T.R.N.C

NEAR EAST UNIVERSITY

INSTITUTE OF HEALTH SCIENCES

THE INTERRELATIONSHIP BETWEEN FETAL HEMOGLOBIN LEVELS AND CLINICAL PHENOTYPES OF BETA-THALASSEMIA

Cornelius Azilabih OYAMAH

MEDICAL BIOCHEMISTRY PROGRAM

MASTER OF SCIENCE GRADUATION PROJECT

NICOSIA

2018

T.R.N.C

NEAR EAST UNIVERSITY

INSTITUTE OF HEALTH SCIENCES

THE INTERRELATIONSHIP BETWEEN FETAL HEMOGLOBIN LEVELS AND CLINICAL PHYNOTYPE OF BETA-THALASSEMIA

Cornelius Azilabih OYAMAH

MEDICAL BIOCHEMISTRY PROGRAM

MASTER OF SCIENCE GRADUATION PROJECT

SUPERVISOR

Assist. Prof. Kerem TERALI, MRes, PhD

NICOSIA

2018

DECLARATION

I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

Name, Last Name:

Signature:

Date:

ACKNOWLEDGEMENT

My profound appreciation goes to God almighty for giving me the grace to complete this thesis. I cannot fail to express my sincere appreciation to my supervisor, Assistant Professor Kerem Teralı for his expert advice and guidance throughout the course of this thesis. Thank you for inspiring me.

To the entire staff of Medical Biochemistry Department, Faculty of Medicine, Near East University, I say thank you for your impact on my academic life. You all have left an imprint on me.

I am highly indebted to my sponsor, the Government of Kaduna State, Nigeria for sponsoring my master's program. Without your sponsorship, I would not have been privileged to study under the tutelage of the great lecturers of the Department of Medical Biochemistry, Near East University, Cyprus. Also, I must say thank you to all the staff of Kaduna State Scholarship Board for their support towards the success of this program.

To my parents Mr. and Mrs. Joseph Oyama Bokah, my brothers; Kennedy, Richard, Neri, Joseph, Martins and my friends; Ceaser Dabour Danladi, Kefas James Konyan, Victor Markus, Solomon Karma, Daniel Bawa, David Johnson, to mention but a few, I really appreciate your love and support. I must equally acknowledge Professor Hamdi Öğüş for his kind words and encouragements.

Lastly but certainly not the least, I must appreciate my wife, Mrs Elizabeth Azilabih Oyamah (My Morning Star) who supports me and brings out the best in me. You are the best, I love you. To my daughter Zillah Azilabih Joseph and the new young man in the family (Alexander Azilabih Joseph), I love you and God bless you.

ABSTRACT

Cornelius, A.O. "The interrelationship between fetal hemoglobin levels and clinical phenotypes of beta-thalassemia". Near East University, Institute of Health Sciences, M.Sc. Graduation Project in Medical Biochemistry Program, Nicosia, 2018.

Beta-thalassemia (β-thalassemia), a common inherited monogenic disorder, is caused by reduction (β^+) or absence (β^0) in the synthesis of the beta-globin chains of the hemoglobin tetramer. There are three phenotypes of beta-thalassemia based on hematological and clinical conditions of increasing severity, *i.e.* β-thalassemia minor (β-thalassemia trait), β-thalassemia intermedia, and β-thalassemia major. However, of the three phenotypes only two are symptomatic: β-thalassemia intermedia (TI) and β-thalassemia major (TM). Fetal hemoglobin (HbF) is the primary hemoglobin molecule present in fetus, and it persists in the blood of newborn babies until about six months after birth. A number of quantitative trait loci (QTL) have been linked to variable HbF levels and shown to influence the clinical phenotype of the disease by altering the expression of globin genes or playing a role in erythropoiesis. In this review entitled "The interrelationship between HbF levels and clinical phenotypes of β-thalassemia", a systematic search of relevant scientific literatures was performed, and the findings were expressed mostly in the form of tables showing percent HbF levels and other disease-associated parameters in various populations. Accordingly, a negative linear correlation was found to exist between the levels of HbF and the severity of the clinical phenotype of β-thalassemia from the reports of relevant scientific literatures. Higher HbF levels were reported to be associated with the milder clinical phenotype of β-thalassemia, while lower HbF levels were reported to be associated with the severe clinical phenotype of β-thalassemia. Treatment of β-thalassemia patients with hydroxyurea has been reported to induce the synthesis of γ-globin chains, thereby increasing the levels of HbF. Therefore, hydroxyurea represents a promising drug in the management of β-thalassemia as revealed by the reports of relevant scientific literatures.

Keywords: fetal hemoglobin; beta-thalassemia; clinical phenotype; quantitative trait loci; thalassemia treatment.

ÖZET

Cornelius, A.O. "Fetal hemoglobin düzeyleri ile beta-talaseminin klinik fenotipleri arasındaki ilişki". Yakın Doğu Üniversitesi, Sağlık Bilimleri Enstitüsü, Tıbbi Biyokimya Yüksek Lisans Programı Mezuniyet Projesi, Lefkoşa, 2018.

Yaygın görülen kalıtsal bir monogenik bozukluk olan beta-talasemi (β-talasemi), hemoglobin tetramerini meydana getiren beta-globin zincirlerinin sentezindeki düşüşten (β^+) veya eksiklikten (β^0) ileri gelir. Hematolojik ve klinik durumlara göre beta-talaseminin azdan çoğa doğru şiddet gösteren üç fenotipi vardır: β-talasemi minör (β-talasemi taşıyıcılığı), β-talasemi intermedia ve β-talasemi majör. Buna karşın bu üç fenotipten sadece ikisi, β-talasemi intermedia (TI) ve β-talasemi majör (TM), semptomatik özellik gösterir. Fetal hemoglobin (HbF), fetüste bulunan başlıca hemoglobin molekülüdür ve doğumdan sonraki altı ay süresince yenidoğan bebeklerin kanındaki varlığını devam ettirir. Çok sayıda kantitatif özellik lokusu (QTL), değişken HbF düzeyleri ile ilişkilendirilmiş olup bunların globin genlerinin ifadesini değiştirerek ya da eritropoezde rol oynayarak hastalığın klinik fenotipini etkileyebildikleri gösterilmiştir. "Fetal hemoglobin düzeyleri ile beta-talaseminin klinik fenotipleri arasındaki ilişki" başlıklı bu derlemede ilgili bilimsel literatür sistematik şekilde taranmış ve bulgular sıklıkla farklı toplumlardaki HbF yüzdeleri ile diğer hastalık ilişkili parametreleri gösteren tablolar şeklinde sunulmuştur. Buna göre ilgili bilimsel literatürde HbF düzeyleri ile β-talaseminin klinik fenotiplerinin şiddeti arasında negatif doğrusal bir korelasyon olduğu görülmektedir. Yüksek HbF düzeyleri β-talaseminin daha hafif seyreden TI klinik fenotipi ile ilişkilendirilirken, düşük HbF düzeyleri β-talaseminin ağır seyreden TM klinik fenotipi ile ilişkilendirilmektedir. β-talasemi hastalarının hidroksiüre ile tedavi edilmesinin γ-globin zincirlerinin sentezini indüklediği ve böylelikle HbF düzeylerini artırdığı rapor edilmiştir. Dolayısı ile ilgili bilimsel literatüre dayanarak hidroksiürenin β-talaseminin yönetiminde umut vadeden bir ilaç olduğu söylenebilir.

Anahtar kelimeler: fetal hemoglobin; beta-talasemi; klinik fenotip; kantitatif özellik lokusu; talasemi tedavisi

TABLE OF CONTENTS

ABBREVIATIONS

2,3-BPG: 2,3-Bisphosphorglycerate.

ACE: Angiotensin Converting Enzymes

AHSP: Alpha Hemoglobin Stabilizing Protein.

AR: Autosomal Recessive.

ARMS-PCR: Amplification Refractory Mutation System-Polymerase Chain Reaction.

BCL11A: B-cell lymphoma/leukemia 11A.

GWAS: Genome-Wide Association Studies.

Hb: Hemoglobin.

HbA: Adult Hemoglobin.

HbF: Fetal Hemoglobin.

HbS: Sickle Hemoglobin.

HPFH: Hereditary Persistence Fetal Hemoglobin.

HPLC: High Performance Liquid Chromatography.

HS40: Hypersensitive Site 40.

HSC: Hemopoietic Stem Cell.

Jak2: Janus Kinase 2.

KLF1 : Kruepple-like factor 1.

LCR: Locus Control Region.

LIC: Liver Iron Concentration.

LRF: Leukamia/lymphoma-related factor.

MCH: Mean corpuscular hemoglobin.

MCV: Mean corpuscular volume.

MRI: Magnetic Resonance Imaging.

mRNA: Messenger Ribonucleic Acid.

MYB: Myeloblastasis (Myb proto-oncogene protein).

NCBI: National Center for Biotechnology Information.

NTDT: Non-Transfusion Dependent Thalassemia.

PHN: Paroxsymal Nocturnal Hemoglobin.

RBC: Red Blod Cell.

ROS: Reactive Oxygen Species.

SNP: Single Nucleotide Polymorphism.

TI: Thalassemia intermedia.

TM: Thalassemia major.

WHO: World Health Organisation

LIST OF FIGURES

LIST OF TABLES

Page No

1.0.INTRODUCTION

Hemoglobin (Hb) the main constituent of human blood is responsible for the transport of oxygen (O_2). Hb is a tetrameric protein synthesized within the Red Blood Cells (RBCs), it carries out the transport of molecular O_2 to the peripheral organs of the body that are dependent on oxygen from the lungs. Due to hemoglobin's affinity for oxygen, carbondioxide (CO_2) is been transported from the peripheral organs of the body to the lungs where it is exhaled (Edoh *et al.,* 2006). Hb is composed of an assembly of four subunits of globular proteins (two alpha and two beta globular subunits) with an embedded heme group within each subunit. Each heme group binds to a molecule of O_2. Amid the major types of Hb, hemoglobin A (HbA) is the normal adult hemoglobin, and hemoglobin F (HbF) is the main Hb in the fetus, also known as fetal hemoglobin. The unusual forms of Hb include; HbS and HbC. All the Hb variants are electrically charged, thus they can be identified and measured by hemoglobin electrophoresis procedures in the laboratory (Chernecky *et al.,* 2003).

The multisubunit protein evolution that is required by advanced organisms for buffering of acidic metabolic by-products and maximum oxygen homeostasis have been exploited and brought forward by molecular engineering. Each of the globin subunit forms a stable bond with heme (ferroprotoporphyrin IX) so as to allow the reversible binding of oxygen in the cytosolic RBCs to the iron atoms of heme. Moreso, the hydrophobic pocket where the heme molecule is inserted protects the reduced heme iron (Fe^{2+}) from oxidation to Fe^{3+} which cannot bind oxygen (Dailey & Meissner, 2013). For the efficient binding and unloading of oxygen in a cooperative manner by Hb tetramer ($\alpha_2\beta_2$), electrostatic interactions between unlike subunits of globin are required, thereby allowing optimal transport to actively metabolizing cells. The binding and unloading of molecular oxygen is demonstrated by the sigmoid shaped oxygen-binding curve which is dependent on the two quaternary structures of Hb tetramer. The deoxy or tensed (T) conformer has a low affinity for O_2 whereas the oxy or relaxed (R) conformer has a higher O_2 affinity. Furthermore, the triggering of allosteric actions of two minor effector molecules 2,3-bisphosphoglycerate (2,3-BPG) and protons (H^+) binding specifically on the deoxy conformer sites which is away from the heme groups gives Hb it functions. Hb needs to be packed into the flexible circulating RBCs to provide the blood with the capacity of carrying high oxygen molecules. For the intracellular concentration of Hb to reach 5 mM or 34 g/dl, an unusual high

solubility is necessary. To attain a high Hb corpuscular concentration as such, it is important for α-globin and β-globin/γ-globin mRNA to be expressed at an elevated levels of erythrocytes differentiation (Schechter, 2013).

In healthy state of embryonic and fetal development, there is sequential expression of globin genes at every stage. The type of Hb produced depends on the site of erythropoiesis. Ageing RBCs are regularly catabolized and exchanged by fresh RBCs synthesized from hemopoietic stem cells (HSCs). Synthesis of Hb is regulated via two multigene clusters; on chromosome sixteen that codes the synthesis of α-like globins; α and zeta (ζ), and chromosome eleven that codes the synthesis of the non-α-like globins, beta (β) globin , gamma (γ), epsilon (ε) and delta (δ). The β-globin gene cluster comprises five useful genes; A-γ, G-γ, δ, β and ε. The α-like globin genes experience a single switch from the embryonic to fetal/adult while the β-like globin experience double switches from embryonic then to fetal to adult. The synthesis of adult β-globin gene is dependent on the absence of γ gene competition (Jennifer, 2015).

During fetal development, HbF makes up around 90% of total Hb. At birth, the blood of the newborn contains about 70% HbF. However, HbF begins to decrease rapidly as the newborn bone marrow starts to form new RBCs. Usually, only 2% or less of total Hb is found as HbF after six months and throughout childhood; also, only 0.5% or less are found in total Hb in adults (Fischback *et al.,* 2004). HbF is distributed heterogeneously among erythrocytes in normal adults, although the synthesis is only limited to a minor group of cells, known as the F-cells (Franco *et al.,* 2006).

HbF ($\alpha_2\gamma_2$) is composed of two α- and two γ-globin subunits containing of 141 and 146 residues of amino acid in that order. The α-subunits are similar to those obtained in the hemoglobins of adult, HbA ($\alpha_2\beta_2$) and HbA_2 ($\alpha_2\delta_2$), while the γ-subunits are only found in HbF and vary from the β-subunits by 39 residues. There are two types of γ-subunits which can be found in HbF, they are; G-γ and A-γ. These two γ-subunits have similar function but are different in the amino acids sequence at position 136 which either contains the amino acid residue, alanine or glycine. On the functional basis, HbF differs mostly from HbA in its slightly high O_2 affinity, described by its low interaction with 2,3-BPG. This feature allows easy transport of oxygen through the placenta, supplying the fetus with oxygen from the maternal bloodstream (Schechter, 2008).

Clinically, the measurement of HbF is essential in the diagnosis and study of some vital globin gene disorders. Similarly, the levels of HbF may differ significantly in some of genetically inherited conditions associated with mild elevation in HbF levels such as hereditary persistent fetal hemoglobin (HPFH) mainly β- and δβ-thalasemias. HbF has been reported to prevent the polymerization of HbS and other agents capable of increasing the synthesis of HbF have been introduced for therapeutic use (Platt, 2008).

More frequent HbF persistence can be seen in some diseases associated with abnormal Hb synthesis (hemoglobinopathy). The occurrence of these is a marker of dysfunction or disease. The non-α-globin and α-globin synthesis must be strictly complemented. Crucial to the pathophysiology of the thalassemias is imbalance in subunits (Nienhuis & Nathan, 2012). Principally, free α-globin subunits are harmful to RBCs. The existence of alpha (α) hemoglobin stabilizing protein (AHSP) mitigates this threat. AHSP is a molecular chaperone that binds tightly and specifically to heme-intact α-globin subunits and is expressed in large amounts in erythroid cells (Mollan *et al.,* 2012). AHSP shields the cell from oxidized heme which is potentially toxic until its reduction to the functional Fe^{2+} heme in a reaction catalyzed by cytochrome b5 reductase. The dissociated α-globin from AHSP forms the very stable dimer (αβ) upon its encounter with an unbound heme-intact β-globin subunit. Electrostatic interaction between α-globin subunits (positively charged) and β-globin subunits (negatively charged) facilitates this process. Varying degree of thalassemia and anemia arise from mutations on the globin genes that alter their synthesis. In addition, mutations that are capable of altering the structure of globin subunits are associated with well-defined clinical and hematological phenotypes (Thom *et al.,* 2013).

The most common hereditary blood disorders worldwide are inherited Hb disorders and they are responsible for nearly 3.4% of mortality in kids below 5 years of age (Modell & Darlison, 2008). Mutations in the human globin genes are accountable for these forms of diseases which are classified into two groups, viz; those characterized by globin synthesis that are impaired (thalassemia) and those characterized by the abnormal globin molecules (Hb variants) production.

Thalassemias are identified as a result of the lack or reduced synthesis of one or more of the globin subunits of Hb tetramer. The commonest forms of thalassemia are alpha (α)-thalassemia and beta (β)-thalassemia which alters the production of α- and β-globin subunits in that order. Over 200 thalassemia mutations have been recognized and known to disturb some of the phases of α- and β-globin synthesis, from RNA transcription to the translation of β-globin mRNA. These mutations are typically point mutations, deletions at regulatory regions and small deletions (Thein, 2013). Complete inhibition of β-globin expression is known as β^0-thalassemia whereas decreased synthesis of structurally normal β-globin is known as β^+-thalassemia. Other structural Hb variants like hemoglobin E can result to a thalassemic effect due to their synthesis at a decreased rate resulting in serious clinical conditions (Jennifer, 2015).

Normally, there is equilibrium in the synthesis of α- and β-globin chains. On the other hand, in β-thalassemia, there is excessive synthesis of α-chains which cluster in precursors of RBCs to form inclusion bodies. This causes damage leading to untimely destruction of the RBCs precursor, thus leading to unproductive erythropoiesis (Higgs *et al.,* 2012).

Globin chain disparity is directly connected to the severity of thalassemias, any factor that reduces this disparity will improve the phenotype. Excess α-globin genes have adverse effect. Coinheritance of α- thalassemia will reduce the excess amount of α-globin. A mutation that affects one gene (β-thalassemia trait) often has no clinical significance however, when both genes are affected by a similar or completely different mutation, it leads to lack or decreased synthesis of the β-globin chains often resulting to serious anemia. A common condition of the thalasemias is the β-thalassemia minor also referred to as β-thalassemia trait (Thein, 2004). According to Thein, (2004); rare deletion forms of β-thalassemia have likewise been recognized. The uneven crossing-over among the partially and linked homologous β- and δ-globin genes is caused by one of these deletions, which results in the merging of δ- and β-globin genes to form δβ-globin gene, and also the Lepore gene which is poorly expressed. The Εγδβ-thalassemias, the δβ-thalassemias and the (HPFH) syndromes are initiated by large deletions that involve the entire β-globin gene cluster or part of it.

Clinically, the phenotype of these syndromes is comparatively homogeneous regardless of the striking heterogeneity of the β-thalassemias molecular basis; this is as a result of their common pathophysiology. In this case, there is a relative lack of HbA tetramers and buildup of unbound excess α-globin subunits that are not capable of forming Hb tetramers due to the relative lack of β-like globin subunits (Nienhuis & Nathan, 2012). Also, in β-thalassemia minor or β-thalassemia trait (heterozygotes), a minor to moderate hypochromic microcytic anemia with no indication of hemolysis can be seen; however, in compound heterozygotes or homozygotes (β-thalassemia major), serious transfusion-dependent hemolytic anemia related to marked unproductive erythropoiesis leading to annihilation of erythroid precursor cells can be seen in the bone marrow (Bernard & Frankling, 2016).

A moderate and incompletely compensated hemolytic anemia which doesn't necessitate regular transfusion therapy to conserve an adequate level of circulating Hb in the affected patient can be seen in a clinical phenotype known as β-thalassemia intermedia. Occasional transfusion may be needed to restore normal levels of Hb if the level of anemia gets worse as a result of associated complications. Notably, there is a milder disease in β-thalassemia intermedia (TI) patients due to fewer severe α- to non-α-globin subunit disproportion than in a usual β-thalassemia major (TM) patient, leading to lesser accumulation of free α-subunits which causes the unproductive erythropoiesis (Bernard & Frankling, 2016). This drop in non-α-globin to α-globin subunits imbalance may be caused by different possibilities such as;

i. Inheritance of the milder forms of the β^{+}-thalassemia mutations having less severe clinical phenotype than the typical β-globin subunit deficiency.
ii. Coinheritance of other genetic traits linked with improved synthesis of β-subunit in HbF.
iii. Coinheritance of a form of α-thalassemia.

Two mutant β-globin genes can be seen in most patients with TI, these patients carry a genotype characteristic of TM with the phenotype improved by one of the factors outlined above. Heterozygosity for one β-globin gene mutant linked with the synthesis of an extremely unstable β-globin subunit capable of causing RBCs destruction is responsible for rare cases of TI (Thom *et al.,* 2013).

Overall β-globin subunit synthesis deficiency are associated with δβ-thalassemias, but clinically, they are milder than usual cases of β^0-thalassemia. Also, in δβ-thalassemias, there is a corresponding persistent expression of the γ-subunit of HbF in high levels thereby reducing the amount of α-subunit in excess. Neonatal hemolytic anemias are linked with Ɛγδβ-thalassemias and it resolves within the first few months after birth. The corresponding phenotype in adults is typical of β-thalassemia minor or β-thalassemia trait. Elevated levels of persistent γ-globin production are characterized as HPFH syndrome which is often considered within the spectrum of δβthalassemia (Thom *et al.,* 2013).

Furthermore, thalassemias are autosomal recessive (AR) genetic disorders. Clinically, thalassemia carriers appear to be normal. Nevertheless, for every single conception, there exists is a 25% tendency that the baby will be thalassemic, a 50% tendency that the baby will be a carrier of thalassemia, and only a 25% tendency that the baby will be normal if both parents are thalassemia carriers. Till date, one of the ways to prevent the birth of thalassemia affected child is by prenatal diagnosis. Today, clinical representations of TM are recorded in developing nations that lack sufficient resources for treatment such as regular blood transfusions and iron chelation therapy to cater for affected individuals (Cappellini *et al.,* 2008).Screening and identification of high-risk couples both being carriers, prior to conception and prenatal diagnosis during pregnancy is therefore a perfect and effective strategy for reducing birth of thalassemia patients in highly prevalent regions. Also, new born screening is aimed at detecting the most important structural hemoglobin variant (Lal *et al.,* 2011).

The existence of β-thalassemia trait is variably associated with increase in HbF level, and is more common in δβ thalassemia and HPFH. Also, inconsistent HbF levels are linked with the occurrence of the polymorphic γ-globin chains in normal healthy subjects. Thus, an increased expression of γ-globin gene has clinical relevance in the treatment of diseases related to the β-globin gene (Andre *et al.,* 2009).

This literature review is aimed at investigating the interrelationship between HbF levels and clinical phenotypes of β-thalassemia; and to suggest possible treatment/management for β-thalassemia conditions.

2.0. GENERAL INFORMATION

2.1. Hemoglobin Structure, Functions and Variants

Hemoglobin (Hb) is a tetrameric allosteric protein. It is the red blood pigment found only in the RBCs. Hemoglobin is a conjugated protein that contains globin; the apoprotein and heme; the non-protein part (prosthetic group). The normal Hb concentration in males ranges between 14–16g/dl and ranges between 13–15g/dl in females (Satyanarayana & Chakrapani, 2009). There are two important biological functions of hemoglobin involved in respiration, these include;

1. Carrying of molecular oxygen from the lungs to peripheral organs
2. Carrying of CO_2 and H^+ from peripheral organs back to the lungs where excretion occurs.

Hb a heterotetrameric spherical super molecule consisting of two α-chains and two non-α-chains (typically β-chains) of simple subunits of globin each with 16 kDa mass. The complete molecule of Hb is formed by nearly six hundred amino acid residues in which the four subunits of globin are folded into spherical (globular) shapes and connected to form a 5.5 nm diameter structure (Nelson & Cox, 2008). The four subunits of the globin are control along by noncovalent interactions. The α-globin and non-α-globin subunits have different amino acids sequences folded in the same manner (Koolman & Roehm, 2005; Nelson & Cox, 2008). There are 141 amino acids residues on the α-globin subunits while on the β-globin subunits; there are 146 amino acids residues. On every four subunits of the globin, a heme (ferroprotoporphyrin IX) prosthetic cluster is attached. This has an iron atom present in the ferrous form (Fe^{2+}). Therefore, it is made up of four heme groups suppressed in four globin chains hydrophobic pockets of the Hb which are dependent on the heme group of the four iron atoms in the ferrous state. The Fe^{2+} ions set up only 0.3% of its mass. The Hb has a relative molecular mass of 64,500 Da, has an isoelectric point of 6.8 and is soluble in water (Nelson & Cox, 2008). In typical Hb, every of the α-globin subunit is matched with a β-globin subunit in a duplicate symmetric manner. Hence, Hb molecule can be seen also as a dimer of αβ-protomers. Every subunit globin of the Hb has a different structure thus, having a different O_2 affinity, having a dissimilar electrical charge and therefore, different electrophoretic motion (Tangvarasittichai, 2011; Koolman & Roehm, 2005).

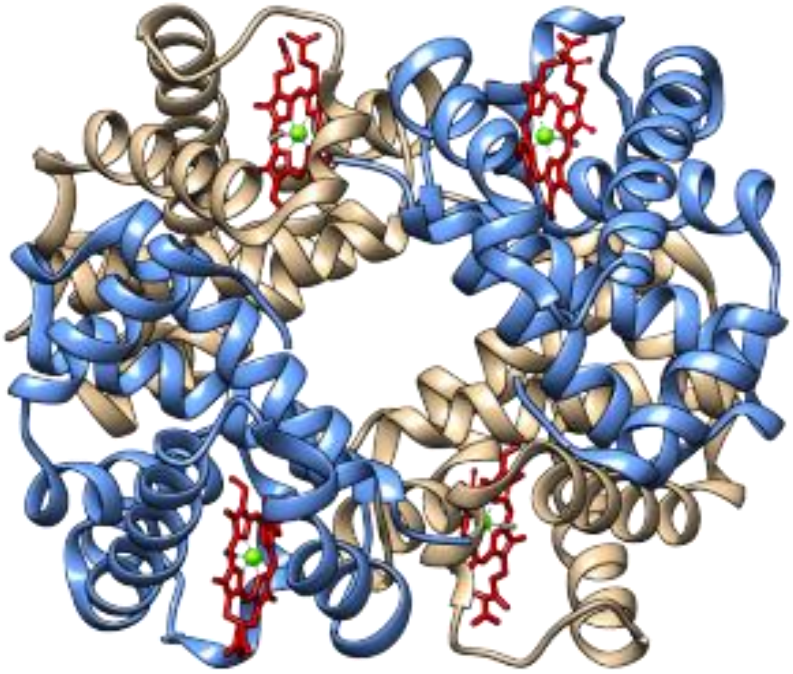

Figure 2.1. Three dimensional Hb molecule structure displaying the α- and β-globin subunits in brown and blue respectively, with heme moiety in red and Fe^{2+} ions in green (Taken from Sabia, 2015).

In the mammalian RBCs, there are two other forms of Hb which exist in equilibrium. These two other forms include; the tensed form (T-form) which correspond to deoxyhemoglobin (deoxyHb) and the relaxed form (R-from) which correspond to oxyhemoglobin (oxyHb). The molecule of Hb is ideally in the T-form in the absence of a ligand, due to the existence of extra salt bridges and alternative noncovalent interactions within the interface between the two dimers (αβ). There is a reform in the tertiary structure of the Hb molecule in the presence of a ligand, as a result of the progressive loosening of the noncovalent bonds holding the tetramer of the Hb together in the T-form thus, resulting to the R-form which has elevated O_2 affinity (Perutz, 1970; Jensen *et al.*, 1998).

In the RBCs, Hb molecule plays transport, metabolic, homeostatic and buffering roles. For metabolic oxidation, O_2 is required in mammalian tissues. The products of oxidation in these mammalian tissues such as CO_2 also need to be expelled so as sustain optimum homeostasis. Therefore, Hb is needed to supply these tissues with O_2 and get rid of CO_2 (Koolman & Roehm, 2005; Nelson & Cox, 2008).

The transport of molecular O_2 occurs when it binds to the molecule of Hb reversibly at the heme group thus, ensuring that the heme iron is kept in the Fe^{2+} state. Therefore, O_2 binding is more favored when compared with the binding of different potential heme ligands (Koolman & Roehm, 2005; Nelson & Cox, 2008).

Hemoglobin affinity to molecular O_2 varies considerably with the structure of globin. This is allosterically controlled when allosteric co-factors such as H^+, organic phosphates, chlorides bind specifically to the binding sites of Hb molecule therefore, lowering the affinity of O_2 of Hb heme groups. These allosteric effectors favorably bind to the T-form of Hb and making them stable as a result of extra bonds formation. In this case, the binding of molecular O_2 is cooperative, which means, the binding of molecular O_2 to one subunit of the Hb molecule eases the binding of next molecular O_2 to the other T-form subunits. The O_2 equilibrium curve exhibit a sigmoid shape which describes this interaction. Also, the cooperative binding of O_2 in the mammalian Hb is not dependent of the pH values however, the cooperative binding of O_2 in the lower living organisms like pishes is largely dependent on the value of pH (Perutz, 1990; Riggs, 1988; Antonini & Brunori, 1971).

For the transportation of CO_2 that is metabolically produced from the tissues into the lungs for elimination, CO_2 is bound to Hb in a reaction expedited by H^+ binding to the Hb at its allosteric sites. The H^+ binding initiates CO_2 hydration in the RBCs towards the formation of bicarbonate (HCO_3^-) in a reaction catalyzed by carbonic anhydrase. The formed HCO_3^- is then transported by HCO_3^-/Cl^- to the plasma which is exchange through the membranes of the RBCs. Both H^+ and HCO_3^- formed are eliminated in this way and there is shift in equilibrium further to the right supporting the binding of carbondioxide as blood flows through the capillaries within the peripheral tissues as presented in the chemical equation below;

$$CO_2 + H_2O \quad \longleftrightarrow \quad H^+ + HCO_3^-$$

On the other hand, carbondioxide reacts with α-amino groups of globin subunits (uncharged) of Hb to produce carbamic acids. Also, if α-amino groups are charged, the carbamic acid produced dissociates to give carbamate at physiological pH as shown by the equations beneath;

$$Hb\text{-}NH_3^+ \longleftrightarrow Hb\text{-}NH_2 + H^+$$

$$Hb\text{-}NH_2 + CO_2 \longleftrightarrow Hb\text{-}NHCOOH$$

$$Hb\text{-}NHCOOH \longleftrightarrow Hb\text{-}NHCOO^- + H^+$$

In mammals, carbamate is highly formed in the deoxyhemoglobin than in the oxyhemoglobin and this has a biological importance. For instance in humans, the binding of CO_2 to deoxyhemoglobin accounts for 87% exchange of CO_2 whereas the binding of CO_2 to oxyhemoglobin only accounts for 13% exchange of CO_2 (Klocke, 1988).

The Hb molecule configuration and role is primarily dependent on its equilibrium. The interchange of H^+ between Hb and its plasma is vital. Therefore, for this to be achieved; the binding/release of H^+ by Hb in the RBCs required for the hydration–dehydration of CO_2 must be ensured. This exchange of H^+ makes Hb an active non-bicarbonate buffer. This function limits the insignificant alterations in the pH of the blood upon fluctuations in the concentration of blood acidity or basicity. The Hb molecule total charge defines the pH of the RBCs by allotting the H^+ transversely in the RBCs membranes. This H^+ circulation is essential in the formation of intra-subunit and inter-subunit salt bridges in Hb. This is equally essential for ligands binding like organic phosphates and chlorides to Hb (Jensen *et al.,* 1998). Disproportion in the α-globin and non-α-globin (β-like globins) subunits of Hb makes the unbound α-globin subunits to precipitate, resulting to loss of natural functions and later on resulting to the pathophysiology of thalassemias (Nienhuis & Nathan, 2012).

In adults, a small percentage of Hb (<5%) known as the minor adult hemoglobin (HbA_2) is made up of two α- and two δ-chains. HbF is produced during the development of the fetus and some of it may persist in adult life. Glycosylated hemoglobin (HbA_1C) synthesized via the covalent binding of a molecule of glucose to Hb also exists in low concentrations. High levels of HbA_1C are seen in diabetes mellitus patients, this is successfully utilized for the prognosis of these patients (Satyanarayana & Chakrapani, 2009). **Table 2.1** below shows the major types of normal hemoglobin and their percentages in the body.

Table 2.1. Normal major types of hemoglobins (Modified from Satyanarayana & Chakrapani, 2009)

Hb variants	**Composition & symbol**	**% in total Hb**
HbF	$\alpha_2 \gamma_2$	<2
HbA_1	$\alpha_2 \beta_2$	90%
HbA_2	$\alpha_2 \delta_2$	<5%
HbA_1C	$\alpha_2 \beta_2$-glucose	<5%

At present, quite a thousand conditions of Hb production and/or structure are known and well-studied thus, giving an understanding on how these mutant genotypes change the synthesized Hb molecule functions and its clinical phenotype. This relationship amid the genotype and phenotype of these mutant hemoglobins has explained pathophysiologically the mechanisms of the related hemoglobinopathies (Forget & Bunn, 2016).

Genetic variations results to these mutant hemoglobins, otherwise known as Hb variants. Some of these Hb variants give rise to diseases and are noted as pathological Hb variants whereas others have no noticeable pathology and are noted as non-pathological Hb variants (Forget & Bunn, 2016).

Furthermore, some non-pathological Hb variants are; hemoglobin A (HbA) constituting 95–98% of the Hb in adult, hemoglobin A_2 (HbA_2) an insignificant Hb constituting 2–3% Hb in adult and hemoglobin F (HbF) the fetal Hb which is produced during pregnancy by the fetus and is tailored for economical O_2 transportation in low oxygen surrounding, constituting 2.5% Hb in adult (Peter & Victor, 2009).

Hemoglobinopathological Hb variants consist of; sickle hemoglobin (HbS) in which there is a replacement of glutamine (Gln) with valine (Val) at position 6 of the β-globin subunit (β-Gln6⟶ Val6). The diverse forms of this variant sickle cell trait (HbAS) gives survival benefit against complications of Falciparum malaria in sickle cell patients because of the fact that HbAS has 40% HbS and 56–68% HbA. Also, hemoglobin H (HbH) is commonly produced in reaction as a result of severe deficiency of α-globin subunits; HbH has an uncommon high oxygen affinity. This can be seen in α-thalassemia patients which is made up of four β-globin subunits (β_4). Hemoglobin M (HbM) is described by the replacement of histidines (His) to tyrosines (Tyr) in either the α-globin, β-globin or γ-globin subunits within the heme hydrophobic pockets causing the iron ion in the heme pocket to remain in the Fe^{3+} state (Forget & Bunn, 2016).

2.2. Adult and Fetal Hemoglobin

In a healthy state of embryonic and fetal development, there is sequential expression of globin genes at every developmental stage. Variations in the erythropoiesis site are complemented by variations in the type of hemoglobin that is synthesized. Matured RBCs are constantly catabolized and substituted by new RBCs synthesized from HSCs. The synthesis of hemoglobin is regulated by two multigene clusters; on chromosome 16 that codes the α-like globins, α- and zeta (ζ) and chromosome 11 that codes the β-like globins, gamma (γ), epsilon (ε), delta (δ) and β). During human development, these genes are set out alongside the chromosomes in the order in which they are expressed as shown in the **table 2.2** below (Jennifer, 2015).

Furthermore, Ɛ, G-γ, A-γ, δ, β are the five functional genes that constitute the β-globin gene cluster. The α-like genes experience a single switch from embryonic to fetal/adult while the β-like genes experience double switches from embryonic to fetal then to adult. The adult β-globin gene expression is dependent on the absence of competition from the γ-gene (Jennifer, 2015).

Table 2.2. Subunits making up the different hemoglobin isoforms (modified from Jennifer, 2015).

Human hemoglobin variants		
Embryonic hemoglobins	**Fetal hemoglobin**	**Adult hemoglobins**
Gower 1-($\zeta_2\varepsilon_2$)	Hemoglobin F-($\alpha_2\gamma_2$)	Hemoglobin A-($\alpha_2\beta_2$)
Gower 2-($\alpha_2\varepsilon_2$)		Hemoglobin A_2-($\alpha_2\delta_2$)
Portland-($\zeta_2\gamma_2$)		

2.3. The Genetic Structure of the Hb Gene Clusters

In humans, Hb molecules are tetramers consisting of globin chains (two pairs); a pair of α-globin chains and a pair of β-like globin chains. Hb synthesis is regulated at the molecular level by two clusters of multigene (**Figure 2.2.A**). The α-gene cluster consists of one embryonic gene (ζ_2), two fetal/adult α-genes (α_2 & α_1), two pseudo genes ($\Psi\zeta_1$ & $\Psi\alpha_1$), and two minor globin-like genes ($\Psi\alpha_2$ & θ), decided in the sequential order: 5'- ζ_2- $\Psi\zeta_1$- $\Psi\alpha_2$- $\Psi\alpha_1$-α_2-α_1-θ-3'. The α-globin cluster has a major regulatory element known as *HS-40* (Shang & Xu, 2016). The β-cluster has an embryonic gene (Ɛ), two fetal genes (G-γ & A-γ), one (1) pseudo gene (Ψβ), and two adult genes (δ & β), decided in the following order: 5'-Ɛ- G-γ- A-γ- Ψβ- δ- β-3'. The β-globin gene cluster has the locus control region (LCR) as an essential regulatory region on the upstream (Shang & Xu, 2017).

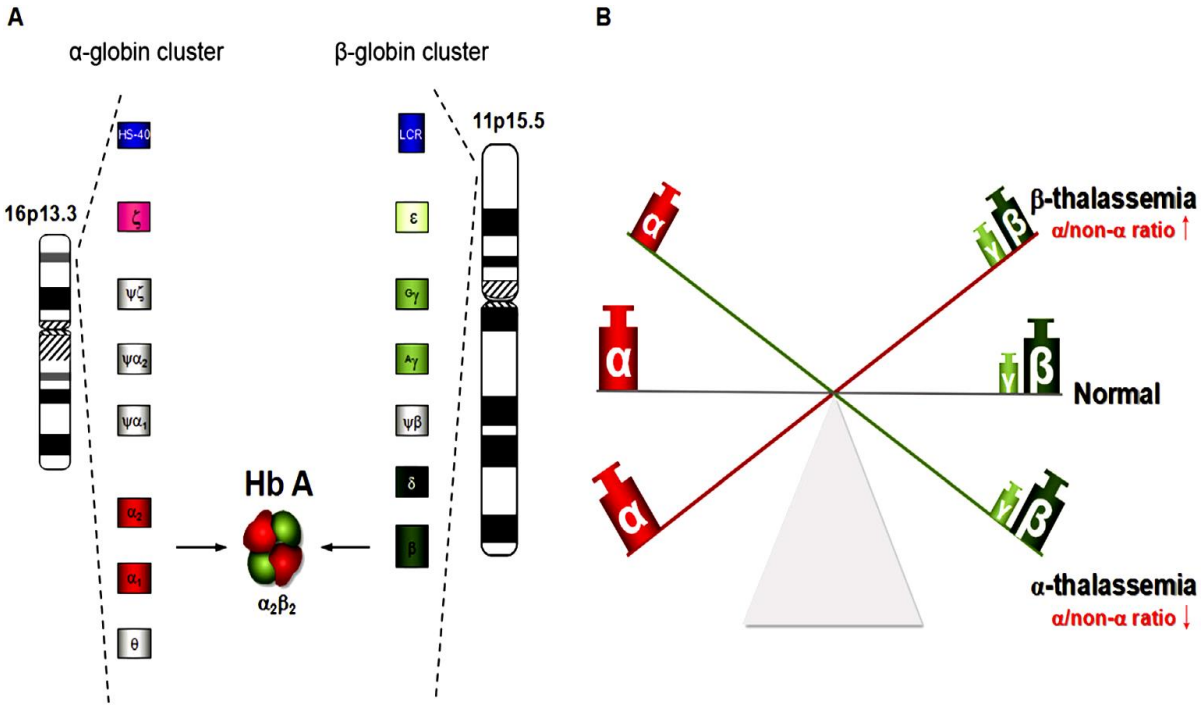

Figure 2.2. Diagrammatic illustration of synthesis of Hb at molecular level controlled by two multigene clusters **(A)** Structure of the α-globin and β-globin gene clusters and **(B)** their pathophysiological roles in thalassemia (Taken from Shang & Xu, 2017).

Thalassemias show a broad range of clinical phenotypes that ranges from asymptomatic to the fatal phenotype. In a typical Hb production, the proportion of α- to non-α subunits is 1:1 as shown above **(Figure 2.2.B)**, but in α-thalassemia, the amount of β-globin like chains is more when compared to that of α-globin chains. In contrast, in β-thalassemia, the amount of β-globin like chains is lower when compared to that of α-globin chains. The extent of disproportion is in proportion to the disease severity (Shang & Xu, 2017). In patients with Hb Bart's hydrops fetalis, due to lack of α-globin chains, the blood of the fetus comprises primarily Hb Bart (γ_4) which cannot release O_2 even in a state of severe oxygen demand. This causes the fetus to suffer severe anemia and hypoxia often leading to the development of fetal abnormalities. Such fetuses most often die either in the uterus within the first and second trimesters or soon after their birth. In Southeast Asia, the disease accounts for up to 90% of all fetal hydrops (Chui, 2005). In patients with HbH disease, which is the intermediate of the clinical form of α-thalassemia, the patients generally produce less than 30% of the required quantity of α-globin, with β-globins relatively in excess forming HbH (β_4). The HbH precipitates in the RBCs and get destroyed prematurely

causing mild hemolysis due to its instability. Hemolysis and ineffective erythropoiesis are the main pathophysiological mechanisms underlying β-thalassemia. Insufficient β-globin chains lead to excess free α-globin chains which are unstable and form alpha (α)-hemichromes, generating reactive oxygen species (ROS) thereby triggering reaction cascades leading to hemolysis and unproductive erythropoiesis. Other complications which are clinically known include; deformation of skeletal tissues, iron overload, splenomegaly and expansion of erythroid bone marrow (Chui, 2005).

2.4. Hemoglobin Switching Process

HbS are normally tetramers consisting of four globin chains. In every developmental stage, the synthesis of α-like globin chains and β-like globin chains is proportionally balanced. The changes in the structure of Hb in humans during development, is shown in (**Figure 2.3.)** below. In the first phase (embryonic phase), there are three variants of Hb, viz; Hb Gower 1 (ζ_2 ε_2), Hb Gower 2 (α_2 ε_2), and Portland ($\zeta_2\gamma_2$). All these embryonic Hb variants are exclusively found in the yolk-sac and then replaced subsequently by the HbF ($\alpha_2\gamma_2$). HbF is the principal Hb in the uterus. It is replaced by HbA ($\alpha_2\beta_2$, approximately 97%) after birth and HbA_2 ($\alpha_2\delta_2$, approximately 2–3%) after a year older. During the first 6 months after birth, HbF is present in the blood of the babies to prevent them from developing β-thalassemia at birth. HbF normally remains in adult blood constituting about 1% of the entire Hb (Higgs, 2012). This whole process is termed the Hb switch.

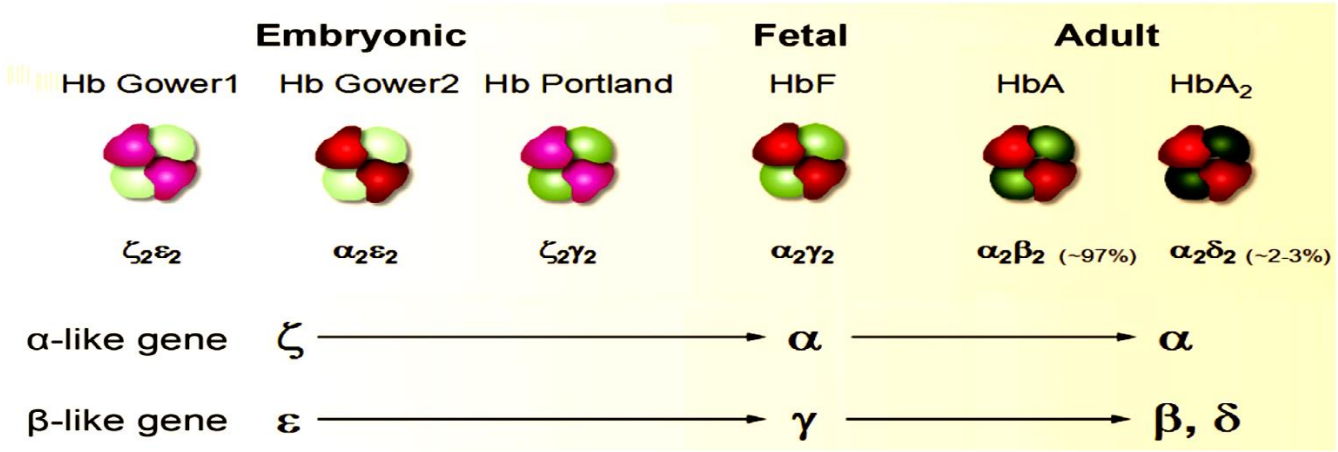

Figure 2.3. Diagrammatic illustration of the process of globins switching from embryonic stage to fetal stage and from fetal to adult stage (Taken from Shang & Xu, 2017).

The α- and β-globin gene clusters are organized along the chromosome in the sequential order of their expression during development as shown in **figure 2.3.** The sequential silencing and activation of these genes are specifically regulated. Previous studies on the expression of these genes showed that the HS-40 region of α-cluster and LCR of β-cluster function as similar regulatory regions (Weatherall, 2001). Each of these regions is held by a complex numerous proteins which function as trans-acting factors (Piel & Weatherall, 2014).

In the α-cluster, gene switching is comparatively simple. Throughout life, the two α genes are unceasingly expressed with the exception of during embryogenesis in which ζ proteins are synthesized. Whereas, switching of genes is more complex in the β-cluster. This comprises of a switch from Ɛ→γ→β. The γ to β switch in particular has more clinical significance as high level of HbF is used as a diagnostic tool for β-thalassemia (Pace *et al.,* 2015). Reactivation and binding of γ-genes to the surplus α-globin is among the leading strategies employed in the treatment of thalassemia. Previous investigations have proved two key mechanisms for silencing γ-globin gene in adults. This includes the γ- and β-globin genes interaction with the LCR (which is competitive) during the switch from fetal to adult Hb and gene-autonomous silencing of γ-globin (Pace *et al.,* 2015). Gene-autonomous γ-globin silencing mechanism offers the origin for a

gene-based methodology for increasing the level of HbF after birth in the management of thalassemia major patients (Sankara & Weiss, 2015). Many transcriptional factors such as *BCL11A, HBSIL–MYB, KLF1, LRF*, and others are involved in this mechanism (Masuda *et al.,* 2016).

BCL11A gene is a key repressor of the expression of γ-globin. Irrespective of whether *BCL11A* is present in transgenic mice or in human erythroid precursor, loss of function of *BCL11A* is enough to prevent γ-globin repression (Bauer & Orkin, 2015). At a distance, it seems to apply its repressive function. It binds to the LCR instead of binding the β-globin gene or γ-globin. It participates in the configuration of the β-locus. It stimulates distant interactions between the β-globin gene and the LCR. Also, the LCR act on γ-globin genes in place of the β-globin gene and knocked it out thus the γ-globin expression is reactivated (Bauer & Orkin, 2015).

The main regulator of transcription of adult β-globin is the *KLF1*. Deactivation of the *KLF1* gene in mice revealed that *KLF1* is vital in activating β-globin expression (Perkins *et al.,* 2016). *KLF1* facilitates the switch from γ to β by the binding the *BCL11A* gene promoter thereby triggering the transcription of *BCL11A*. When the *KLF1* expression is knocked down, the *BCL11A* gene expression is inhibited and the γ: β proportion in erythroblasts is increased (Zhou *et al.,* 2010). The *KLF1/BCL11A* regulatory axis has been suggested to play an essential role in the Hb switch (Crispino & Weiss, 2014). *KLF1* activates *BCL11A*, which represses the expression of γ-globin gene, thus supporting the switch from HbF ($\alpha_2\gamma_2$) to HbA ($\alpha_2\beta_2$) in the normal developmental process (Crispino & Weiss, 2014). Also, in normal developmental process, *KLF1* itself activates the expression of β-globin (Suzuki *et al.,* 2013). In a few cases of HPFH, *KLF1* insufficiency leads to decreased expression of *BCL11A*, thereby increasing the level of HbF and decreasing HbA level (Crispino & Weiss, 2014).

The mechanism of *HBSIL–MYB* that affects the expression of γ-globin still needs further investigation. Nevertheless, in mice, the inactivation of *HBSIL–MYB* yielded an increase in the expression of Ɛ- and γ-globin signifying that it accounts for the silencing of γ-globin during the developmental process (Masuda *et al.,* 2016). In recent times, *LRF* was acknowledged as a novel transcriptional factor that suppresses the expression γ-globin (Masuda *et al.,* 2016). In adults, *LRF* acts on the γ-globin genes and preserves the density of the nucleosome optimum for the silencing of γ-globin gene (Masuda *et al.,* 2016). The *LRF* function in the repression of γ-globin

is independent on *BCL11A* protein; this proposes the existence of more factors or elements that may contribute to the switching of the hemoglobin (Masuda *et al.*, 2016). In the future, microRNAs and epigenetics alteration should be investigated.

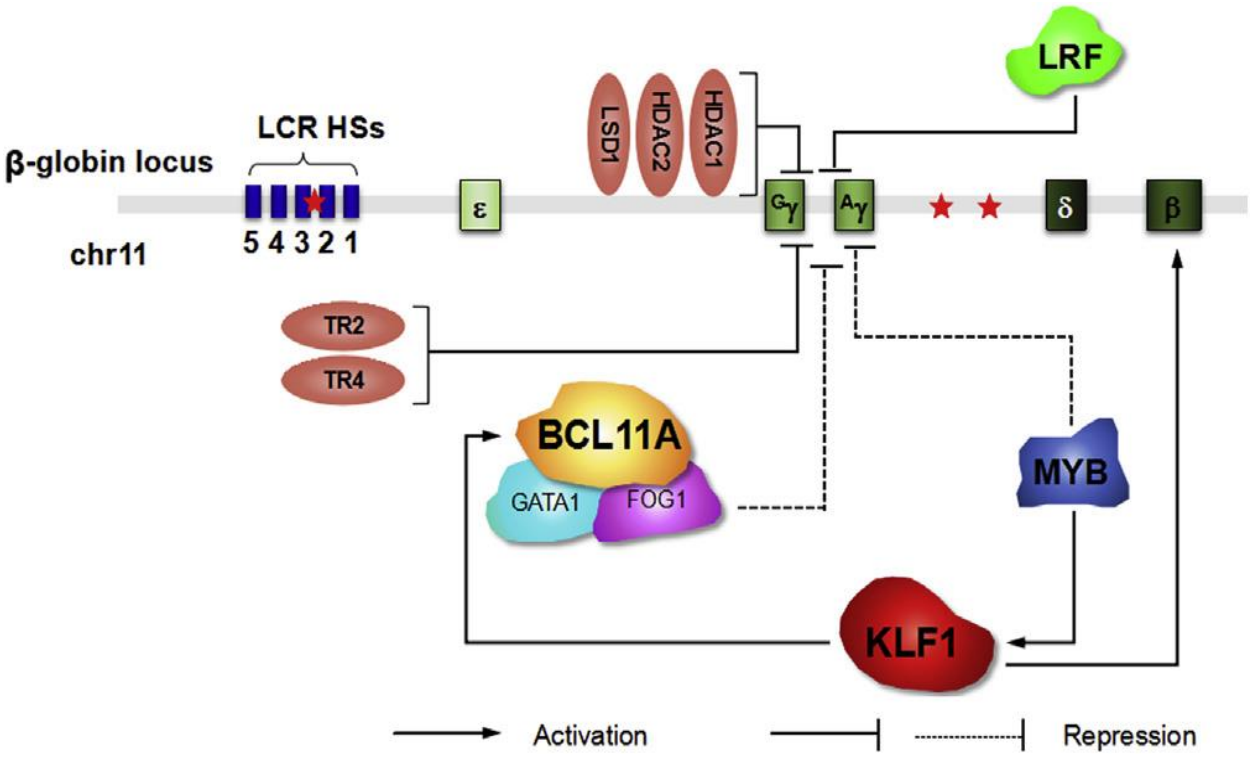

Figure 2.4. A diagrammatic illusrtation showing a key transcriptional factor that is involved in switching process of the γ to β genes. The binding sites of *BCL11A* are shown using red stars. The LCR encompasses the hypersensitive sites numbered 1-5 (blue boxes). *MYB*, *KLF*, *GATA1*, *FOG1*, together with the *BCL11A* complex all repress γ-globin via a mechanism of action which is indirect. These are indicated using dotted lines (Taken from Shang & Xu, 2017).

2.5. Thalassemia, Prevalence and Molecular Basis

Worldwide, thalassemia is among the most prevalent autosomal recessive diseases. However, the prevalence of thalassemia varies according to geographical locations with Cyprus (14%) and Sardinia (12%) having the highest recorded rates (Jennifer, 2015). Thalassemia is predominant in Mediterranean, Central Asian, Middle Eastern, Far East Indian Subcontinent, and African populations. Each year nearly 1.5% of the world's population has been projected by the World

Health Organization (WHO) to be carriers of β-thalassemia with at least sixty thousand (60,000) people born severely affected. Also, migration amongst populations contributes to the widespread of β-thalassemia throughout the world. The most predominant mutations are found in sub-tropical and tropical regions of the world where elevated gene frequencies have been observed in line with the affiliated protection proffered against malaria (Galanello & Cao, 2011).

Furthermore, two forms of mutations in globin genes cause thalassemias. These mutations are: deletion mutations and non-deletion mutations. The deletion mutations usually involve over 1 kb of range whereas non-deletion mutations consist of oligonucleotide deletions/insertions or single nucleotide substitutions (Shang & Xu, 2017). In different populations, another range of α- and β-thalassemia mutations is often found. For molecular diagnosis to be carry out, the patients ethnic origin should be put into consideration because the mutations reference data found in a given populaces are peculiar to these populations (Shang & Xu, 2017).

The majority of β-thalassemia is as a result of non-deletion defects. Non-deletion variants of over 300 have been characterized in diverse populations (Shang *et al.,* 2011). Only minorities of these variants involve minor deletions in the β-globin gene coding regions, but most of them are point mutations (Shang *et al.,* 2011). Mutations of β-thalassemia are categorized into three groups based on the extent of quantitative decrease in the normal β-globin synthesis. These groups include; (1) βeta^{0}-thalassemia mutation (β^{0}), which results to β-globin absence; (2) βeta^{+}-thalassemia mutation (β^{+}), which decreases severely the β-globin output; (3) βeta^{++}-thalassemia mutation (β^{++}, also called silent β-mutation), which slightly decreases the β-globin synthesis. A list of common β-mutations is presented on **table 3.2** below.

Moreover, some variants of Hb are produced at lower rates or are extremely unstable leading to other thalassemia phenotypes like HbE ($\beta^{CD26\ (G>A)}$). This is due to β-codon 26 mutation (GAG>AAG) which results to the substitution of amino acid from glutamine to lysine. Also, it causes the activation of a new splice site responsible for unusual mRNA processing (known as a β^{+}-thalassemia mutation) (Weatherall, 2001). These mutations are further subdivided into different groups based on the mechanisms by which they interfere with the functions of the β-globin gene. These groups are; (1) mutations that interfere with transcription, e.g. $\beta^{CAP\text{þ}39\ (C>T)}$ in the 5'UTR or $\beta^{101\ (C>T)}$ in the promoter; (2) mutations that interfere with the processing the RNA , e.g. $\beta^{Term\ CD+32\ (A>C)}$ in the 3'UTR; $\beta^{PA\ (GATAAG)}$ that reduces the effectiveness of the cleavage-

polyadenylation process and $\beta^{-IVS1-110\ (G>A)}$ that create cryptic splice sites; and (3) mutations that interfere with the translation of RNA, e.g. start codon mutation $\beta^{-(ATG>GTG)}$, frameshift mutation $\beta^{CD41-42\ (-CTTT)}$ and nonsense mutation $\beta^{CD39\ (C>T)}$ (Thein, 2013).

Uncommon β-thalassemia gene deletional mutations have also been recognized. The β-globin gene itself is exclusively restricted to a group of deletions. For instance, the six hundred and nineteen (619) bp deletion, cleaves the β-globin gene 3'-end (Thein, 2013). This mutation is common among Asian-Indian population and is responsible for nearly 30 percent of the β-thalassemia cases recorded in this populace. This particular group of deletions is also commonly known as β^0-mutations. Other groups of deletional mutations include large deletions that involve a fragment of the β-globin gene or a complete β-globin gene cluster. Such large deletions account for HPFH or δβ-thalassemias (Chen *et al.,* 2010).

Table 2.3. Deletional mutations that are common in thalassemia and ethnic group affected. Deletion (β-gene): those deletional mutations that affect β-globin gene and deletion (HPFH/ζβ): those deletional mutations involving fragment or the whole β-globin gene clusters (Modified from Shang & Xu, 2017).

Ethnic group affected	Locus	Mutation/types of deletion	Common mutations
Southeast Asia	β-globin	$\alpha\ \alpha^T$(α1 gene)	HbQ-Thailand
Mediterranean		β^{++}-mutation	$\beta^{-101(C>T)}$
Mediterranean		β^{+}-mutation	$\beta^{IVS1-101(G>A)}$
Southeast Asia			HbE
Mediterranean		β^0-mutation	$\beta^{CD39(C>T)}$
Southeast Asia			$\beta^{CD41-42(-CTTT)}$
Asian Indian		Deletion (β gene)	619 bp deletion
Chinese		Deletion (HPFH/ζ β)	SEA-HPFH
			G-γ^+ $(^\gamma\delta\beta)^0$

2.6. The Genotype–Phenotype Associated with β-Thalassemia

The β-thalassemia is a genetic syndrome of Hb synthesis described by absence (β^0) or reduced (β^+) β-globin subunit production of Hb molecule (Weatherall & Clegg, 2001). Most individuals that are affected with thalassemia acquire this disorder as a Mendelian recessive. Milder anemia and microcytosis can be seen in heterozygous individuals and are characterized as having β-thalassemia minor or trait (Nienhius & Nathan, 2012). While severe anemia of varying degrees can be seen in homozygous individuals who are categorized as homozygous β-thalassemia or TM or TI. According to Thein (1999), a dominantly inherited β-thalassemia (that rarely occurs) that causes disease in heterozygous individuals is due to unstable β-globin variants that are highly synthesized. Frequently, the disruption only affects β-globin synthesis; however there can be unusual cleaveage of one or more of the other genes on chromosome 11 by deletional mutations (Nienhius & Nathan, 2012). This results in other forms of the disease categorized as δβ -, γδβ -, or Ɛγδβ -thalassemia.

2.6.1. Heterozygous β-Thalassemia

Cao & Galanello (2010) described the hematological characteristics of β-thalassemia trait as microcytosis, hypochromia, and there is typically a raise in the percentage of HbA_2. Hb is composed of 92–95% HbA, 3.8% HbA_2, and variable quantities of HbF ranging from 0.5–4%. Coupled to hypochromia and microcytosis, there is noticeable disparity in the shape and size of RBCs. The RBCs of β^0-thalassemia trait have a low mean corpuscular volume (MCV)/ mean corpuscular hemoglobin (MCH) compared to those of β^+-thalassemia trait. Historically, a mild anemia with hypochromic red cells and microcytic, which are typically of β-thalassemia trait have been assumed not to have clinical significance besides being associated with anemia during pregnancy period (White *et al.,* 1985). Nevertheless, a recent research conducted in Sri Lanka recommended that, β-thalassemia trait individuals may show symptoms of anemia such as dizziness, fatigue, headache, lethargy and exercise intolerance in spite of having levels of Hb that overlap the average range. Insignificant difference in the rate of recurrence of these symptoms among the two groups with either mild anemia or normal Hb levels was recorded (Premawardhena *et al.,* 2008).

Also, rate of recurrence of infectious incidents in individuals with β-thalassemia trait was significantly increased. Only men with β-thalassemia trait had lower rate of recurrence of advanced coronary artery disease (Tassiopoulos *et al.,* 2005). Similarly, myocardial infarction is common among men with β-thalassemia trait at older age (Tassiopoulos *et al.*, 2005).

2.6.2. Homozygous β-Thalassemia

There is highly inconsistent clinical range for homozygous β-thalassemia patients (Weatherall & Clegg, 2001; Cao & Galanello, 2010). Numerous individuals with homozygous β-thalassemia show severe anemia at the early stage of life and continue to dependent on transfusion for the rest of their lives. These individuals are diagnosed as TM. Others may have anemia of varying degrees and may need transfusion occasionally. These individuals are diagnosed as TI. In TI patients, the level of anemia is said to be from almost usual levels to sufficiently severe anemia (which requires blood transfusion occasionally). Erythroid hyperplasia results to osteoporosis that may be quite severe and medullary expansion with facial deformities (Nienhius & Nathan, 2012). Also, extramedullary hematopoiesis leads to the expansion of the pulmonary masses of erythroid cells, liver, spleen and paraspinal (Nienhius & Nathan, 2012). Conditions for the diagnosis of both the major and the intermedia syndromes are not well defined, but largely the diagnosis is based on the Hb level. Mostly, a cut off of 7g/dl of Hb is used as a range to differentiate the major and intermedia syndromes. Nevertheless this principle is confusing due to related splenomegaly and the severity of the anemia. Also, abnormal development may differ among patients at different times. Wide-ranging environmental factors, action of many secondary and tertiary modifiers contribute remarkably to the phenotypical multiplicity and the heterogeneity of mutations of the β-globin locus of β-thalassemias (Weatherall, 2001). **Figure 2.5.** below shows increasing severity clinical conditions of β-thalassemia.

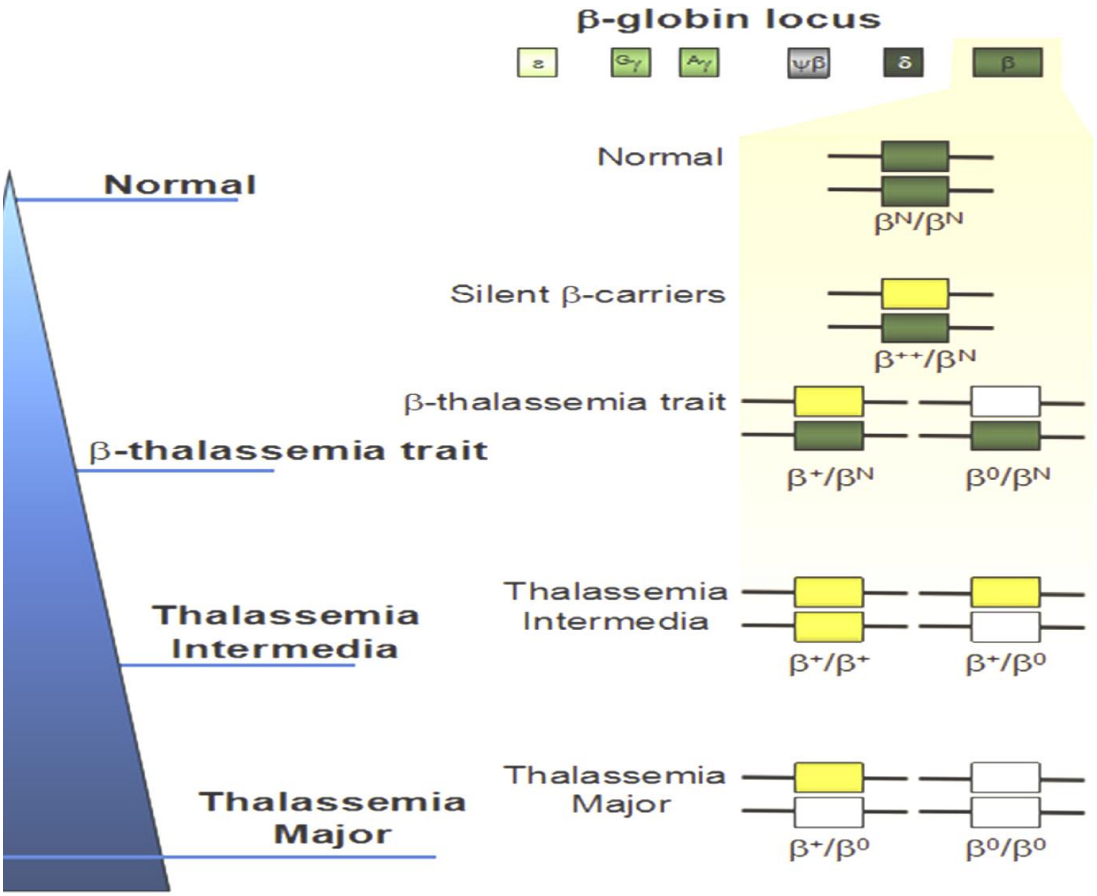

Figure 2.5. β-thalassemia genotype–phenotype correlation and clinical classification (Taken from Shang & Xu, 2017).

2.7. Genetic Modifiers

There is a wide severity in the phenotypes of β-thalassemia which ranges from mild to severe forms. Also, the genotype–phenotype associations of β-globin genes have been pronounced above. Therefore, a wide-ranging phenotypic variability can be seen in individuals that have the same β-thalassemia genotype. This variability in phenotype ranges from mild to severe forms of diseases because of numerous genetic modifiers (associated or not associated to the β-globin locus). Furthermore, Thein (2013) briefly categorized the genetic modifiers basically into two forms: (1) those that acts at the level of the α- and β-chains imbalance known as the primary modifiers and (2) those that acts at the level of the impediments associated to disease and treatment known as the secondary modifiers.

The fundamental pathophysiological mechanisms behind β-thalassemia consist of the extent of imbalance of globin chains and the surplus α-globins. The factors responsible for the reduction in the extent of imbalance would have a substantial effect on the phenotypes (Higgs, 2012). Recognizing these modifiers has a significant role in precisely diagnosing β-thalassemia. The two major groups of modifiers identified are shown in **table 2.4** below.

Table2.4. β-thalassemia genetic modifiers that regulate the synthesis of HbF (modified from Shang & Xu, 2017)

Groups	**Aggravating Factors**	**Ameliorating Factors**
1. Variations that affect HbF synthesis		rs2071348 (A>C) rs766432 (A>C) rs9399137 (T>C) rs11886868 (T>C) rs4895441 (A>G) rs382144 (C>T) *KLF1* (wt/var)
2. α-globin genes copy numbers	α-triplication/α-quadruplication	α-thalassemia mutations

The severity of β-thalassemia can be enhanced by the coinheritance of α-thalassemia which result to lower α-globin synthesis and decreases the damages done to RBCs by free intracellular α-globin. In areas where both α- and β-thalassemia are highly dominant, coinheritance of these thalassemias is common (Weatherall, 2001). The coinheritance of α^0- or α^+-thalassemia (--/αα or -α/αα) which can enhance the severity of patients with β^0/β^0 from TM to TI (Mettananda *et al.*, 2015). Contrarily, coinheritance of α-triplication (αα/ααα) or α-quadruplication (αααα/ααα) can worsen the severity due to the additional α -globin genes in which the synthesis of α-globin is

increased. According to Thein (2013), there would be phenotypic worsening from thalassemia trait to TI when there is coinheritance of heterozygotes for β-thalassemia (β^0/β^N or β^+/β^N). Though, α-triplications carriers are phenotypically normal and therefore, in most populations the occurrence of this variation is not well-known (Thein, 2013).

To modify the clinical severity of β-thalassemia, the synthesis of HbF post birth is an essential factor because the augmented level of γ-globin binds the excess α-globin to form HbF. Several factors found on the β-gene cluster and other locations on other chromosomes are implicated in setting the levels of HbF. A distinguished factor that affects the level of HbF in the β-gene cluster is a polymorphism (C>T) located at position 158 of the G-γ-gene (rs382144) (Khelil, *et al.,* 2011). The polymorphism (C>T) is likewise known as *Xmn*I polymorphism. *Xmn*I polymorphism is relatively common amongst many populations. According to Perkins *et al.,* (2016) *Xmn*I polymorphism seems to exert little effect on individuals that are normal, nevertheless it up-regulates the synthesis of HbF significantly in β^0-thalassemia. In European populations, its genetic impact to the HbF levels is estimated to be around 10%. A polymorphism (A>C) found on the Ψβ gene (rs2071348) has also been reported to improve the levels of HbF, resulting in milder symptoms of β-thalassemia (Giannopoulou *et al.,* 2012).

Similarly, β-thalassemia phenotype is regulated by other factors that control the expression of the γ-gene; these factors also act as genetic modifiers. Data from genome-wide association studies (GWAS) established that two loci unrelated to the β-cluster, that is, *HBS1L–MYB* on 6q23 and *BCL11A* on 2p16, are quantitative trait loci (QTL) that control HbF synthesis. According to Wonkam *et al.,* (2014), Single Nucleotide Polymorphisms (rs4671393, rs6732518, rs766432, rs1427407, rs11886868 and rs7557939) on the *BCL11A* gene were reported to be linked to the levels of HbF in different populations or F-cell numbers. In non-anemic North Europeans, genetic influence is estimated to be around 15% (Menzel *et al.,* 2007) and in Americans of African descent effected by sickle cell disease, it was estimated to be around 7–12% (Menzel & Thein, 2009). The C-allele of rs11886868 is significantly related to increased levels of HbF and is expressed significantly in TI diagnosed patients more than in TM diagnosed patients of Sardinian origin (Uda *et al.,* 2008). The rs766432 "C" allele is related with increased levels of HbF/F-cells in Chinese patients (Sedgewick *et al.,* 2008). Likewise, single nucleotide polymorphisms (rs4895441, rs1320963, and rs9399137) in the *HBS1L–MYB* intergenic region

were reported to be related to HbF synthesis in diverse populations (So *et al.,* 2008), with a genetic influence of about 19% and 3–7% in Europeans and African Americans respectively. Lately, the systematical analysis of the severity of thalassemia in patients that coinherited *KLF1* variants were performed (Yu *et al.,* 2015). The special impacts of *KLF1* variants on hematologic indices of thalassemia patients and individuals that are normal are briefly listed in **table 2.5** below. The relationship between *KLF1* variations and elevated HbA_2 and levels of HbF in carriers of α- and β-thalassemia has been recognized. It was recommended that co-inherited *KLF1* variation ($KLF1^{wt/var}$) possibly could lead to the increase in the synthesis of HbF, which likewise enhances the severity of the clinical phenotype of β-thalassemia (Liu *et al.,* 2014).

Table 2.5. Hematological phenotype of thalassemia and the Impact of *KLF1* Variants on HbF synthesis (Modified from Shang & Xu, 2017).

	Genotype	**Effect of co-inheritance with *KLF1*$^{(wt/var)}$**
Normal	αα/αα, $\beta^N\beta^N$	MCH↓, MCV↓, HbA_2↑, HbF↑
α-thalassemia	-α/αα, β^N/β^N	MCV↓, MCH↓, HbA_2↑, HbF↑
	$\alpha^T\alpha/\alpha\alpha$, β^N/β^N	
	--/-α, -$\alpha^T\alpha$	effects observed were insignificant
β-thalassemia	αα/αα, $\beta^0\beta^N$	HbA_2↑, HbF↑
	αα/αα, $\beta^0\beta^0$	*KLF* is a potent genetic modifier that ameliorate severity

2.8. Laboratory Diagnosis of β-Thalassemia

Laboratory results are used in evaluating a concluding diagnosis in presence of high HbF value by examining the globin genes thereby selecting the correct reference intervals. HPFH diagnosis and δβ-thalassemia syndromes alongside a minimum set of other laboratory tests, HbF measurement has been a tool. A flowchart (**Figure 2.6**) has been deduced summarizing other most significant measurements apart of HbF levels. These hematological indices include; mean corpuscular hemoglobin (MCH), MCV and markers of iron deficiency status.

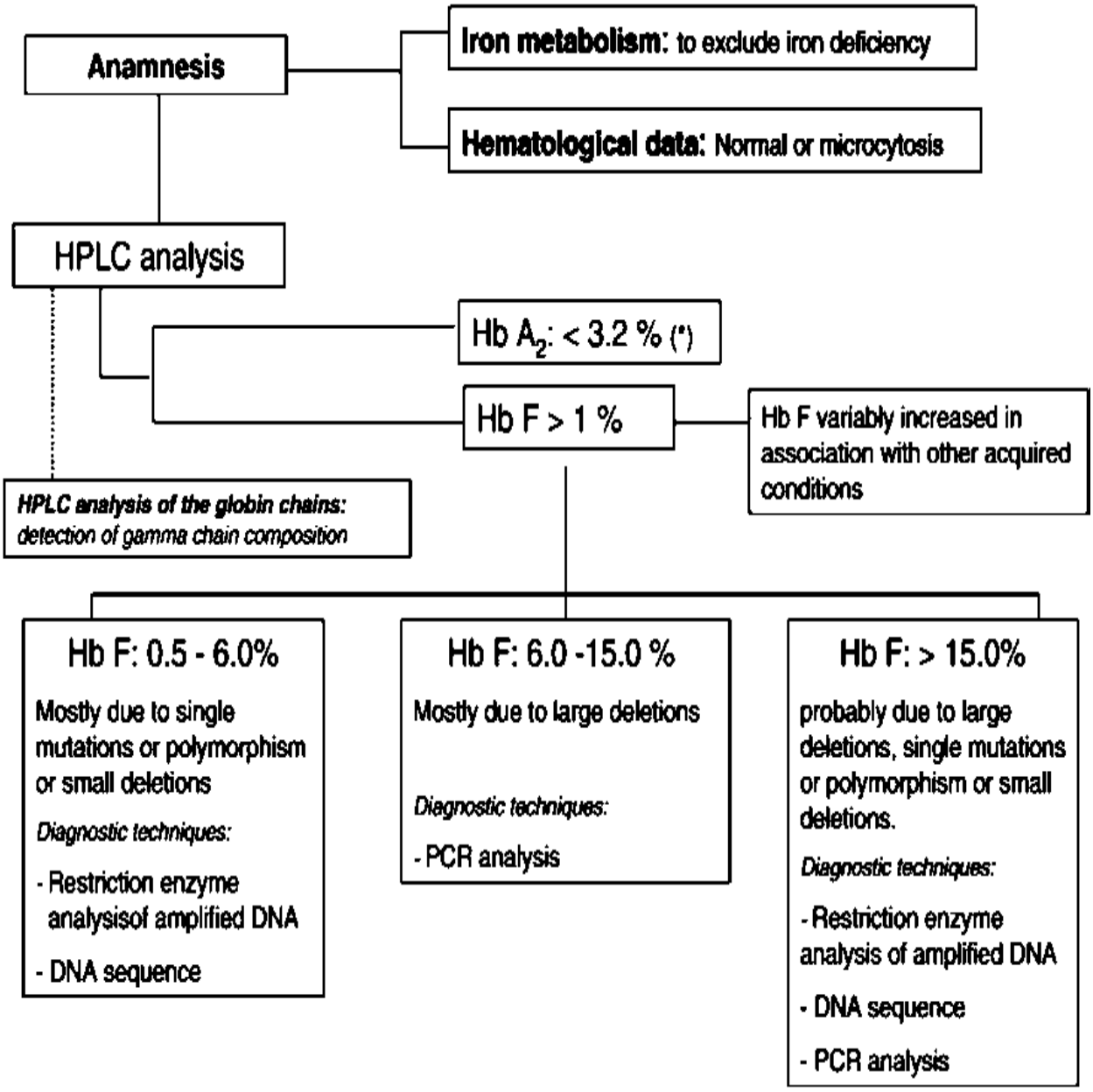

Figure 2.6 Showing the likely genetic causes of increased levels of HbF and the diagnostic methodologies for molecular characterization and quantification steps (Taken from Andrea *et al.*, 2009).

3.0. EVIDENCE

Numerous pre-analytical factors can affect the levels of HbF in the blood, of which most are reported. Elevated levels of HbF can be attributed to defects in γ-genes, acquired conditions, or defects in other genes. Generally, the levels of HbF have been associated generally with the most essential hematological disorders such as anemia, hypochromia, microcytosis (Andrea *et al.*, 2009).

According to Andrea *et al.*, 2009, the unit for the measurement of HbF expression levels is the comparative percentage (%) on total Hb. Although, it is not an SI unit, it is well recognized and regularly used all over the world.

Table 3.1. RBC Indices in β-thalassemia (Modified from Galanello *et al.*, 1979).

RBC Index	**Normal**		**Affected**	**Carrier**
	Male	**Female**	**β-Thalassemia Major**	**β-Thalassemia Minor**
Hb (g/dL)	15.9 ± 1.0	14.0 ± 0.9	<7	Males:11.5–15.3 Females: 9.1–14
MCV (fl)	89.1 ± 5.01	87.6 ± 5.5	50 – 70	<79
MCH (pg)	30.9 ± 1.9	30.2 ± 2.1	12 – 20	<27

• The RBCs morphologic changes of microcytosis, anisocytosis, hypochromia, nucleated RBCs (i.e., erythroblasts) and poikilocytosis (spiculated tear-drop and elongated cells) are demonstrated in affected individuals. The degree of anemia is distinctly elevated following splenectomy is related with the number of erythroblasts.

• Carriers show decreased MCH, MCV (**Table 3.1.**), and in affected individuals RBCs morphologic changes that are less severe.

Table 3.2. Hb patterns in β-thalassemia (Age >12 Months). β^0-thalassemia: lack of β-globin chain synthesis completely; β^+-thalassemia: flexible degree of decrease of β-globin chain production (Modified from Telen & Kaufman, 1999).

Hb	**Normal**	**Affected**	**Carrier**	
		B^0-Thalassemia Homozygotes 2	**β^+-Thalassemia Homozygotes or β^+/β^0 Compound Heterozygotes 3**	**β -Thalassemia Minor**
HbA	96–98%	0	10–30%	92–95%
HbA_2	2–3%	2–5%	2–5%	>3.5%
HbF	<1%	95–98%	70–90%	0.5–4%

The amount and type of Hb present has been identified by the quantitative and qualitative analiysis of Hb (cellulose acetate electrophoresis and DE-52 microchromatography or HPLC). In β-thalassemia, the following types Hb are of relevance. The Hb pattern in β-thalassemia differs by β-thalassemia type as shown in the **table 3.2** above with HbF having the highest percent.

Table 3.3. Hematological and hemoglobinical data of HbF in control subjects and thalassemic patients (Modified from Fabrizio *et al.*, 2002).

Group patients	**No of cases**	**Hb g/dl**	**HbF%**
Sub-silent β-thalassemia. Intermedia	33	10.1 ± 1.0	20.5 ± 18.2
Evident β-thalassemia intermedia	41	8.3 ± 1.16	33.40 ± 28.04
β-thalassemia Intermedia in double heterozygous β-tha+ triplicated alpha	58	10.0 ± 1.38	5.15 ± 3.9
HbH disease	15	9.4 ± 1.2	< 1
Thalassemia major	24	10.4 ± 1.0	7.7 ± 8.5***
Healthy non-thalassemia subjects	53	14.4 ± 1.1	<1
Iron-deficient non-thalassemia subjects	42	8.7 ± 1.2	<1

In 74 patients, investigation of the hematological data and the clinical symptoms were carried out. Patients having sub-silent β-thalassemia intermedia have decreased total Hb content showing very mild or no clinical symptoms. Patients having evident β-thalassemia intermedia showed severe clinical symptoms, which are often related with substantial splenomegaly (and 41 percent of them have undergone splenectomy). Also, the Hb content is considerably lesser when compared to patients with sub-silent β-thalassemia intermedia ($t = 7.44$; gl 72; $p < 0.001$). In patients with evident β-thalassemia intermedia, the level of HbF is higher than in patients with sub-silent β-thalassemia intermedia, and the statistical difference is significant ($t = 2.28$; gl 72; $p < 0.05$).

Table 3.4. The rs11886868 genotypes distribution in patients with β-thalassemia, HPFH subjects and the general population from Ogliastra (Modified from Uda *et al.,* 2008).

	Frequency				**P-value genotypic vs allelic test**			
	n Individuals	C/C	C/T	T/T	HPFH	TI	TM	Sardinia
HPFH	66	0.227	0.546	0.227	-	0.987	3.29×10^{-7}	2.15×10^{-16}
TI	52	0.192	0.597	0.211	0.847	-	2.91×10^{-6}	1.23×10^{-12}
TM	74	0.040	0.355	0.635	1.72×10^{-6}	6.49×10^{-6}	-	0.963
Sardinia	1,412	0.040	0.316	0.644	8.52×10^{-13}	3.16×10^{-12}	0.969	-

Values on the left hand side are genotype frequencies for each group. Also, the upper and lower diagonal on the right represents chi square p-values for the allelic and genotypic tests in that order, between the indicated groups of individuals.

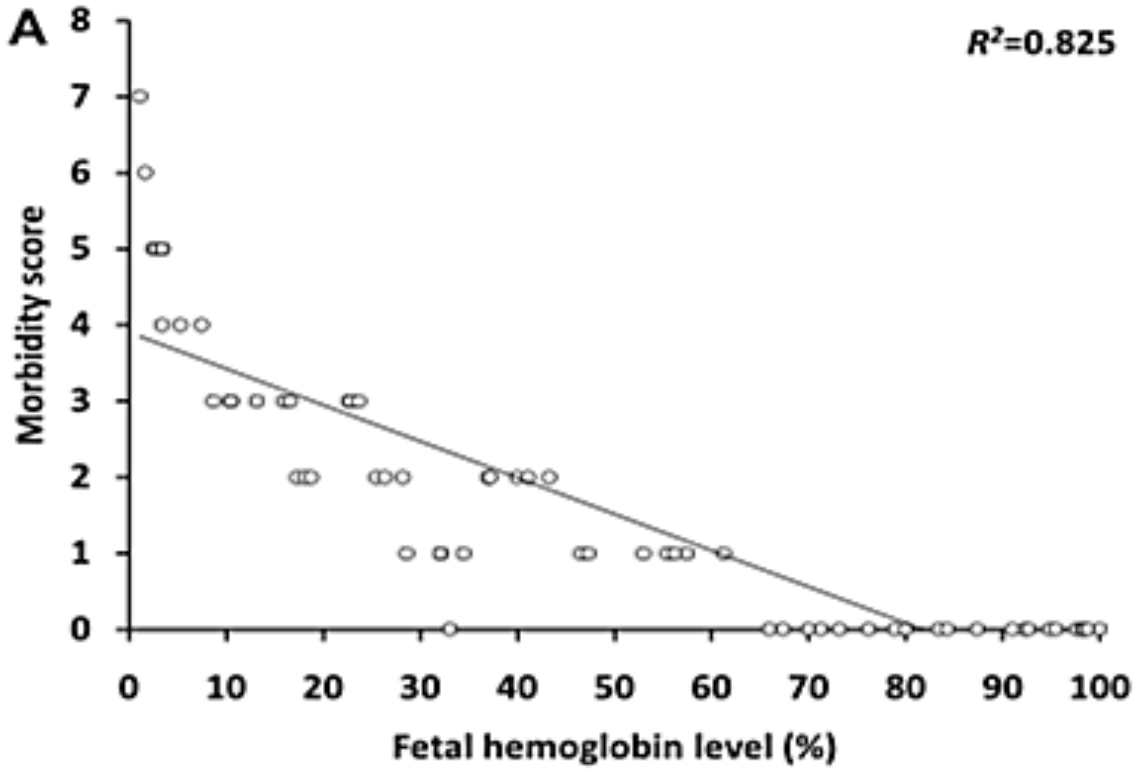

Figure 3.1. Linear regression plot of fetal hemoglobin against morbidity score (Taken from Musallam *et al.*, 2012).

Table 3.5. HbF and HbA_2 values ranging from minimum to maximum in normal infants through the first 2 years (Modified from Andrea *et al.*, 2009).

Age (month)	**N**	**HbF%**	**HbA%**	**HbA_2%**
At birth	1250	58–84	15–40	0–1
1–3	29	29–61	38–70	0.5–1.5
3–5	85	9–40	65–90	1.3–2.1
5–8	50	3–15	83–95	1.6–2.6
8–12	89	1–10	89–96	1.8–2.9
12–24	222	0.5–3.0	94–97	1.9–3.0
>25	3550	0.1–1.2	95–98	2.0–3.3

By measuring Hb fraction in not less than 100 adults who are not iron deficient, and are neither α nor β-thalassemia carriers, all laboratory experts are hypothetically in control of determining their own reference interval values of the HbF. In cases of HbF measurement, the lower values are usually found in subjects that do not have any thalassemia syndromes whereas, the upper limit values of subjects that are normal are often reported. Therefore, as a result of other variations in the arrangement of the laboratory analytical techniques and characteristics of the native populace as regards to iron deficiency or α thalassemia carrier, some laboratories might get slightly different values. HbF level reaches a stable state with respect to proper management during the first year of life, and after the second year HbF reaches adult levels as stated in **table 3.5** above.

Table 3.6. Population group and Frequency of the *Xmn*I polymorphism (-158 C>T) showing the levels of HbF in healthy populations and individuals having hemoglobinopathies (Modified from Rev *et al.,* 2011).

Population group	**Frequency of *Xnm*I polymorphism**	**HbF levels**	**Phenotype expressed**
Hong Kong	14%	0.22±0.03%	Heterozygote β-thalassemia
Caucasian	14%	0.34–1.07%	Healthy
Iran	39–41%	NA	Intermedia β-thalassemia
Iran	70.5%	NA	Major β-thalassemia
Indian	25%	NA	Intermedia and major β-thalassemia
Saudi Arabia(SW)	3.3%	9.3±5.8%	Sickle cell disease
Saudi Arabia (East)	93.2%	11.3±6.2%	Sickle cell disease
Brazil	33.3%	15.48±11.69%	Without complains of anemia

NA–not available; SW–southwest

Table 3.7. Parameters associated with *Xmn*I polymorphism in 51 patients according to independent t-test and Chi-square test (Modified from Majid & Tayyebeh, 2015).

Parameter	*Xmn*I (CC)	*Xmn*I (CT)	P value
Hb (gm/dl)	7.94 ± 1.34	9.58 ± 1.25	0.001
HbF (%)	70.32 ± 40.56	89.30 ± 21.87	0.04
Splenectomy (%)	7 (43.8)	15(42.9)	0.59

Of 51 patients, 35 (68.6%) patients were heterozygous (CT) and 16 (31.4%) patients were homozygous (CC).Of 30 patients under treatment by hydroxyurea, 20 (66.7%) patients were heterozygous (CT) and 10 (33.3%) patients were homozygous (CC). These results demonstrated that in the heterozygous (CT) genotype, the Hb (9.58 ± 1.25 gm/dl) and HbF (89.30 ± 21.87%) levels were significantly higher in comparison with homozygous (CC) genotype (7.94 ± 1.34 gm/dl and , respectively. Furthermore, it was observed that after drug usage, the Hb and HbF levels in patients with heterozygous (CT) genotype (0.7 ± 1.26 gm/dl and 5.95 ± 14.8, respectively) raised more in comparison with homozygous (CC) genotype (0.26 ± 1.43 gm/dl and 0.8 ± 1.31, respectively).

Table 3.8. Effect of hydroxyurea treatment on the Hb and HbF levels of β-thalassemia patients (Modified from Majid & Tayyebeh, 2015)

Mean level	Before treatment	After treatment	P value
Hb* (g/dl)	n = 20(CT), n = 10(CC) 8.47 ± 1.36	n = 20(CT), n = 10(CC) 9.01 ± 1.52	0.03
HbF**	n = 15(CT), n = 7(CC) 81.87 ± 26.95	n = 10(CT), n = 6(CC) 86.11 ± 28.00	0.32

Table 3.9. HbF values of the clinical phenotypes of β-thalassemia (Modified from Teralı *et al.*, 2016).

β-thalassemia	**n**	**Median**	**Mean**	**SD**	**95%cl**
TI	27	15.3	22.1	19.31	12.37-26.25
TM	45	3.0	4.67	4.12	3.71-5.63

A total number of 72 patients were diagnosed of β-thalassemia, 27 were β-thalassemia Intermedia (TI) and 45 were β-thalassemia Major (TM). High level of HbF was found among the TI group which decreased transfusion requirements and ameliorate the severity of clinical phenotype in favour of TI over TM group.

4.0. TREATMENT AND MANAGEMENT

In patients with β-thalassemia particularly non-transfusion dependent thalassemia (NTDT), the clinical morbidities detected may involve several organs and organ systems (Musallam *et al.*, 2012). Also, when there is no proper management the occurrence of these morbidities gets high as age advances. Likewise, the multiplicity of disease in NTDT patients has direct influence on the quality of life patients (Musallam *et al.*, 2011). This surveillance highlights the significance of timely management and prevention in this patient populace. Currently there are no available recommendations for the management of NTDT patients; nevertheless, due to emerging data from current studies together with expert opinion generally help put forward a management structure for NTDT patients.

4.1. Transfusion

Whether and when to initiate transfusion therapy is one among the foremost difficult therapeutic choices for β-thalassemia patients (Borgna-Pignatti, 2007; Taher *et al.*, 2011). β-thalassemia major patients depends on transfusions for survival. For other patients, transfusion may occur rarely or occasionally as shown in **table 4.1.** below. For instance, occasional transfusion therapy may be suitable for β-thalassemia patients that are not transfusion-dependent which provides healthy erythrocytes and reduces the occurrence of ineffective erythropoiesis (Fuchroen & Weatherall, 2012). NTDT patients should also be closely monitored for triggers of regular transfusion, as delayed transfusion can lead to growth retardation, puberty delay, thalassemic facies and hypersplenism.

Table 4.1. Clinical requirements for occasional, intermittent or chronic transfusions in patients with β-thalassemia (Modified from Cappellini *et al.*, 2014).

Transfusion frequency	**Diseases**	**Clinical requirement**
Chronic	β-Thalassemia major or severe HbE/β-thalassemia Severe β-thalassemia intermedia	-Patients require regular blood transfusions for survival. -Frequent blood transfusion may become necessary when symptoms are severe.
Intermittent	Moderate -thalassemia intermedia or HbE β-thalassemia	-Patient requires transfusion as a result of specific clinical features and symptoms. -Reduction in Hb with profound splenomegaly. -Growth failure. -Failure of secondary sexual development. -Poor quality of life. -Patient requires transfusion for the prevention/ management of complications. -Thrombotic or cerebrovascular disease. -Pulmonary hypertension.
Occasional	Mild β-thalassemia intermedia or HbE/ β-thalassemia	-Patients may require a one-off blood transfusion on the occurrence of a specific event. -Anticipated acute stress and/or blood loss (e.g., pregnancy, surgery, infection). -Hb decrease.

Furthermore, when initiating and continuing transfusion in patients with TI or mild/moderate HbE/ β-thalassemia, a number of considerations should be taken into account. Unnecessarily frequent transfusions should be avoided in such patients. The levels of Hb can fluctuate in non-transfusion-dependent patients upon diagnosis, and therefore, patients should be followed carefully over several months before deciding on what treatment should be given to such patients. Quality of life must also be considered as patients can survive and even thrive with an Hb level around 7 g/dl particularly those with HbE/ β-thalassemia (Olivieri, 2012). It is essential that patients are frequently reexamined, following initiation of transfusion, this will help to determine whether continued transfusion is necessary or not.

Due to transfusion, a number of complications can occur. These include iron overload and related complications such as liver, cardiac, and endocrine problems. Additionally, alloimmunization may occur, whereby the recipient mounts an immune response to donor antigens, leading to various clinical consequences.

4.2. Splenectomy

Hypersplenism may be as a result of huge numbers of cells being pooled and destroyed in the spleen's reticuloendothelial system and hemodilution which leads to an elevation in the plasma volume. Therefore, spleen size should be carefully examined in all patients with β-thalassemia.

Many patients with TM require splenectomy. This should be performed in specific, defined clinical circumstances including splenic enlargement accompanied by left upper quadrant pain or early satiety, or leucopenia or thrombocytopenia due to hypersplenism. However, good clinical management practice may delay or prevent hypersplenism, reducing the need for splenectomy (Piga *et al.,* 2011). In contrast, among patients with TI or mild/moderate HbE/β-thalassemia, splenectomy should not be the first management option if others are available as it is associated with multiple adverse outcomes (Rodeghiero & Ruggeri, 2012). Splenectomy ought to be avoided in NTDT patients younger than 5 years of age. In general, splenectomy should be reserved for very specific patients such as; those with worsening anemia resulting to reduced growth and development; where transfusion and iron chelation are not possible or available; and in cases of hypersplenism or splenomegaly/massive splenomegaly.

4.3. Iron overload/Chelation

Patients with transfusion-dependent TM, iron overload occurs due to accumulation of iron from transfusions and, to a lesser extent, increased intestinal absorption. Equally, iron overload in patients with TI or mild/moderate HbE/ β-thalassemia is as a result of increased intestinal absorption secondary to ineffective erythropoiesis and, to a much lesser extent, accumulation of iron from transfusions (Ginzburg & Rivella, 2011).

Iron overload is related with an increased likelihood of various complications. In TM patients, iron overload is fatal early in life, usually due to cardiac failure if left untreated (Zurlo *et al.,* 1989). Notably, access to cardiac MRI techniques has resulted to a substantial decrease in mortality as a result of cardiac iron overload in recent years. However, as the management of cardiac disorders is improving, liver damage is coming to the forefront, and deaths from hepatic complications are increasing relative to other iron overload-related conditions (Chouliaras *et al.,* 2011). Among patients with TI or mild/moderate HbE/ β-thalassemia, there is generally an absence of cardiac siderosis irrespective of liver iron concentration (LIC), and the most common complications include extramedullary hematopoiesis, osteoporosis, cholelithiasis and hypogonadism (Taher *et al.,* 2011). Iron load should be frequently monitored in β-thalassemia patients and iron load values should be used to guide treatment decisions, including initiation and cessation of chelation and dose escalation (Angelucci *et al.,* 2000).

4.4. HbF Induction

In patients with β-thalassemia, reducing agents such as hydroxyurea can be used to increase the synthesis of γ-globin which is a β-globin-like molecule. This binds to α-chains to produce HbF, thus addressing the imbalance in globin chains. This, also, reduces the occurrence of ineffective erythropoiesis, decreases hemolysis and increases total Hb. There is, unfortunately, a lack of randomized clinical trials investigating the efficacy of hydroxyurea treatment. Although there are data available from a large number of single-arm trials or retrospective analyses of hydroxyurea therapy, patient numbers are small and results have not been consistently reproduced (Musallam *et al.,* 2013). In patients with TM, the fraction of patients who are no longer dependent on transfusion after treatment varies greatly between studies from up to 25–80%. About 20–50% of

patients exhibit a 'partial response' whereby transfusion requirements are reduced. Improvements in transfusion requirements are also associated with a reduction in iron overload and hemolytic indices (Musallam *et al*., 2013).

In TI, responses vary greatly and study end points differ according to the severity of the disease before treatment. In a small study of TI patients who were previously transfusion-dependent as a result of the severity of the disease, eight of nine patients showed a good response about > 70% reduction in transfusion requirements (Bradai *et al.,* 2007).

Hb increases of > 10 g/dl were observed in around 60–70% of patients in studies of non-transfusion-dependent TI patients, although this was not always maintained during a follow-up of more than 12 months. Hydroxyurea treatment is associated with a decreased incidence of many of the morbidities associated with this disease (Rigano *et al.,* 2010).

It is suggested that a modulator of HbF is trialed where there is no immediate need for transfusion or splenectomy, and no indication that Hb levels will drop suddenly. If level of Hb drop suddenly, then transfusion prior to splenectomy might be more appropriate.

4.5. Future Treatment Options

Several new molecules and treatment strategies are currently in development, some of which show some promise for β-thalassemia treatment. Gene therapy has been trialed in several exploratory studies, with the aim of transferring β-globin in stem cells to decrease the α-β disproportion in erythroid cells, ultimately resulting to transfusion independence (Yannaki *et al.,* 2013). In one experimental clinical trial, a patient (adult) with severe HbE/β-thalassemia dependent on monthly transfusions since early infancy became transfusion-independent for 21 months following gene therapy (Cavazzana *et al.,* 2010).

Janus kinase 2 (Jak2) inhibitors have also been investigated for β-thalassemia treatment as they may regulate the excessive production of immature erythroid cells in thalassemia, hypothetically reversing extramedullary hematopoiesis (Rivella, 2009). Based on the accessible preclinical evidence, Jak2 inhibitors are anticipated to reduce the occurrence of splenomegaly, transfusion requirements and perhaps iron overload in TI patients, though clinical data are not yet available (Rivella, 2012).

Lastly, techniques that will be helpful for the correction of anemia without transfusions have been explored, including sotatercept (angiotensin-converting enzyme [ACE]-011) and ACE-536, modified activin type IIa or IIb receptor fusion proteins. These proteins inhibit signaling induced by some members that usually transform growth factor β super family, promoting maturation of terminally differentiating erythroblasts (Cappellini *et al.,* 2013).

5.0. DISCUSSION

Basically, the severity of morbidity in the clinical phenotypes of β-thalassemia molecularly is dependent on the degree of the α-globin and non α-globin subunits imbalance that constitute the Hb molecule. β-thalassemia intermedia (TI); the relatively mild clinical phenotype of β-thalassemia is associated with higher HbF levels whereas, β-thalassemia major (TM); the severe clinical phenotype of β-thalassemia is associated with lower levels of HbF.

Teralı *et al.,* (2016) reported a range of HbF levels between 12.37–26.25% for TI patients with a mean HbF level of 22.1% and a range of HbF levels between 3.71–5.63% for TM patients with a mean HbF level of 4.67% (**Table 3.9**) in a study conducted on β-thalassemia patients of Turkish-Cypriot origin. They were able to establish a negative correlation between the levels of HbF and the total number of transfusions in β-thalassemia patients suggesting that TI patients who require less frequent transfusions have higher levels of HbF while the TI patients who require more frequent transfusions have lower levels of HbF. High level of HbF is suggested to clinically improve the clinical phenotypes of β-thalassemia by decreasing the dependence on transfusion (Teralı *et al.,* 2016).

In a genome wide association study conducted by Uda *et al.,* (2008), the C-allele of rs118868686 polymorphism on the *BCL11A* gene known to be strongly associated with high levels of HbF was found to be significantly more frequent in TI patients than in β-thalassemia major patients (**Table 3.4**). This is an indication that that the variants of the rs11886868 polymorphism on the *BCL11A* gene carrying the C-allele play a vital role in ameliorating the clinical phenotype of β-thalassemia by increasing the levels of HbF.

Although the mechanism of action for the regulation of HbF levels by *BCL11A* gene is yet to be clearly understood, it is speculated that the *BCL11A* gene binds to the regulatory regions on the γ-globin gene cluster thereby playing a regulatory role in Hb switch and subsequently determining the relative levels of HbF and HbA being synthesized (Quek &Thein, 2007).

High levels of HbF have been reported to be linked with decreased rate of mortality and morbidity in β-thalassemia as well as in sickle cell anemia patients (Platt *et al.,* 1994; Platt *et al.,* 1991; Musallam *et al.,* 2012). In a study carried out by Musallam *et al.,* (2012) to evaluate the relationship between the level of HbF and the morbidity in TI patients, a strong negative linear

correlation was established between HbF level and the morbidity score suggesting that higher levels of HbF ameliorates the clinical phenotype of the TI patients (**Figure 3.1**).

In a study carried out to analyse the hematological data and the clinical symptoms in β-thalassemia patients by Fabrizio *et al.,* (2002), HbF level was found to higher in evident β-thalassemia intermedia patients than in sub-silent β-thalassemia intermedia patients. Heterozygous β-thalassemia patients with inherited triplication of the α-globin gene had even much lower level of HbF (**Table 3.3**) suggesting that higher level of HbF is associated with the milder clinical phenotype of β-thalassemia.

β-thalassemia is the most common monogenic disease in humans. Genetic and non-genetic factors such as (C→T) polymorphism and administration of hydroxyurea have been stated to influence γ-globin gene expression and the severity of clinical symptoms of β-thalassemia (Miller *et al.,* 1988). *Xmn*Iγ-G affects Hb and HbF levels only in erythropoietic stress conditions (Sampietro *et al.,* 1992). Some studies have reported that there is no association between the presences of T allele at this site and the reduction of the clinical symptoms in TI patients (Neishabury *et al.,* 2010; Miller *et al.,* 1987).

In a study by Majid & Tayyebeh (2015), to determine the association between *Xmn*Iγ-G polymorphism and Hb/HbF levels and the effects of hydroxyurea on TI patients in Isfahani population were studied by the Tetra-Primer ARMSPCR technique). The frequency of T allele at the *Xmn*I polymorphic site has been reported differently in various populations, varying from 10–76.9% (Miller *et al.,* 1987; Arab *et al.,* 2011). However, the frequency of T allele at *Xmn*I polymorphic site in 51 patients with TI was found 34% in the study. Different studies have proved that the existence of T allele at *Xmn*I polymorphic site is associated with an increased amount of total Hb and HbF in intermediate β-thalassemia patients (Arab *et al.,* 2011; Gibney *et al.,* 2008). The presence of T allele in *Xmn*I polymorphic site reduces the binding of transcription silencers to the γ-globin gene promoter. Therefore, the γ-globin gene is reactivated in adult life in erythropoietic stress conditions (Bank, 2006; Schechter, 2008). Several studies have shown that there is a significant association between the occurrence of T allele at *Xmn*I polymorphic site and increased amount of HbF and even reduction of severity of clinical symptoms in patients (Qatanani *et al.,* 2000; Akbari *et al.,* 2008; Hamid *et al.,* 2009; Haj *et al.,* 2010; Chinelato *et al.,* 2011). However, some other studies have indicated that there is no association between the

presence of T allele at this site and increased HbF level. It has also been reported that there is no correlation between the presence of T allele at this site and the reduction of clinical symptoms in TI patients (Neishabury *et al.*, 2010). In line with the majority of the first group, it was found that the levels of Hb and HbF are significantly increased in the presence of T allele at the *Xmn*I polymorphic site. These different results in various studies could be caused by the complexity of gene regulation pathways for γ-globin gene expression and also HbF levels (Neishabury *et al.*, 2010). Hydroxyurea is a chemical agent that may increase Hb and HbF levels. This effect can be exerted through γ-globin expression, and which is associated with allele at the *Xmn*I polymorphic site. The study by Majid &Tayyebeh (2015) has shown the frequency of *Xmn*I polymorphic site in 51 patients with TI that was determined, and its correlation with levels of Hb and HbF was analyzed (**Table 3.5**). The results indicated that in the presence of T allele at *Xmn*I polymorphic site, the Hb and HbF levels were increased. In addition, the association between *Xmn*I γ-G polymorphism and the effect of hydroxyurea was studied. In the current investigation, it has been demonstrated that in the patients carrying T allele, Hb and HbF levels are increased statistically, and they also response to hydroxyurea treatment better than patients without the T allele (**Table 3.6**).

6.0. CONCLUSION

Thalassemia is known to be the commonest cause of inherited anemia worldwide. This study aimed at investigating the interrelationship between HbF levels and the clinical phenotypes of β-thalassemia has shown that a negative linear correlation exits between the levels of HbF and the severity of morbidity in the clinical phenotypes of the β-thalassemia. Higher HbF levels are correlated with TI which is the milder clinical phenotype whereas, lower levels of HbF are associated with TM which is the severe clinical phenotype of β-thalassemia.

Treatment with hydroxyurea of β-thalassemia patients induces the synthesis of γ-globin chains thereby increasing the level of HbF has been proved to be promising in the management of β-thalassemia patients. Clinical trials involving sufficiently large number of patients should be carried out to validate the effectiveness of hydroxyurea in β-thalassemia treatment. On the other hand, more research should be carried out in the field of gene therapy aimed at gene modification for the treatment of β-thalassemia. With respect to the current advances in understanding the expression of HbF, the mechanisms underlying this observation should be evaluated. This could lead to other potential therapeutic interventions.

REFERENCES

Akbari, M.T., Izadi, P., Izadyar, M., Kyriacou, K. & Kleanthous, M. (2008) Molecular basis of thalassemia intermedia in Iran. Hemoglobin, 32(5), pp.462-470.

Andre, M., Renata, P., Daniel, L. & Giovanni, I. (2009) The relevance of hemoglobin F measurement in the diagnosis of thalassemia and related hemoglobinopathies.Clinical biochemistry. 42,1797-1801.

Angelucci, E., Brittenham, G.M., Mclaren, C.E., Ripalti, M., Baronciani, D., Giardini, C., Galimberti, M., Polchi, P. & Lucarelli, G. (2000) Hepatic iron concentration and total body iron stores in thalassemia major. New England Journal of Medicine, 343(5), pp.327-331.

Antonini, E. & Brunori, M. (1971) Haemoglobin and Myoglobin in Their Reactions with Ligands. North-Holland, Amsterdam, Pp. 1-45.

Arab, A., Karimipoor, M., Rajabi, A., Hamid, M., Arjmandi, S. & Zeinali, S. (2011) Molecular characterization of β-thalassemia intermedia: a report from Iran. Molecular biology reports, 38(7), pp.4321-4326.

Bank, A., (2006) Regulation of human fetal hemoglobin: new players, new complexities. Blood, 107(2), pp.435-443.

Bauer, D. E. & Orkin, S. H. (2015) Hemoglobin switching's surprise: the versatile transcription factor *BCL11A* is a master repressor of fetal hemoglobin. Current Opinion in Genetic Development. 33, 62-70.

Bernard, G., Forget, & Frankling, B. (2016) Gold Spring Harbor Perspectives in Medicine: Haematology Division, Department of Medicine. Brigham and women Hospital,Harvard Medical School, Boston Massachusettes.022115.http://perspectiveinmedicinecship.org/.

Borgna-Pignatti, C. (2007) Modern treatment of thalassemia intermedia. Bristish Journal of Haematology. 138, 291–304.

Bradai, M., Pissard, S., Abad, M.T., Dechartres, A., Ribeil, J.A., Landais, P. & De Montalembert, M. (2007) Decreased transfusion needs associated with hydroxyurea therapy in Algerian patients with thalassemia major or intermedia. Transfusion, 47(10), pp.1830-1836.

Cappellini, M. D., Cohen, A. & Eleftheriou, A. (2008) Guidelines for the Clinical Management of Thalassemia. 2nd edition. Thalassemia International Federation.

Cappellini, M.D., Viprakasit, V. & Taher, A.T. (2014). An overview of current treatment strategies for β-thalassemia. Expert Opinion on Orphan Drugs, 2(7), pp.665-679.

Cavazzana-Calvo, M., Payen, E., Negre, O., Wang, G., Hehir, K., Fusil, F., Down, J., Denaro, M., Brady, T., Westerman, K. & Cavallesco, R. (2010) Transfusion independence and HMGA2 activation after gene therapy of human β-thalassaemia. Nature, 467(7313), p.318.

Chen, W., Zhang, X., Shang, X., Cai, R., Li, L., Zhou, T., Sun, M., Xiong, F. & Xu, X. (2010) The molecular basis of beta-thalassemia intermedia in southern China: genotypic heterogeneity and phenotypic diversity. BMC medical genetics, 11(1), p.31.

Chernecky, C. C. (2003) Laboratory Tests and Diagnostic Procedures. Philadelphia, PA: Elsevier—Health Sciences Division.

Chinelato, I.S., Carrocini, G.C.D.S. & Bonini-Domingos, C.R. (2011) *Xmn*I polymorphism frequency in heterozygote beta thalassemia subjects and its relation to Fetal hemoglobin levels. Revista brasileira de hematologia e hemoterapia, 33(6), pp.483-483.

Chouliaras, G., Berdoukas, V., Ladis, V., Kattamis, A., Chatziliami, A., Fragodimitri, C., Karabatsos, F., Youssef, J. & Karagiorga-Lagana, M. (2011) Impact of magnetic resonance imaging on cardiac mortality in thalassemia major. Journal of Magnetic Resonance Imaging, 34(1), pp.56-59.

Chui, D. H. (2005) Alpha-thalassemia: Hb H disease and Hb Barts hydrops fetalis. Annals of New York Academy Science. 1054, 25-32.

Crispino, J. D. & Weiss, M.J. (2014) Erythro-megakaryocytic transcription factors associated with hereditary anemia. Blood. 123, 3080-8.

Dailey, H. A. & Meissner P. N. (2013) Erythroid heme biosynthesis and its disorders. Cold Spring Harbor Perspective in Medicine. doi: 10.1101/cshperspect.a011676.

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

Fischback, F. T., Lippincott, W. & Wilkins (2004) Manual of Laboratory and Diagnostic Tests: Foetal hemoglobin in normally healthy adults: relationship with polymorphic sequence cis to the β-globin gene. European Journal of Human Genetics. 10, 320-326.

Forget, B. G. & Bunn, H. F. (2016) Classification of the clinical disorders of hemoglobin. Cold harbor perspective medicine. 3, 011684.

Franco, R. S., Yasin, Z., Palascak, M. B., Ciraolo, P., Jointer, C. H. & Rucknagel, D. L. (2006) The effect of fetal hemoglobin on the survival characteristics of sickle cells. Blood. 108(3), 1073–6.

Fucharoen, S. & Weatherall, D.J. (2012) The hemoglobin E thalassemias. Cold Spring Harbor Perspective in Medicine. 2(8), a011734.

Galanello, R. & Cao, A. (2011) Gene test review. Alpha-thalassemia. Genetics in Medicine. 13, 83-8.

Galanello, R., Melis, M.A., Ruggeri, R., Addis, N., Scalas, M.T., Maccioni, L., Furbetta, M., Angius, A., Tuveri, T. & Cao, A. (1979) β°-Thalassemia Trait in Sardinia. Hemoglobin, 3(1), pp.33-46.

Giannopoulou, E., Bartsakoulia, M., Tafrali, C., Kourakli, A., Poulas, K., Stavrou, E.F., Papachatzopoulou, A., Georgitsi, M. & Patrinos, G.P. (2012) A single nucleotide polymorphism in the HBBP1 gene in the human β-globin locus is associated with a mild β-thalassemia disease phenotype. Hemoglobin, 36(5), pp.433-445.

Gibney, G.T., Panhuysen, C.I., So, J.C., Ma, E.S., Ha, S.Y., Li, C.K., Lee, A.C., Li, C.K., Yuen, H.L., Lau, Y.L. & Johnson, D.M. (2008) Variation and heritability of Hb F and F-cells among β-thalassemia heterozygotes in Hong Kong. American journal of hematology, 83(6), pp.458-464.

Ginzburg, Y. & Rivella, S. (2011) Beta-thalassemia. A model for elucidating the dynamic regulation of ineffective erythropoiesis and iron metabolism. Blood. 118(16), 4321-30.

Hamid, M., Mahjoubi, F., Akbari, M.T., Arab, A., Zeinali, S. & Karimipoor, M. (2009) Molecular analysis of γ-globin promoters, HS-111 and 3′ HS1, in β-thalassemia intermedia patients associated with high levels of HbF. Hemoglobin, 33(6), pp.428-438.

Higgs, D. R., Engel, J. D. & Stamatoyannopoulos, G. (2012) Thalassemia. Lancet; 379, 373-83.

Jennifer, W. (2015) Diagnosis and management of thalassemia: Paediatrics and child health. Haematology symposium. 25(8), 360-366.

Jensen, F. B., Fago, A. & Weber, R. E. (1998) Hemoglobin structure and function. In: Perry, S. F. & Tufts, B. L. (eds.) Fish Respiration. Academy press, San Diego, pp. 1-40.

Khelil, A.H., Morinière, M., Laradi, S., Khelif, A., Perrin, P., Chibani, J.B. & Baklouti, F. (2011) *Xmn*I polymorphism associated with concomitant activation of G-γ and A-γ globin gene transcription on a β^0-thalassemia chromosome. Blood Cells, Molecules, and Diseases, 46(2), pp.133-138.

Klocke, R. A. (1988). Velocity of CO_2 exchange in blood. Annual Review of Physiology. 50, 625-637.

Koolman, J. & Roehm, K., (2005) Color Atlas of Biochemistry. 2nd edition, Revised and Enlarged. Thieme, Stuttgart. p 280.

Lal, A., Goldrich, M. L. & Haines, D. A. (2011) Heterogeneity of hemoglobin disease in childhood. New England Journal of Medicine. 364, 710-8.

Liu, D., Zhang, X., Yu, L., Cai, R., Ma, X., Zheng, C., Zhou, Y., Liu, Q., Wei, X., Lin, L. & Yan, T. (2014) Erythroid Krüppel-like factor mutations are relatively more common in a thalassemia endemic region and ameliorate the clinical and hematological severity of β-thalassemia. Blood, pp.blood-2014.

Majid, M. & Tayyebeh, G. (2015) Role of XmnIγ^{G} polymorphism in hydroxyurea treatment and fetal hemoglobin levels at Isfahanain patients with beta thalassemia intermedia. Iranian biomedical journal. 19(3), 177-180.

Masuda, T., Wang, X., Maeda, M., Canver, M.C., Sher, F., Funnell, A.P., Fisher, C., Suciu, M., Martyn, G.E., Norton, L.J. & Zhu, C. (2016) Transcription factors *LRF* and *BCL11A* independently repress expression of fetal hemoglobin. Science, 351(6270), pp.285-289.

Menzel, S. & Thein, S.L. (2009) Genetic architecture of hemoglobin F control. Current Opinion in Hematology. 16, 179-86.

Menzel, S., Garner, C., Gut, I., Matsuda, F., Yamaguchi, M., Heath, S., Foglio, M., Zelenika, D., Boland, A., Rooks, H. & Best, S. (2007) A QTL influencing F cell production maps to a gene encoding a zinc-finger protein on chromosome 2p15. Nature genetics, 39(10), p.1197.

Mettananda, S., Gibbons, R.J. & Higgs, D.R. (2015) Alpha-Globin as a molecular target in the treatment of beta-thalassemia. Blood. 125, 3694-701.

Miller, B.A., Olivieri, N., Salameh, M., Ahmed, M., Antognetti, G., Huisman, T.H., Nathan, D.G. & Orkin, S.H. (1987) Molecular analysis of the high-hemoglobin-F phenotype in Saudi Arabian sickle cell anemia. New England journal of medicine, 316(5), pp.244-250.

Miller, S. A., Dykes, D. D. & Polesky, H. F. (1988) A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Resource. 16(3), 1215.

Miller, S.A., Dykes, D.D. & Polesky, H.F.R.N. (1988) A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic acids research, 16(3), p.1215.

Modell, B. & Darlison, M. (2008) Global epidemiology of hemoglobin disorders and derived service indicators. Bull World Health Organisation.86, 480-7.

Mollan, T. L., Khandros, E., Weiss, M. J. & Olson, J. S. (2012) The kinetics of a-globin binding to a hemoglobin stabilizing protein (AHSP) indicate preferential stabilization of a hemichrome folding intermediate. Journal of Biological Chemistry. 287, 11338–11350.

Musallam, K. M., Taher, A. T. & Rachmilewitz, E. A. (2012) Beta-Thalassemia Intermedia. A Clinical Perspective. Cold Spring Harbor Perspective in Medicine. 2(7), a013482.

Musallam, K.M., Khoury, B., Abi-Habib, R., Bazzi, L., Succar, J., Halawi, R., Hankir, A., Koussa, S. & Taher, A.T. (2011) Health-related quality of life in adults with transfusion-

independent thalassaemia intermedia compared to regularly transfused thalassaemia major: new insights. European journal of haematology, 87(1), pp.73-79.

Musallam, K.M., Taher, A.T., Cappellini, M.D. & Sankaran, V.G. (2013) Clinical experience with fetal hemoglobin induction therapy in patients with β-thalassemia. Blood, pp.blood-2012.

Neishabury, M., Azarkeivan, A. & Najmabadi, H. (2010) Frequency of Positive XmnIγG polymorphism and coinheritance of common alpha thalassemia mutations do not show statistically significant difference between thalassemia major and intermedia cases with homozygous IVSII-1 mutation. Blood Cells Molecular Diseases. 44(2), 95-9.

Nelson, D. L. & Cox, M. M. (2008) Lehninger principles of biochemistry. 5th edition. W. H. Freeman and company, New York, USA, pp. 10,13, 52, 87.

Nienhuis, A. W. & Nathan, D. G. (2012) Pathophysiology and clinical manifestations of the β-thalassemias. Cold Spring Harbor Perspective Medicine. doi a01172.

Oberoi, S., Das, R., Panigrahi, I., Kaur, J. & Marwaha, R.K. (2011) *Xmn*I-G γ polymorphism and clinical predictors of severity of disease in β-thalassemia intermedia. Pediatric Blood Cancer. 57(6), 1025-8.

Oberoi, S., Das, R., Panigrahi, I., Kaur, J. & Marwaha, R.K. (2011) *Xmn*I-Gγ polymorphism and clinical predictors of severity of disease in β-thalassemia intermedia. Pediatric blood & cancer, 57(6), pp.1025-1028.

Olivieri, N. (2012) Treatment strategies for hemoglobin E beta-thalassemia. Blood Review. 26(Suppl 1), 28-30.

Pace, B.S., Liu, L., Li, B. & Makala, L.H. (2015) Cell signaling pathways involved in drug-mediated fetal hemoglobin induction: Strategies to treat sickle cell disease. Experimental biology and medicine, 240(8), pp.1050-1064.

Perkins, A., Xu, X., Higgs, D.R., Patrinos, G.P., Arnaud, L., Bieker, J.J. & Philipsen, S. (2016) " Krüppeling" erythropoiesis: an unexpected broad spectrum of human red blood cell disorders due to *KLF1* variants unveiled by genomic sequencing. Blood, pp.blood-2016.

Perutz, M. F. (1970) Stereochemistry of cooperative effects in hemoglobin. Nature. 228, 726-739.

Peter, J. K. & Victor, W. R. (2009) Proteins: Myoglobin and Hemoglobin. In. Robert, K. M., David, A. B., Kathleen, M. B., Peter, J. K., Victor, W. R. & Anthony, P. W. (eds.) Harper's illustrated biochemistry. 28th edition. McGraw-Hill publishers, New York, p.45.

Piel, F.B. & Weatheral, D.J. (2014) The alpha-thalassemias. New England Journal of Medicine. 371, 1908-16.

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), pp.808-810.

Platt, O. S. (2008) Hydroxyurea for the treatment of sickle cell anemia. New England Jorunal of Medicine. 358, 1362–9.

Porter, J., Origa, R., Forni, G.L., Laadem, A., Galacteros, F., Miteva, D., Sung, V., Chopra, R., Arlet, J.B., Ribeil, J.A. & Klesczewski, K. (2013) A phase 2a, open-label, dose-finding study to determine the safety and tolerability of sotatercept (ACE-011) in adults with beta (β)-thalassemia: interim results.

Premawardhena, A., Arambepola, M., Katugaha, N. & Weatherall, D. J. (2008) Is the β-thalassaemia trait of clinical importance? British Journal Haematology. 141, 407–410.

Qatanani, M., Taher, A., Koussa, S., Naaman, R., Fisher, C., Rugless, M., Old, J. & Zahed, L. (2000) β-Thalassaemia intermedia in Lebanon. European journal of haematology, 64(4), pp.237-244.

Quek, L. & Thein, S.L. (2007) Molecular therapies in β-thalassaemia. British journal of haematology, 136(3), pp.353-365.

Rev, B. H. H. (2011) What influences Hb Fetal production in adulthood. 33(3), 231-6.Doi:10.5581/1516.8484.

Rigano, P., Pecoraro, A., Calzolari, R., Troia, A., Acuto, S., Renda, D., Pantalone, G.R., Maggio, A. & Marzo, R.D. (2010) Desensitization to hydroxycarbamide following long-term treatment of thalassaemia intermedia as observed in vivo and in primary erythroid cultures from treated patients. British journal of haematology, 151(5), pp.509-515.

Riggs, A. F. (1988). The Bohr effect. Annual Review of Physiology. 50, 181-204.

Rivella, S. (2009) Ineffective erythropoiesis and thalassemias. Current Opinion in Hematology. 16(3), 187-94.

Rivella, S. (2012) The role of ineffective erythropoiesis in non-transfusion-dependent thalassemia. Blood Review. 26(Suppl 1), 12-15.

Rodeghiero, F. & Ruggeri, M. (2012) Short- and long-term risks of splenectomy for benign haematological disorders: Should we revisit the indications? British Journal of Haematology. 158(1), 16-29.

Sampietro, M., Thien, S. L. & Pazmany, L. (1992) Variation of HbF and F-cells number with G-gamma *Xmn*I polymorphism (C>T) in normal individuals. Blood. 79(3),832-833.

Sankaran, V.G. & Weiss, M.J. (2015). Anemia: progress in molecular mechanisms and therapies. Nature medicine, 21(3), p.221.

Sankaran, V.G., Menne, T.F., Xu, J., Akie, T.E., Lettre, G., Van Handel, B., Mikkola, H.K., 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), pp.1839-1842.

Satyanarayana, U. & Chackrapani, U. (2009) Hemoglobin and porphyrins. In:Satyanarayana U.(eds) Biochemistry. 3rd edition. Nagarajuna Nagar, Mahanadu Road, Vijayawada, India. Pp 196-220.

Schechter, A. N. (2008) Hemoglobin research and origins of molecular medicine. Blood. 112, 39–38.

Schechter, A. N. (2013) Hemoglobin functions. Cold Spring Harbor Perspective Medicine. doi a011650.

Sebia, (2015) Hemoglobinopathies: Hemoglobin variants and thalassemias, a growing public health problem. Available from: http://www.sebia.com/enEN/produits/hemoglobin-disorders [accessed 20th December, 2017].

Sedgewick, A.E., Timofeev, N., Sebastiani, P., So, J.C., Ma, E.S., Chan, L.C., Fucharoen, G., Fucharoen, S., Barbosa, C.G., Vardarajan, B.N. & Farrer, L.A. (2008) *BCL11A* is a major HbF quantitative trait locus in three different populations with β-hemoglobinopathies. Blood Cells, Molecules, and Diseases, 41(3), pp.255-258.

Shang, X. & Xu, X. (2017). Update in the genetics of thalassemia: What clinicians need to know. Best Practice & Research Clinical Obstetrics & Gynaecology, 39, pp.3-15.

Shang, X., Rao, Z., Lou, J., Li, W., Xiong, F., Zhou, H.P. & Xu, X. (2011) Compound heterozygosity for a rare small deletion and a common point mutation in the beta-globin gene: report of two Chinese families. International journal of laboratory hematology, 33(1), pp.79-84.

So, J., Song, Y.Q., Tsang, S., Tang, L.F., Chan, A., Ma, E. & Chan, L.C. (2008) The *HBS1L-MYB* intergenic region on chromosome 6q23 is a quantitative trait locus controlling foetal haemoglobin level in beta thalassaemia carriers. Journal of medical genetics.

Suzuki, M., Yamazaki, H., Mukai, H.Y., Motohashi, H., Shi, L., Tanabe, O., Engel, J.D. & Yamamoto, M. (2013) Disruption of the Hbsl1-Myb locus causes hereditary persistence of fetal hemoglobin in mouse. Molecular and cellular biology, pp.MCB-01617.

Taher, A.T., Musallam, K.M., Karimi, M., El-Beshlawy, A., Belhoul, K., Daar, S., Saned, M.S., El-Chafic, A.H., Fasulo, M.R. & Cappellini, M.D. (2010) Overview on practices in thalassemia

intermedia management aiming for lowering complication rates across a region of endemicity: the OPTIMAL CARE study. Blood, 115(10), pp.1886-1892.

Tangvarasittichai, S. (2011) Thalassemia Syndrome. In. Kenji, I. (Ed.) Advances in the Study of Genetic Disorders, ISBN: 978-953-307-305-7, InTech. Available from:http://www.intechopen.com/books/advances-in-the-studyof-genetic-disorders/thalassemia-syndrome. [Accessed 13th October, 2017].

Tassiopoulos, S., Deftereos, S., Konstantopoulos, K., Farmakis, D., Tsironi, M., Kyriakdis, M. & Aessopos, A. (2005) Does heterozygous β-thalassemia confera protection against coronary artery disease? Annals of New York Academy of Science. 1054, 467–470.

Telen, M.J. & Kaufman, R.E. (1999) The mature erythrocyte. In: Lee GR, Paraskevas F, Foerster J, Lukens J, eds. Wintrobe's Clinical Hematology. 10 ed. Baltimore, MD: Lippincott Williams & Wilkins.pp207.

Terali, K., Ozkan, O. C., Cetin, O., Ugurlu, K. C. & Akdeniz, E. (2016) The contribution of elevated foetal haemoglobin levels to a relatively mild clinical phenotype in Turkish Cypriot patients with beta thalassemia. Federation of European Biochemical Society Journal. 283, 103-103.

Thein, S.L. (1999) Is it dominantly inherited β thalassaemia or just a β-chain variant that is highly unstable? British journal of haematology, 107(1), pp.12-21.

Thein, S. L. (2004) Genetic insights into the clinical diversity of β -thalassemia. Britrish Journal of Haematology. 124(3), 264-74.

Thein, S. L. (2013) The molecular basis of beta-thalassemia. Cold Spring Harbor Perspective Medicine. 3, a011700.

Thein, S. L., Stephens, A. D. & Okpala, I. (2004) The genetics and multiple phenotypes of beta thalassaemia, and The diagnosis and significance of alpha thalassaemia: Practical Management of Haemoglobinopathies. Blackwell Publishing Ltd. 26-39 and 40-44.

Thein, S.L., Menzel, S., Lathrop, M. & Garner, C. (2009) Control of fetal hemoglobin: new insights emerging from genomics and clinical implications. Human molecular genetics, 18(R2), pp.R216-R223.

Thom, C. S., Dickson, C. F., Gell, D. A. & Weiss, M. J. (2013) Hemoglobin variants. Biochemical properties and clinical correlates. Cold Spring Harbor Perspective Medicine. doi:10.1101/cshperspect.a011858.

Uda, M., Galanello, R., Sanna, S., Lettre, G., Sankaran, V.G., Chen, W., Usala, G., Busonero, F., Maschio, A., Albai, G. & Piras, M.G. (2008) Genome-wide association study shows *BCL11A* associated with persistent fetal hemoglobin and amelioration of the phenotype of β-thalassemia. Proceedings of the National Academy of Sciences, 105(5), pp.1620-1625.

Weatherall, D. J. & Clegg, J. B. (2001) The thalassemia syndromes, 4th ed. Blackwell Science, Oxford.

Weatherall, D. J., (2001) Phenotype-genotype relationships in monogenic disease: lessons from the thalassaemias. Nature Review Genetics. 2:245-55.

White, J. M., Richards, R., Byrne, M., Buchanan, T., White, Y. S. & Jelenski, G. (1985) Thalassemia trait and pregnancy. Journal in Clinical Pathology. 38, 810–817.

Wonkam, A., Bitoungui, V.J.N., Vorster, A.A., Ramesar, R., Cooper, R.S., Tayo, B., Lettre, G. & Ngogang, J. (2014) Association of variants at *BCL11A* and *HBS1L-MYB* with hemoglobin F and hospitalization rates among sickle cell patients in Cameroon. PloS one, 9(3), p.e92506.

Yannaki, E., Karponi, G., Zervou, F., Constantinou, V., Bouinta, A., Tachynopoulou, V., Kotta, K., Jonlin, E., Papayannopoulou, T., Anagnostopoulos, A. & Stamatoyannopoulos, G. (2013) Hematopoietic Stem Cell Mobilization for Gene Therapy: Superior Mobilization by the Combination of Granulocyte–Colony Stimulating Factor Plus Plerixafor in Patients with β-Thalassemia Major. Human gene therapy, 24(10), pp.852-860.

Yu, L.H., Liu, D., Cai, R., Shang, X., Zhang, X.H., Ma, X.X., Yan, S.H., Fang, P., Zheng, C.G., Wei, X.F. & Liu, Y.H. (2015) Changes in hematological parameters in α-thalassemia individuals co-inherited with erythroid Krüppel-like factor mutations. Clinical genetics, 88(1), pp.56-61.

Zhou, D., Liu, K., Sun, C. W. (2010) *KLF1* regulates *BCL11A* expression and gamma- to beta-globin gene switching. Nature Genetics. 42, 742-4.

Zurlo, M., De Stefano, P., Borgna-Pignatti, C., Di Palma, A., Melevendi, C., Piga, A., Di Gregorio, F., Burattini, M. & Terzoli, S. (1989) Survival and causes of death in thalassaemia major. The Lancet, 334(8653), pp.27-30.