta, P. (2021). Experience with combination of hydroxyurea and low-dose thalidomide in transfusion-dependent beta thalassemia patients. *Annals of Hematology*, *100*(6), 1417–1427. https://doi.org/10.1007/s00277-021-04501-3

11. Borgna-Pignatti, C., Rugolotto, S., De Stefano, P., Piga, A., Di Gregorio, F., Gamberini, M. R., Sabato, V., Melevendi, C., Cappellini, M. D., & Verlato, G. (1998). Survival and Disease Complications in Thalassemia Major. *Annals of the New York Academy of Sciences*, *850*(1), 227–231. https://doi.org/10.1111/j.1749-6632.1998.tb10479.x

12. Bouguila, J., Besbes, G., & Khochtali, H. (2015). Skeletal facial deformity in patients with β thalassemia major: Report of one Tunisian case and a review of the literature. *International Journal of Pediatric Otorhinolaryngology*, *79*(11), 1955–1958. https://doi.org/10.1016/j.ijporl.2015.08.037

13. Brancaleoni, V., Di Pierro, E., Motta, I., & Cappellini, M. D. (2016). Laboratory diagnosis of thalassemia. *International Journal of Laboratory Hematology*, *38*(S1), 32–40. https://doi.org/10.1111/ijlh.12527

14. Cao, A., & Galanello, R. (2010). Beta-thalassemia. *Genetics in Medicine*, *12*(2), 61–76. https://doi.org/10.1097/GIM.0b013e3181cd68ed

15. Cappellini, M. D., Farmakis, D., Porter, J., & Taher, A. (2021). Guidelines for the Management of Transfusion Dependent Thalassaemia (4th Edition – 2021). *TIF*. https://thalassaemia.org.cy/publications/tif-publications/guidelines-for-the-management-of-transfusion-dependent-thalassaemia-4th-edition-2021/

16. Cappellini, M. D., Robbiolo, L., Bottasso, B. M., Coppola, R., Fiorelli, G., Mannucci, & M, P. (2000). Venous thromboembolism and hypercoagulability in splenectomized patients with thalassaemia intermedia. *British Journal of Haematology*, *111*(2), 467–473. https://doi.org/10.1111/j.1365-2141.2000.02376.x

17. Cappellini, M. D., & Taher, A. T. (2021). The use of luspatercept for thalassemia in adults. *Blood Advances*, *5*(1), 326–333. https://doi.org/10.1182/bloodadvances.2020002725

18. Cappellini, M. D., Viprakasit, V., Taher, A. T., Georgiev, P., Kuo, K. H. M., Coates, T., Voskaridou, E., Liew, H.-K., Pazgal-Kobrowski, I., Forni, G. L., Perrotta, S., Khelif, A., Lal, A., Kattamis, A., Vlachaki, E., Origa, R., Aydinok, Y., Bejaoui, M., Ho, P. J., … Piga, A. (2020). A Phase 3 Trial of Luspatercept in Patients with Transfusion-Dependent β-Thalassemia. *New England Journal of Medicine*, *382*(13), 1219–1231. https://doi.org/10.1056/NEJMoa1910182

19. Carter, D., Chakalova, L., Osborne, C. S., Dai, Y., & Fraser, P. (2002). Long-range chromatin regulatory interactions in vivo. *Nature Genetics*, *32*(4), 623–626. https://doi.org/10.1038/ng1051

20. Chaisiripoomkere, W., Jootar, S., Chanjarunee, S., & Ungkanont, A. (1999). Serum erythropoietin levels in thalassemia major and intermedia. *The Southeast Asian Journal of Tropical Medicine and Public Health*, *30*(4), 786–788.

21. Chen, H.-Q., Wu, L.-S., Jiang, F., & Li, D.-Z. (2021). Dominant β-Thalassemia Phenotype Caused by Hb Dieppe (HBB: C.383A>G): Another Case Report. *Hemoglobin*, 1–3. https://doi.org/10.1080/03630269.2021.2016441

22. Chonat, S., & Quinn, C. T. (2017). Current Standards of Care and Long Term Outcomes for Thalassemia and Sickle Cell Disease. *Advances in Experimental Medicine and Biology*, *1013*, 59–87. https://doi.org/10.1007/978-1-4939-7299-9_3

23. Cohen, A., Martin, M., Mizanin, J., Konkle, D. F., & Schwartz, E. (1990). Vision and hearing during deferoxamine therapy. *The Journal of Pediatrics*, *117*(2 Pt 1), 326–330. https://doi.org/10.1016/s0022-3476(05)80556-6

24. Croteau, S. E., Luo, H.-Y., Lehmann, L. E., Chui, D. H. K., & Neufeld, E. J. (2013). Novel dominant β-thalassemia: Hb Boston-Kuwait [codon 139/140(+T)]. *Pediatric Blood & Cancer*, *60*(10), E131-134. https://doi.org/10.1002/pbc.24611

25. Dahlbäck, B., & Villoutreix, B. O. (2005). Regulation of Blood Coagulation by the Protein C Anticoagulant Pathway. *Arteriosclerosis, Thrombosis, and Vascular Biology*, *25*(7), 1311–1320. https://doi.org/10.1161/01.ATV.0000168421.13467.82

26. Davies, P., Robertson, S., Hegde, S., Greenwood, R., Massey, E., & Davis, P. (2007). Calculating the required transfusion volume in children. *Transfusion*, *47*(2), 212–216. https://doi.org/10.1111/j.1537-2995.2007.01091.x

27. Deng, W., Lee, J., Wang, H., Miller, J., Reik, A., Gregory, P. D., Dean, A., & Blobel, G. A. (2012). Controlling long range genomic interactions at a native

locus by targeted tethering of a looping factor. *Cell*, *149*(6), 1233–1244. https://doi.org/10.1016/j.cell.2012.03.051

28. Deng, W., Rupon, J. W., Krivega, I., Breda, L., Motta, I., Jahn, K. S., Reik, A., Gregory, P. D., Rivella, S., Dean, A., & Blobel, G. A. (2014). Reactivation of developmentally silenced globin genes by forced chromatin looping. *Cell*, *158*(4), 849–860. https://doi.org/10.1016/j.cell.2014.05.050

29. Devlin, T. M. (2011). *Textbook of biochemistry: With clinical correlations* (7th ed..). John Wiley & Sons.

30. Doerfler, P. A., Feng, R., Li, Y., Palmer, L. E., Porter, S. N., Bell, H. W., Crossley, M., Pruett-Miller, S. M., Cheng, Y., & Weiss, M. J. (2021). Activation of γ-globin gene expression by GATA1 and NF-Y in hereditary persistence of fetal hemoglobin. *Nature Genetics*, *53*(8), 1177–1186. https://doi.org/10.1038/s41588-021-00904-0

31. Drissen, R., Palstra, R.-J., Gillemans, N., Splinter, E., Grosveld, F., Philipsen, S., & de Laat, W. (2004). The active spatial organization of the β-globin locus requires the transcription factor EKLF. *Genes & Development*, *18*(20), 2485–2490. https://doi.org/10.1101/gad.317004

32. Edoh, D., Antwi- Bosaiko, C., & Amuzu, D. (2006). Fetal hemoglobin during infancy and in sickle cell adults. *African Health Sciences*, *6*(1), 51–54.

33. Engle, M. A. (1964). Cardiac Involvement in Cooley's Anemia. *Annals of the New York Academy of Sciences*, *119*(2), 694–702. https://doi.org/10.1111/j.1749-6632.1965.tb54070.x

34. Felicetti, L., Novelletto, A., Benincasa, A., Terrenato, L., & Colombo, B. (1984). The HbA/HbA2 ratio in newborns and its correlation with fetal maturity. *British Journal of Haematology*, *56*(3), 465–471. https://doi.org/10.1111/j.1365-2141.1984.tb03976.x

35. Frangoul, H., Altshuler, D., Cappellini, M. D., Chen, Y.-S., Domm, J., Eustace, B. K., Foell, J., de la Fuente, J., Grupp, S., Handgretinger, R., Ho, T. W., Kattamis, A., Kernytsky, A., Lekstrom-Himes, J., Li, A. M., Locatelli, F., Mapara, M. Y., de Montalembert, M., Rondelli, D., … Corbacioglu, S. (2021). CRISPR-Cas9 Gene Editing for Sickle Cell Disease and β-Thalassemia. *New England Journal of Medicine*, *384*(3), 252–260. https://doi.org/10.1056/NEJMoa2031054

36. Freson, K., Matthijs, G., Thys, C., Mariën, P., Hoylaerts, M. F., Vermylen, J., & Van Geet, C. (2002). Different substitutions at residue D218 of the X-linked transcription factor GATA1 lead to altered clinical severity of

macrothrombocytopenia and anemia and are associated with variable skewed X inactivation. *Human Molecular Genetics*, *11*(2), 147–152. https://doi.org/10.1093/hmg/11.2.147

37. Galanello, R., Agus, A., Campus, S., Danjou, F., Giardina, P. J., & Grady, R. W. (2010). Combined iron chelation therapy. *Annals of the New York Academy of Sciences*, *1202*(1), 79–86. https://doi.org/10.1111/j.1749-6632.2010.05591.x

38. Galanello, R., & Origa, R. (2010). Beta-thalassemia. *Orphanet Journal of Rare Diseases*, *5*(1), 11. https://doi.org/10.1186/1750-1172-5-11

39. Gammella, E., Diaz, V., Recalcati, S., Buratti, P., Samaja, M., Dey, S., Noguchi, C. T., Gassmann, M., & Cairo, G. (2015). Erythropoietin's inhibiting impact on hepcidin expression occurs indirectly. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology*, *308*(4), R330–R335. https://doi.org/10.1152/ajpregu.00410.2014

40. Ganz, T., & Nemeth, E. (2012). Hepcidin and iron homeostasis. *Biochimica et Biophysica Acta*, *1823*(9), 1434–1443. https://doi.org/10.1016/j.bbamcr.2012.01.014

41. George, E., & Ann, T. J. A. M. (2010). Genotype-phenotype diversity of beta-thalassemia in Malaysia: Treatment options and emerging therapies. *The Medical Journal of Malaysia*, *65*(4), 256–260.

42. Gilman, J. G. (1988). Expression of G gamma and A gamma globin genes in human adults. *Hemoglobin*, *12*(5–6), 707–716. https://doi.org/10.3109/03630268808991664

43. Gujja, P., Rosing, D. R., Tripodi, D. J., & Shizukuda, Y. (2010). Iron Overload Cardiomyopathy, Better Understanding of An Increasing Disorder. *Journal of the American College of Cardiology*, *56*(13), 1001–1012. https://doi.org/10.1016/j.jacc.2010.03.083

44. Haase, V. H. (2013). Regulation of erythropoiesis by hypoxia-inducible factors. *Blood Reviews*, *27*(1), 41–53. https://doi.org/10.1016/j.blre.2012.12.003

45. Hashemieh, M., & Jafari, N. (2022). Vascular Brain Damage in Thalassemia Syndrome: An Emerging Challenge. *Iranian Journal of Child Neurology*, *16*(1), 19–29. https://doi.org/10.22037/ijcn.v16i3.33794

46. Hoffman, R., Benz, E. J., Silberstein, L. E., Heslop, H., Weitz, J. I., Anastasi, J., Salama, M. E., & Abutalib, S. A. (2018). *Hematology: Basic principles and practice* (Seventh edition..). Elsevier.

47. Hsu, E., & Moosavi, L. (2022). Biochemistry, Antithrombin III. In *StatPearls*. StatPearls Publishing. http://www.ncbi.nlm.nih.gov/books/NBK545295/

48. Jaing, T.-H., Chang, T.-Y., Chen, S.-H., Lin, C.-W., Wen, Y.-C., & Chiu, C.-C. (2021). Molecular genetics of β-thalassemia: A narrative review. *Medicine*, *100*(45), e27522. https://doi.org/10.1097/MD.0000000000027522

49. Jawad, M., Aftab, I., Saeed, M., Mumtaz, G., Iram, S., & Shahida, M. (2018). *Hepcidin Levels in Multi Transfused β Thalassemia Major Patients | Journal of Rawalpindi Medical College*. https://journalrmc.com/index.php/JRMC/article/view/176

50. Kaddah, A. M., Abdel-Salam, A., Farhan, M. S., & Ragab, R. (2017). Serum Hepcidin as a Diagnostic Marker of Severe Iron Overload in Beta-thalassemia Major. *The Indian Journal of Pediatrics*, *84*(10), 745–750. https://doi.org/10.1007/s12098-017-2375-4

51. Karakas, S., Tellioglu, A. M., Bilgin, M., Omurlu, I. K., Caliskan, S., & Coskun, S. (2016). Craniofacial Characteristics of Thalassemia Major Patients. *The Eurasian Journal of Medicine*, *48*(3), 204–208. https://doi.org/10.5152/eurasianjmed.2016.150013

52. Karim, Md. F., Ismail, Md., Hasan, A. M., & Shekhar, H. U. (2016). Hematological and biochemical status of Beta-thalassemia major patients in Bangladesh: A comparative analysis. *International Journal of Hematology-Oncology and Stem Cell Research*, *10*(1), 7–12.

53. Karimi, M., Emadmarvasti, V., Hoseini, J., & Shoja, L. (2011). Major Causes of Hospital Admission in Beta Thalassemia Major Patients in Southern Iran. *Iranian Journal of Pediatrics*, *21*(4), 509–513.

54. Khandros, E., & Blobel, G. A. (2021). Heterogeneity of Fetal Hemoglobin Production in Adult Red Blood Cells. *Current Opinion in Hematology*, *28*(3), 164–170. https://doi.org/10.1097/MOH.0000000000000640

55. Kountouris, P., Michailidou, K., Christou, S., Hadjigavriel, M., Sitarou, M., Kolnagou, A., Kleanthous, M., & Telfer, P. (2020). Effect of HBB genotype on survival in a cohort of transfusion-dependent thalassemia patients in Cyprus. *Haematologica*, *106*(9), 2458–2468. https://doi.org/10.3324/haematol.2020.260224

56. Koury, S. T., Koury, M. J., Bondurant, M. C., Caro, J., & Graber, S. E. (1989). Quantitation of Erythropoietin-Producing Cells in Kidneys of Mice by In Situ Hybridization: Correlation With Hematocrit, Renal Erythropoietin mRNA, and

Serum Erythropoietin Concentration. *Blood*, *74*(2), 645–651. https://doi.org/10.1182/blood.V74.2.645.645

57. Ladis, V., Chouliaras, G., Berdousi, H., Kanavakis, E., & Kattamis, C. (2005). Longitudinal study of survival and causes of death in patients with thalassemia major in Greece. *Annals of the New York Academy of Sciences*, *1054*, 445–450. https://doi.org/10.1196/annals.1345.067

58. Liu, N., Hargreaves, V. V., Zhu, Q., Kurland, J. V., Hong, J., Kim, W., Sher, F., Macias-Trevino, C., Rogers, J. M., Kurita, R., Nakamura, Y., Yuan, G.-C., Bauer, D. E., Xu, J., Bulyk, M. L., & Orkin, S. H. (2018). Direct Promoter Repression by BCL11A Controls the Fetal to Adult Hemoglobin Switch. *Cell*, *173*(2), 430-442.e17. https://doi.org/10.1016/j.cell.2018.03.016

59. Liu, N., Xu, S., Yao, Q., Zhu, Q., Kai, Y., Hsu, J. Y., Sakon, P., Pinello, L., Yuan, G.-C., Bauer, D. E., & Orkin, S. H. (2021). Transcription factor competition at the γ-globin promoters controls hemoglobin switching. *Nature Genetics*, *53*(4), 511–520. https://doi.org/10.1038/s41588-021-00798-y

60. Liu, Y., Parkes, J. G., & Templeton, D. M. (2003). Differential accumulation of non-transferrin-bound iron by cardiac myocytes and fibroblasts. *Journal of Molecular and Cellular Cardiology*, *35*(5), 505–514. https://doi.org/10.1016/S0022-2828(03)00072-5

61. Locatelli, F., Thompson, A. A., Kwiatkowski, J. L., Porter, J. B., Thrasher, A. J., Hongeng, S., Sauer, M. G., Thuret, I., Lal, A., Algeri, M., Schneiderman, J., Olson, T. S., Carpenter, B., Amrolia, P. J., Anurathapan, U., Schambach, A., Chabannon, C., Schmidt, M., Labik, I., … Walters, M. C. (2022). Betibeglogene Autotemcel Gene Therapy for Non–β0/β0 Genotype β-Thalassemia. *New England Journal of Medicine*, *386*(5), 415–427. https://doi.org/10.1056/NEJMoa2113206

62. Martyn, G. E., Wienert, B., Yang, L., Shah, M., Norton, L. J., Burdach, J., Kurita, R., Nakamura, Y., Pearson, R. C. M., Funnell, A. P. W., Quinlan, K. G. R., & Crossley, M. (2018). Natural regulatory mutations elevate the fetal globin gene via disruption of BCL11A or ZBTB7A binding. *Nature Genetics*, *50*(4), 498–503. https://doi.org/10.1038/s41588-018-0085-0

63. Mast, A. E., & Murphy, E. L. (2017). The price of blood is measured in iron. *Lancet (London, England)*, *390*(10110), 2331–2333. https://doi.org/10.1016/S0140-6736(17)32156-6

64. Masuda, T., Wang, X., Maeda, M., Canver, M. C., Sher, F., Funnell, A. P. W., Fisher, C., Suciu, M., Martyn, G. E., Norton, L. J., Zhu, C., Kurita, R., Nakamura, Y., Xu, J., Higgs, D. R., Crossley, M., Bauer, D. E., Orkin, S. H., Kharchenko, P.

V., & Maeda, T. (2016). Transcription factors LRF and BCL11A independently repress expression of fetal hemoglobin. *Science (New York, N.Y.), 351*(6270), 285–289. https://doi.org/10.1126/science.aad3312

65. Mathias, L. A., Fisher, T. C., Zeng, L., Meiselman, H. J., Weinberg, K. I., Hiti, A. L., & Malik, P. (2000). Ineffective erythropoiesis in β-thalassemia major is due to apoptosis at the polychromatophilic normoblast stage. *Experimental Hematology, 28*(12), 1343–1353. https://doi.org/10.1016/S0301-472X(00)00555-5

66. Matthes-Martin, S., Pötschger, U., Barr, R., Martin, M., Boztug, H., Klingebiel, T., Attarbaschi, A., Eibler, W., & Mann, G. (2012). Costs and Cost-Effectiveness of Allogeneic Stem Cell Transplantation in Children Are Predictable. *Biology of Blood and Marrow Transplantation*, *18*(10), 1533–1539. https://doi.org/10.1016/j.bbmt.2012.04.002

67. Merchant, R., Choudhari, A. J., Verma, M., Patkar, D. P., & Doctor, P. (2018). Intracranial Hematopoiesis in Beta Thalassemia: A Case Series. *The Indian Journal of Pediatrics*, *85*(8), 679–681. https://doi.org/10.1007/s12098-018-2618-z

68. Modell, B., & Darlison, M. (2008). Global epidemiology of haemoglobin disorders and derived service indicators. *Bulletin of the World Health Organization*, *86*(6), 480–487. https://doi.org/10.2471/BLT.06.036673

69. Murphy, C. J., & Oudit, G. Y. (2010). Iron-Overload Cardiomyopathy: Pathophysiology, Diagnosis, and Treatment. *Journal of Cardiac Failure, 16*(11), 888–900. https://doi.org/10.1016/j.cardfail.2010.05.009

70. Musallam, K. M., Bou-Fakhredin, R., Cappellini, M. D., & Taher, A. T. (2021). 2021 update on clinical trials in β-thalassemia. *American Journal of Hematology, 96*(11), 1518–1531. https://doi.org/10.1002/ajh.26316

71. Musallam, K. M., Sankaran, V. G., Cappellini, M. D., Duca, L., Nathan, D. G., & Taher, A. T. (2012). Fetal hemoglobin levels and morbidity in untransfused patients with β-thalassemia intermedia. *Blood, 119*(2), 364–367. https://doi.org/10.1182/blood-2011-09-382408

72. Musallam, K. M., Vitrano, A., Meloni, A., Pollina, S. A., Karimi, M., El-Beshlawy, A., Hajipour, M., Di Marco, V., Ansari, S. H., Filosa, A., Ricchi, P., Ceci, A., Daar, S., Vlachaki, E., Singer, S. T., Naserullah, Z. A., Pepe, A., Scondotto, S., Dardanoni, G., … Maggio, A. (2021). Survival and causes of death in 2,033 patients with non-transfusion-dependent β-thalassemia. *Haematologica*, *106*(9), 2489–2492. https://doi.org/10.3324/haematol.2021.278684

73. Nagababu, E., Fabry, M. E., Nagel, R. L., & Rifkind, J. M. (2008). Heme Degradation and Oxidative Stress in Murine Models for Hemoglobinopathies: Thalassemia, Sickle Cell Disease and Hemoglobin C Disease. *Blood Cells, Molecules & Diseases*, *41*(1), 60–66. https://doi.org/10.1016/j.bcmd.2007.12.003

74. Needs, T., Gonzalez-Mosquera, L., & Lynch, D. (2022). Beta Thalassemia. *StatPearls*. https://www.statpearls.com/ArticleLibrary/viewarticle/30017

75. Nemeth, E. (2010). Hepcidin in β-thalassemia. *Annals of the New York Academy of Sciences*, *1202*, 31–35. https://doi.org/10.1111/j.1749-6632.2010.05585.x

76. Nemeth, E., Tuttle, M. S., Powelson, J., Vaughn, M. B., Donovan, A., Ward, D. M., Ganz, T., & Kaplan, J. (2004). Hepcidin Regulates Cellular Iron Efflux by Binding to Ferroportin and Inducing Its Internalization. *Science*, *306*(5704), 2090–2093. https://doi.org/10.1126/science.1104742

77. Nienhuis, A. W., & Nathan, D. G. (2012). Pathophysiology and Clinical Manifestations of the β-Thalassemias. *Cold Spring Harbor Perspectives in Medicine*, *2*(12), a011726. https://doi.org/10.1101/cshperspect.a011726

78. Oevermann, L., Schulte, J. H., Hundsdörfer, P., Hakimeh, D., Kogel, F., Lang, P., Corbacioglu, S., Eggert, A., & Sodani, P. (2019). HLA-haploidentical hematopoietic stem cell transplantation in pediatric patients with hemoglobinopathies: Current practice and new approaches. *Bone Marrow Transplantation*, *54*(2), 743–748. https://doi.org/10.1038/s41409-019-0598-x

79. Oikonomopoulou, C., & Goussetis, E. (2021). HSCT remains the only cure for patients with transfusion-dependent thalassemia until gene therapy strategies are proven to be safe. *Bone Marrow Transplantation*, *56*(12), 2882–2888. https://doi.org/10.1038/s41409-021-01461-0

80. Oldfield, A. J., Yang, P., Conway, A. E., Cinghu, S., Freudenberg, J. M., Yellaboina, S., & Jothi, R. (2014). Histone-fold domain protein NF-Y promotes chromatin accessibility for cell type-specific master transcription factors. *Molecular Cell*, *55*(5), 708–722. https://doi.org/10.1016/j.molcel.2014.07.005

81. Oostenbrink, L. V. E., Pool, E. S., Jol-van der Zijde, C. M., Jansen-Hoogendijk, A. M., Vervat, C., van Halteren, A. G. S., Bredius, R. G. M., Smiers, F. J. W., van Tol, M. J. D., Schilham, M. W., Lankester, A. C., & Mohseny, A. B. (2021). Successful mismatched hematopoietic stem cell transplantation for pediatric hemoglobinopathy by using ATG and post-transplant cyclophosphamide. *Bone Marrow Transplantation*, *56*(9), 2203–2211. https://doi.org/10.1038/s41409-021-01302-0

82. Origa, R. (2017a). β-Thalassemia. *Genetics in Medicine*, *19*(6), 609–619. https://doi.org/10.1038/gim.2016.173

83. Origa, R. (2017b). β-Thalassemia. *Genetics in Medicine*, *19*(6), 609–619. https://doi.org/10.1038/gim.2016.173

84. Origa, R., Galanello, R., Ganz, T., Giagu, N., Maccioni, L., Faa, G., & Nemeth, E. (2007). Liver iron concentrations and urinary hepcidin in β-thalassemia. *Haematologica*, *92*(5), 583–588. https://doi.org/10.3324/haematol.10842

85. Oudit, G. Y., Trivieri, M. G., Khaper, N., Liu, P. P., & Backx, P. H. (2006). Role of L-type Ca2+ channels in iron transport and iron-overload cardiomyopathy. *Journal of Molecular Medicine (Berlin, Germany)*, *84*(5), 349–364. https://doi.org/10.1007/s00109-005-0029-x

86. Pace, B. S., & Zein, S. (2006). Understanding mechanisms of γ-globin gene regulation to develop strategies for pharmacological fetal hemoglobin induction. *Developmental Dynamics*, *235*(7), 1727–1737. https://doi.org/10.1002/dvdy.20802

87. Palstra, R.-J., de Laat, W., & Grosveld, F. (2008). Beta-globin regulation and long-range interactions. *Advances in Genetics*, *61*, 107–142. https://doi.org/10.1016/S0065-2660(07)00004-1

88. Palstra, R.-J., Tolhuis, B., Splinter, E., Nijmeijer, R., Grosveld, F., & de Laat, W. (2003). The beta-globin nuclear compartment in development and erythroid differentiation. *Nature Genetics*, *35*(2), 190–194. https://doi.org/10.1038/ng1244

89. Philippe, M. A., Ruddell, R. G., & Ramm, G. A. (2007). Role of iron in hepatic fibrosis: One piece in the puzzle. *World Journal of Gastroenterology : WJG*, *13*(35), 4746–4754. https://doi.org/10.3748/wjg.v13.i35.4746

90. Piga, A. (2017). Impact of bone disease and pain in thalassemia. *Hematology: The American Society of Hematology Education Program*, *2017*(1), 272–277.

91. Piga, A., Serra, M., Longo, F., Forni, G., Quarta, G., Cappellini, M. D., & Galanello, R. (2011). Changing patterns of splenectomy in transfusion-dependent thalassemia patients. *American Journal of Hematology*, *86*(9), 808–810. https://doi.org/10.1002/ajh.22102

92. Pirastu, M., Kan, Y. W., Galanello, R., & Cao, A. (1984). Multiple mutations produce delta beta 0 thalassemia in Sardinia. *Science (New York, N.Y.)*, *223*(4639), 929–930. https://doi.org/10.1126/science.6198720

93. Rachmilewitz, E. A., & Giardina, P. J. (2011). How I treat thalassemia. *Blood*, *118*(13), 3479–3488. https://doi.org/10.1182/blood-2010-08-300335

94. Ranjbaran, R., Okhovat, M. A., Mobarhanfard, A., Aboualizadeh, F., Abbasi, M., Moezzi, L., Golafshan, H. A., Behzad-Behbahani, A., Bagheri, M., & Sharifzadeh, S. (2014). Relationship between AHSP Gene Expression, β/α Globin mRNA Ratio, and Clinical Severity of the β-thalassemia Patients. *Annals of Clinical & Laboratory Science*, *44*(2), 189–193.

95. Ravangard, R., Mirzaei, Z., Keshavarz, K., Haghpanah, S., & Karimi, M. (2018). Blood transfusion versus hydroxyurea in beta-thalassemia in Iran: A cost-effectiveness study. *Hematology*, *23*(7), 417–422. https://doi.org/10.1080/10245332.2017.1404262

96. Ribeil, J.-A., Arlet, J.-B., Dussiot, M., Cruz Moura, I., Courtois, G., & Hermine, O. (2013). Ineffective Erythropoiesis in β-Thalassemia. *The Scientific World Journal*, *2013*, 394295. https://doi.org/10.1155/2013/394295

97. Riewpaiboon, A., Nuchprayoon, I., Torcharus, K., Indaratna, K., Thavorncharoensap, M., & Ubol, B. (2010). Economic burden of beta-thalassemia/Hb E and beta-thalassemia major in Thai children. *BMC Research Notes*, *3*(1), 29. https://doi.org/10.1186/1756-0500-3-29

98. Rizzuto, V., Koopmann, T. T., Blanco-Álvarez, A., Tazón-Vega, B., Idrizovic, A., Díaz de Heredia, C., Del Orbe, R., Pampliega, M. V., Velasco, P., Beneitez, D., Santen, G. W. E., Waisfisz, Q., Elting, M., Smiers, F. J. W., de Pagter, A. J., Kerkhoffs, J.-L. H., Harteveld, C. L., & Mañú-Pereira, M. del M. (2021). Usefulness of NGS for Diagnosis of Dominant Beta-Thalassemia and Unstable Hemoglobinopathies in Five Clinical Cases. *Frontiers in Physiology*, *12*. https://www.frontiersin.org/article/10.3389/fphys.2021.628236

99. Rochette, J., Craig, J. E., Thein, S. L., & Rochette, J. (1994). Fetal hemoglobin levels in adults. *Blood Reviews*, *8*(4), 213–224. https://doi.org/10.1016/0268-960X(94)90109-0

100. Rorsman, P., & Ashcroft, F. M. (2018). Pancreatic β-Cell Electrical Activity and Insulin Secretion: Of Mice and Men. *Physiological Reviews*, *98*(1), 117–214. https://doi.org/10.1152/physrev.00008.2017

101. Rund, D., & Rachmilewitz, E. (2005). β-Thalassemia. *New England Journal of Medicine*, *353*(11), 1135–1146. https://doi.org/10.1056/NEJMra050436

102. Sankaran, V. G., Menne, T. F., Xu, J., Akie, T. E., Lettre, G., Van Handel, B., Mikkola, H. K. A., Hirschhorn, J. N., Cantor, A. B., & Orkin, S. H. (2008).

Human Fetal Hemoglobin Expression Is Regulated by the Developmental Stage-Specific Repressor BCL11A. *Science*, *322*(5909), 1839–1842. https://doi.org/10.1126/science.1165409

103. Sawado, T., Halow, J., Bender, M. A., & Groudine, M. (2003). The β-globin locus control region (LCR) functions primarily by enhancing the transition from transcription initiation to elongation. *Genes & Development*, *17*(8), 1009–1018. https://doi.org/10.1101/gad.1072303

104. Shah, F. T., Sayani, F., Trompeter, S., Drasar, E., & Piga, A. (2019). Challenges of blood transfusions in β-thalassemia. *Blood Reviews*, *37*, 100588. https://doi.org/10.1016/j.blre.2019.100588

105. Shah, N., Mishra, A., Chauhan, D., Vora, C., & Shah, N. R. (2010). Study on effectiveness of transfusion program in thalassemia major patients receiving multiple blood transfusions at a transfusion centre in Western India. *Asian Journal of Transfusion Science*, *4*(2), 94. https://doi.org/10.4103/0973-6247.67029

106. Shah, S., Sheth, R., Shah, K., & Patel, K. (2020). Safety and effectiveness of thalidomide and hydroxyurea combination in β-thalassaemia intermedia and major: A retrospective pilot study. *British Journal of Haematology*, *188*(3), e18–e21. https://doi.org/10.1111/bjh.16272

107. Sharma, D. C., Singhal, S., Woike, P., Rai, S., Yadav, M., & Gaur, R. (2020). Hereditary persistence of fetal hemoglobin. *Asian Journal of Transfusion Science*, *14*(2), 185–186. https://doi.org/10.4103/ajts.AJTS_71_16

108. Shyamala, V. (2014). Transfusion transmitted infections in thalassaemics: Need for reappraisal of blood screening strategy in India. *Transfusion Medicine*, *24*(2), 79–88. https://doi.org/10.1111/tme.12110

109. Siatecka, M., & Bieker, J. J. (2011). The multifunctional role of EKLF/KLF1 during erythropoiesis. *Blood*, *118*(8), 2044–2054. https://doi.org/10.1182/blood-2011-03-331371

110. Singer, S. T., Olivieri, N. E., Sweeters, N., Rodwell, C., Oliver, C., Vichinsky, E. P., & Kuypers, F. A. (2004). Biotin-Labeled RBC Survival in Thalassemia and Impact of Treatment. *Blood*, *104*(11), 3616. https://doi.org/10.1182/blood.V104.11.3616.3616

111. Sohawon, D., Lau, K. K., Lau, T., & Bowden, D. K. (2012). Extra-medullary haematopoiesis: A pictorial review of its typical and atypical locations. *Journal of*

Medical Imaging and Radiation Oncology, *56*(5), 538–544. https://doi.org/10.1111/j.1754-9485.2012.02397.x

112. Soler, E., Andrieu-Soler, C., de Boer, E., Bryne, J. C., Thongjuea, S., Stadhouders, R., Palstra, R.-J., Stevens, M., Kockx, C., van IJcken, W., Hou, J., Steinhoff, C., Rijkers, E., Lenhard, B., & Grosveld, F. (2010). The genome-wide dynamics of the binding of Ldb1 complexes during erythroid differentiation. *Genes & Development*, *24*(3), 277–289. https://doi.org/10.1101/gad.551810

113. Subahi, E. A., Ata, F., Choudry, H., Iqbal, P., AlHiyari, M. A., Soliman, A. T., De Sanctis, V., & Yassin, M. A. (2022). Extramedullary haematopoiesis in patients with transfusion dependent β-thalassaemia (TDT): A systematic review. *Annals of Medicine*, *54*(1), 764–774. https://doi.org/10.1080/07853890.2022.2048065

114. Sultan, S. (2018). *Prothrombotic markers in Thalassemia major patients: A paradigm shift*. *73*(4), 5.

115. Susanah, S., Rakhmilla, L. E., Ghozali, M., Trisaputra, J. O., Moestopo, O., Sribudiani, Y., Idjradinata, P. S., & Maskoen, A. M. (2021). Iron Status in Newly Diagnosed β-Thalassemia Major: High Rate of Iron Status due to Erythropoiesis Drive. *BioMed Research International*, *2021*, 5560319. https://doi.org/10.1155/2021/5560319

116. Taher, A. T., Cappellini, M. D., Bou-Fakhredin, R., Coriu, D., & Musallam, K. M. (2018). Hypercoagulability and Vascular Disease. *Hematology/Oncology Clinics of North America*, *32*(2), 237–245. https://doi.org/10.1016/j.hoc.2017.11.001

117. Taher, A. T., Musallam, K. M., & Cappellini, M. D. (2021). β-Thalassemias. *New England Journal of Medicine*, *384*(8), 727–743. https://doi.org/10.1056/NEJMra2021838

118. Tamaddoni, A., Khabaz Astaneh, S., Tabaripour, R., & Akhavan-Niaki, H. (2019). Krüppel-Like Factor 1 Gene Mutations in Thalassemia Patients from North Iran: Report of a New Mutation Associated with β-Thalassemia Intermedia. *Hemoglobin*, *43*(1), 12–17. https://doi.org/10.1080/03630269.2019.1567528

119. Tan, W., Li, C., Wu, X., He, Y., Feng, X., Liao, J., Liu, H., Xu, W., & Ren, Y. (2015). Cost Comparison of Hematopoietic Stem Cell Transplantation and Conventional Therapy for Patients with Thalassemia Major in China. *Blood*, *126*(23), 2082. https://doi.org/10.1182/blood.V126.23.2082.2082

120. Tanno, T., Bhanu, N. V., Oneal, P. A., Goh, S.-H., Staker, P., Lee, Y. T., Moroney, J. W., Reed, C. H., Luban, N. L., Wang, R.-H., Eling, T. E., Childs, R.,

Ganz, T., Leitman, S. F., Fucharoen, S., & Miller, J. L. (2007). High levels of GDF15 in thalassemia suppress expression of the iron regulatory protein hepcidin. *Nature Medicine*, *13*(9), 1096–1101. https://doi.org/10.1038/nm1629

121. Tavazzi, D., Duca, L., Graziadei, G., Comino, A., Fiorelli, G., & Cappellini, M. D. (2001). Membrane-bound iron contributes to oxidative damage of β-thalassaemia intermedia erythrocytes. *British Journal of Haematology*, *112*(1), 48–50. https://doi.org/10.1046/j.1365-2141.2001.02482.x

122. Thein, S. L. (1992). Dominant beta thalassaemia: Molecular basis and pathophysiology. *British Journal of Haematology*, *80*(3), 273–277. https://doi.org/10.1111/j.1365-2141.1992.tb08132.x

123. Thein, S. L. (2013). The Molecular Basis of β-Thalassemia. *Cold Spring Harbor Perspectives in Medicine*, *3*(5), a011700. https://doi.org/10.1101/cshperspect.a011700

124. Thein, S. L., & Menzel, S. (2009). Discovering the genetics underlying foetal haemoglobin production in adults. *British Journal of Haematology*, *145*(4), 455–467. https://doi.org/10.1111/j.1365-2141.2009.07650.x

125. Thomas, E. D., Sanders, J., Buckner, C. D., Papayannopoulou, T., Borgna-Pignatti, C., Stefano, P. D., Clift, R., Sullivan, K., & Storb, R. (1982). Marrow Transplantation for Thalassemia. *The Lancet*, *320*(8292), 657–229. https://doi.org/10.1016/S0140-6736(82)90319-1

126. Thuret, I., Ruggeri, A., Angelucci, E., & Chabannon, C. (2022). Hurdles to the Adoption of Gene Therapy as a Curative Option for Transfusion-Dependent Thalassemia. *Stem Cells Translational Medicine*, *11*(4), 407–414. https://doi.org/10.1093/stcltm/szac007

127. Vento, S., Cainelli, F., & Cesario, F. (2006). Infections and thalassaemia. *The Lancet Infectious Diseases*, *6*(4), 226–233. https://doi.org/10.1016/S1473-3099(06)70437-6

128. Voskou, S., Aslan, M., Fanis, P., Phylactides, M., & Kleanthous, M. (2015). Oxidative stress in β-thalassaemia and sickle cell disease. *Redox Biology*, *6*, 226–239. https://doi.org/10.1016/j.redox.2015.07.018

129. Walters, M. C., Smith, A. R., Schiller, G. J., Esrick, E. B., Williams, D. A., Gogoleva, T., Rouy, D., Cockroft, B. M., & Vercellotti, G. M. (2021). *Updated Results of a Phase 1/2 Clinical Study of Zinc Finger Nuclease-Mediated Editing of Poster 3974 BCL11A in Autologous Hematopoietic Stem Cells for Transfusion-Dependent Beta Thalassemia*. 1.

130. Weatherall, D. J. (2012). The definition and epidemiology of non-transfusion-dependent thalassemia. *Blood Reviews*, *26*, S3–S6. https://doi.org/10.1016/S0268-960X(12)70003-6

131. Weidlich, D., Kefalas, P., & Guest, J. F. (2016). Healthcare costs and outcomes of managing β-thalassemia major over 50 years in the United Kingdom. *Transfusion*, *56*(5), 1038–1045. https://doi.org/10.1111/trf.13513

132. Xu, J., Bauer, D. E., Kerenyi, M. A., Vo, T. D., Hou, S., Hsu, Y.-J., Yao, H., Trowbridge, J. J., Mandel, G., & Orkin, S. H. (2013). Corepressor-dependent silencing of fetal hemoglobin expression by BCL11A. *Proceedings of the National Academy of Sciences of the United States of America*, *110*(16), 6518–6523. https://doi.org/10.1073/pnas.1303976110

133. Xu, J., Sankaran, V. G., Ni, M., Menne, T. F., Puram, R. V., Kim, W., & Orkin, S. H. (2010). Transcriptional silencing of γ-globin by BCL11A involves long-range interactions and cooperation with SOX6. *Genes & Development*, *24*(8), 783–798. https://doi.org/10.1101/gad.1897310

134. Yang, Y., Ren, R., Ly, L. C., Horton, J. R., Li, F., Quinlan, K. G. R., Crossley, M., Shi, Y., & Cheng, X. (2021). Structural basis for human ZBTB7A action at the fetal globin promoter. *Cell Reports*, *36*(13), 109759. https://doi.org/10.1016/j.celrep.2021.109759

135. Zhang, H., Zhabyeyev, P., Wang, S., & Oudit, G. Y. (2019). Role of iron metabolism in heart failure: From iron deficiency to iron overload. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease*, *1865*(7), 1925–1937. https://doi.org/10.1016/j.bbadis.2018.08.030

136. Zivot, A., Lipton, J. M., Narla, A., & Blanc, L. (2018). Erythropoiesis: Insights into pathophysiology and treatments in 2017. *Molecular Medicine*, *24*(1), 11. https://doi.org/10.1186/s10020-018-0011-z

Curriculum Vitae