GENETIC POLYMORPHISM IN HIGH HbF ANEMIC PREGNANT WOMEN AND THE EFFECT OF STEM CELL FACTOR AND ERYTHROPOIETIN-TREATED K562 CELLS ON ERK SIGNALING OF MAPK PATHWAY

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by

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TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF ABBREVIATIONS

AIHA	Autoimmune hemolytic anemia
ARMS	Amplification Refractory Mutation System
ARMS-PCR	Amplification Refractory Mutation System-PCR
ATCC	American Type Culture Collection
BCL11A	B-cell lymphoma/leukemia 11A
BMT	Bone marrow Transplantation
BSA	Bovine serum albumin
CBC	Complete blood counts
CDC	Centers for Disease Control
cDNA	Complementary DNA
CKD	Chronic kidney disease
CNVs	Copy Number Variants
CREB1	cAMP Response Element Binding Protein 1
DMSO	Dimethyl Sulfoxide
DNMT	DNA Methyl-Transferase
EB	Elution Buffer
EGFR	Epithelial Growth Factor Receptor
EKLF	Erythroid Krüppel-like factor
EPO	Erythropoietin
ERK1 and 2	Extracellular signal-regulated kinases 1 and 2
FBS	Fetal bovine serum
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
gap-PCR	gap polymerase chain reaction
GWAS	Genome-wide association studies
Hb	Hemoglobin

HbA	Adult hemoglobin
HbF	Fetal hemoglobin
HbH	Hemoglobin H
HbS	Sickle hemoglobin
HIV	Human Immunodeficiency Virus
HMIP	HBS1L-MYB Intergenic Region
HPFH	Hereditary persistence of fetal hemoglobin
HPLC	High-Performance Liquid Chromatography
HSCs	Hematopoietic Stem Cells
HU	Hydroxyurea
HUSM	Hospital Universiti Sains Malaysia
HWE	Hardy–Weinberg Equilibrium
IDA	Iron deficiency anemia
JAK/STAT	Janus kinase/signal transducers and activators of transcription
KLF1	Kruppel-like factor 1
LCR	Locus Control Region
MAF	Minor Allele Frequency
MAPK	Mitogen-Activated Protein Kinase
MCH	Mean Corpuscular Hemoglobin
MCHC	Mean Corpuscular Hemoglobin Concentration
MCV	Mean Corpuscular Volume
MLPA	Multiplex ligation-dependent probe amplification
mRNA	Messenger RNA
NIH	National Institutes of Health
O&G	Obstetrics and Gynecology
O_2	Oxygen
PBS	Phosphate-buffered saline

PCR	Polymerase Chain Reaction
PSG	Penicillin-Streptomycin with glutamine
qPCR	Quantitative Polymerase Chain Reaction
QTL	Quantitative Trait Loci
RBC	Red blood cell
RPMI	Roswell-Park Memorial Institute medium
RT-qPCR	Quantitative Real-Time Polymerase Chain Reaction
SCA	Sickle Cell Anemia
SCF	Stem Cell Factor
SEM	Standard error of the mean
SNP	Single Nucleotide Polymorphism
TBE	Tris-Borate-EDTA
TBST	Tris-buffered saline with Tween 20
WHO	World Health Organization

LIST OF APPENDICES

GENETIK POLIMORFISMA PADA ANEMIA HbF TAHAP TINGGI DALAM WANITA HAMIL DAN KESAN FAKTOR SEL STEM DAN ERITROPOIESIS-TERAWAT SEL K562 PADA ISYARAT ERK LALUAN MAPK

ABSTRAK

Anemia adalah salah satu keadaan biasa berlaku pada wanita hamil disebabkan oleh perolehan atau pewarisan genetik. Kecelaruan hemoglobin (Hb) termasuk hemoglobin fetus (HbF) yang disebabkan oleh polimorfisma nukleotida tunggal (SNP) telah dilaporkan boleh menyebabkan anemia semasa hamil. Beberapa lokus genetik, termasuk polimorfisma DNA pada *BCL11A*, *HMIP-2*, dan *XmnI*,mempunyai pengaruh yang signifikan terhadap tahap HbF. Selain itu, sel manusia leukemia myelogenous kronik K562 mempunyai fenotip embryo HbF dan digunakan secara meluas sebagai model bagi saringan penggalak HbF. Pembezaan sel K562 dikaitkan dengan peningkatan ekspresi gen globin embryo-fetal seperti gen γ-globin. Krüppel-like factor 1 (*KLF1*), *BCL11A*, and *MYB* memainkan peranan dalam regulasi genγ-globin. Oleh itu, kajian ini ingin melihat hubungan antara tahap HbF dan polimorfisme DNA dalam *BCL11A* rs1186868, rs6545816, dan rs1427407, *HMIP-2* rs9376090, dan *XmnI* rs7482144 pada wanita hamil yang mengalami anemia di Hospital Universiti Sains Malaysia. Kajian ini melibatkan 164 wanita hamil yang mengalami anemia (Hb < 11 g/dl) dan 27% mempunyai tahap HbF yang tinggi (> 1%). HPLC digunakan untuk menentukan tahap HbF dan HbA2. Multiplex ARMS-PCR dan gap-PCR digunakan untuk mengesan mutasi dan pemotongan pada gen β-globin di dalam 44 sampel yang mempunyai tahap HbF yang tinggi. Daripada 22 sampel, 15 sample mempunyai mutasi pada gen β-globin manakala tiada mutasi pada gen *δδ*β-globin dikesan. Sampel

tanpa mutasi ditentukan genotip dengan diskriminasi alel pada *BCL11A* rs1186868, *HMIP-2* rs9376090, dan *XmnI* rs7482144 dengan menggunakan PCR Real-time, manakala penjujukan DNA Sanger dilakukan untuk *BCL11A* rs6545816 dan *BCL11A* rs1427407. Berdasarkan SNPs yang dikaji, alel G pada *BCL11A* rs1186868 SNP mungkin merupakan alel penggalak untuk HbF. Tambahan pula, tidak terdapat perbezaan yang signifikan pada tahap HbF dan genotip pada *HMIP-2* rs9376090, *XmnI* rs7482144, *BCL11A* rs6545816, dan *BCL11A* rs1427407 SNP. Selain itu, faktor tumbesaran seperti faktor sel stem (SCF) dan erythropoietin (EPO) memainkan peranan penting dalam proses penghasilan darah, dan bahagian kedua kajian ini bertujuan untuk mengenalpasti kesan faktor-faktor ini kepada tahap mRNA γ-globin, protein *BCL11A*, *KLF1*, dan ERK melalui laluan MAPK ke atas sel K562. Sel K562 didedahkan selama 24 jam dengan EPO, SCF, atau kombinasi keduanya dan ekspresi mRNA γ-globin, protein *BCL11A*, *KLF1*, dan laluan MAPK (ERK dan pERK) ditentukan. Kombinasi SCF dan EPO mampu menambahkan ekspresi gen γ-globin. Tambahan pula, faktor-faktor ini dilihat mampu menggalakkan ekspresi gen *BCL11A* dan *KLF1* secara berbeza, walaupun melalui laluan yang sama iaitu laluan MAPK. Kesimpulannya, tahap HbF yang tinggi dalam wanita hamil yang mengalami anemia tidak berkitan dengan polimorfisme genetik *BCL11A*, *HMIP-2*, dan *XmnI* dan faktor tumbesaran seperti SCF dan EPO berpotensi untuk digunakan dalam pengurusan hemoglobinopati melalui laluan MAPK.

GENETIC POLYMORPHISM IN HIGH HbF ANEMIC PREGNANT WOMEN AND THE EFFECT OF STEM CELL FACTOR AND ERYTHROPOIETIN-TREATED K562 CELLS ON ERK SIGNALING OF MAPK PATHWAY

ABSTRACT

Anemia is one of the most common conditions in pregnant women due to acquired or genetic dysregulation. Dysregulation of hemoglobin (Hb) including fetal hemoglobin (HbF) due to single-nucleotide polymorphism (SNP) have been reported to cause anemia in pregnancy. Several genetic loci, including DNA polymorphisms at B-cell lymphoma 11A (*BCL11A)*, *HMIP-2*, and *XmnI*, have a significant influence on HbF levels. K562 human chronic myelogenous leukemia cells, on the other hand, have an embryonic HbF phenotype and are widely used as a screening model for HbF inducers. K562 cell differentiation is associated with an increase in the expression of embryo-fetal globin genes such as γ-globin genes. Krüppel-like factor 1 (*KLF1*), *BCL11A*, and *MYB* regulate the γ-globin gene. Hence, this study intended to determine the association of HbF level and DNA polymorphism at *BCL11A* rs1186868, rs6545816, and rs1427407, *HMIP-2* rs9376090, and *XmnI* rs7482144 in anemic pregnant women visiting Hospital Universiti Sains Malaysia. In this study, a total of 164 anemic pregnant women (Hb <11 g/dl) were recruited and 27% exhibited high level of HbF (> 1%). High-performance liquid chromatography was used to determine the HbF and HbA2 levels. Multiplex ARMS-PCR and gap-PCR were used to detect mutations and deletion at the β-globin gene cluster in 44 samples with high HbF levels respectively. From 22 samples, 15 mutations were detected at the β-globin gene while no mutation at the δβ-globin gene. Samples without these mutations were genotyped for allelic discrimination for *BCL11A*

rs1186868, *HMIP-2* rs9376090, and *XmnI* rs7482144 by using real-time PCR, while Sanger DNA sequencing was performed for the *BCL11A* gene at rs6545816 and rs1427407. Based on SNPs, allele G at SNP *BCL11A* rs1186868 could possibly contribute as HbF-promoting alleles. Furthermore, there was no significant difference in HbF levels between genotypes for the SNPs *HMIP-2* rs9376090, *XmnI* rs7482144, *BCL11A* rs6545816, and *BCL11A* rs1427407. On the other hand, growth factors such as stem cell factor (SCF) and erythropoietin (EPO) are important for erythropoiesis, and the second part of this study was aimed to elucidate the effect of these growth factors on the expression of γ-globin mRNA levels,*BCL11A*, *KLF1*, and the ERK of the mitogen-activated protein kinase (MAPK) pathway, on the K562 cell line. K562 cells were treated for 24 hours with SCF, EPO, or a combination of both and expression of γ-globin mRNA, *BCL11A* and *KLF-1* as well as theexpression of the MAPK pathway (ERK and pERK) were determined. A combination of SCF and EPO treatment increased γ-globin expression. Furthermore, these growth factors were demonstrated to promote *BCL11A* and *KLF1* protein expressions differently, although via the same signaling pathway which is the MAPK pathway. In conclusion, highHbF levels in anemic pregnant women was not associated by *BCL11A*, *HMIP-2*, and *XmnI* genetic polymorphisms and growth factors such as SCF and EPO has the potentialto be included in management of hemoglobinopathies through its promoting effect via MAPKpathway.

CHAPTER 1

INTRODUCTION

1.1 Background of the study

Hemoglobin (Hb) is biologically in charge of carrying oxygen (O_2) from the lungs to peripheral tissues and is present in high amounts in erythrocytes (S. Jorge et al., 2018a). The four polypeptide subunits that make up human Hb each contain a heme molecule that binds to the O_2. In healthy individuals, Hb consists of adult hemoglobin (HbA) (α2β2), HbA2 (α2δ2), and fetal hemoglobin (HbF) (α2γ2) which represents 96%, < 3.5%, and < 1%, respectively (Al-Amodi et al., 2018). During gestation, HbF containing the γ-globin predominates while a transition in gene expression from γ-globin to β-globin during birth replaces HbF and at six months of age, the primary type of Hb is HbA (Wang & Thein, 2018).

HbF is a tetramer of two adult α-globin polypeptides and two fetal γ-globin polypeptides (Pang, van Weerd, Hamoen, & Snyder, 2022). HbF levels, which are controlled by a number of genes and are influenced by the environment, are largely responsible for reducing the morbidity and mortality of the major congenital Hb diseases, such as sickle cell anemia (SCA) (Okeke, Silas, Nnodu, & Clementina, 2022). Three major quantitative trait loci (QTLs), the *XmnI*, *HMIP-2* intergenic region on chromosome 6q23, and B-cell lymphoma/leukemia 11A (*BCL11A*) on chromosome 2p16, were identified through genome-wide association studies (GWAS). These QTLs account for 20% to 50% of the typical HbF variability in SCA and β-thalassemia (Bhanushali, Patra, Nair, Verma, & Das, 2015).

Thalassemia, SCA, severe anemia, and hereditary persistence of fetal hemoglobin (HPFH) have all been linked to high HbF during pregnancy. *XmnI* polymorphism (Basak & Sankaran, 2016), B-cell lymphoma/leukemia 11A (*BCL11A*), and *HMIP-2* are a few variables that may increase HbF levels (Vinjamur et al., 2016). Kruppel-like factor 1 (*KLF1*), a transcription factor specific to erythroids, has been postulated as a pro-*BCL11A* expression regulator in both mouse models and human cells (Basak & Sankaran, 2016).

HbF is present mainly during fetal and neonatal life but gradually disappears until the first year after birth. However, HbF levels may vary depending on the γ-globin gene mutation or differences in γ-globin arrangement (Martin & Thein, 2013). Additionally, γ-globin levels range from 5 to 30% of total Hb in newborns (Martin & Thein, 2013).

The *XmnI* polymorphism, also known as the sequence mutation (C-T) at position 158 in the globin gene, predisposes to increased HbF production in maturity, especially in settings of erythropoietic stress, as seen in β-thalassemia or SCA (Grosso et al., 2008). It has been previously reported that patients with HbF have multiple *XmnI* polymorphic sites approximately 60% more than normal individuals (Carrocini, Zamaro, & Bonini-Domingos, 2011a). Hence, this research investigated the relationship between high HbF and hematological parameters, as well as the presence of *BCL11A* rs1186868, *BCL11A* rs6545816, *BCL11A* rs1427407, *HMIP-2* rs9376090, and *Xmn1* rs7482144 in anemic pregnant women with acquired causes.

The K562 cells were first isolated from a 53-year-old female's pleural effusion. The cells are proteomically related to undifferentiated granulocytes and erythrocytes

(Bharatkumar, 2018). To better understand the processes involved in erythroid differentiation, K562 cells have been used extensively (Li et al., 2012). For assessing the *in vitro* cytotoxicity or cell survival of various drugs on K562 cells, various techniques are available (Bharatkumar, 2018).

Human cancer cells rely on the mitogen-activated protein kinase (MAPK) cascade for survival, spread, and resistance to pharmacological therapy (Siwei et al., 2019). Numerous biological processes, such as cell survival and proliferation, differentiation, and programmed cell death, are regulated by these active genes (Elghobashy, Assar, Mahmoud, Eltorgoman, & Elmasry, 2020). Although there has been evidence of interaction between cAMP-dependent and MAPK pathways in nonerythroid cells, MAPKs don't seem to be dependent on cAMP-dependent processes to control HbF. Erythroid cells have been used in a number of studies to examine the role of MAPKs in HbF production (Gift, Shaheen, Nicolas, Charles & Ambroise, 2015).

It has been demonstrated that a number of therapeutic substances, including the chemotherapy drug hydroxyurea (HU), increase the level of γ-globin and HbF (Khan, Ali & Musharraf, 2020). The main way that HU has an impact is through causing a dose-dependent increase in HbF. As a result of reactivating the fetal γ-globin gene and producing HbF with anti-sickle action, HU is crucial in reducing the symptoms of SCA (Zhu et al., 2017). For optimal erythropoiesis, growth factors including erythropoietin (EPO) and stem cell factor (SCF) are crucial. Important growth hormones EPO and SCF bind to EpoR and c-Kit, respectively, to control erythroid progenitor proliferation, survival, and differentiation (Aguilar, Lopez-Marure, Jiménez-Sánchez, & Rocha-Zavaleta, 2014). EPO is a hematological cytokine that affects the synthesis of red blood cells (RBCs) (Kaddam, 2017). EPO also

promotes the differentiation, survival, and proliferation of erythroid precursors (Lavrinenko, Zinabadinova, Chaikovsky, Sokurenko, & Shobat, 2016). SCF, on the other hand, activates γ-globin expression by initiating downstream intracellular signaling pathways. Furthermore, SCF substantially stimulates HbF reactivation in normal adult erythroid cells. SCF levels have been reported to be elevated in β-thalassemia patients (Sripichai & Fucharoen, 2016).

1.2 Rationale of the study

Anemia in pregnancy is mainly caused by iron and other nutrients deficiency due to the growing needs from developing fetus. However, dysregulation in Hb production due to single-nucleotide polymorphism (SNP) has been reported to cause anemia in pregnancy (Mohammad et al., 2022). However, the underlying cause of SNPs causing anemia during pregnancy is not clear. Genes such as *BCL11A* and *KLF1* are important in erythroid differentiation. Therefore, this study intended to determine the SNPs of *BCL11A*, *HMIP-2*, and *XmnI* polymorphisms among anemic pregnant women. Since growth factors such as SCF and EPO are important for erythropoiesis, this study intended to determine the effect of these growth factors on K562 cells, a hematopoietic cancer cell line, on the expression of several genes *BCL11A, KLF1*, and ERK of MAPK pathway as well as the quantity of γ-globin mRNA.

1.3 Hypothesis

This study hypothesized that the genetic polymorphisms in the *BCL11A, HMIP-2,* and *XmnI* genes significantly impact the level of HbF in anemic pregnant women. Additionally, treatment of K562 cells with SCF and EPO would alter the expression of the γ-globin gene as well as *BCL11A*, *KLF1*, and ERK of the MAPK pathway.

1.4 Objectives

1.4.1 General objective

This study aims to determine the genetic polymorphism in anemic pregnant women with high HbF level and to determine the effect of SCF and erythropoietin-treated K562 cells on ERK and pERK signaling of the MAPK pathway.

1.4.2 Specific objectives

i. To assess the HbF and HbA2 in an anemic pregnant woman.

ii. To screen for thalassemia and the relationship between high HbF and the existence of SNPs of *BCL11A*, *HMIP-2*, and *XmnI* in anemic pregnant women.

iii. To determine the expression of γ-globin mRNA in K562 cells following stem cell factor and erythropoietin treatment.

iv. To determine the expression of *BCL11A*, *KLF1,* and ERK and pERK of MAPK on K562-treated cells.

CHAPTER 2

LITERATURE REVIEW

2.1 Red blood cells

Erythrocytes or red blood cells (RBCs) are the most abundant cells in the blood that mainly transports oxygen (O_2). RBCs are formed in the bone marrow and mature in the circulation, RBCs interact with other blood components. Human RBCs are flexible, oval, biconcave disks with no nuclei, organelles, or ribosomes. RBC transfer carbon dioxide from the tissues to the lungs and oxygen from the lungs to the tissues (Mahmood, Lim, Mazalan, & Razak, 2013; Nombela & Ortega-Villaizan, 2018). The number of RBCs varied at different stage of life as newborn, child, woman, and man has an average of 4.8-7.2 million RBCs/mm^3, 3.8-5.5 million RBCs/mm^3, 4.2-5.0 million RBCs/mm^3, and 4.6-6.0 x 10^6 RBCs/mm^3, respectively (Mahmood & Mansor, 2012). RBCs count is the total amount of RBCs in the whole blood and it is a good predictor of the O_2 level in the body. As blood O_2 level falls when the heart and lungs fail to function properly and to restore normal O_2 supply, the body increases RBC production (Mahmood & Mansor, 2012). Hence, RBCs count is crucial for identifying disorders such as leukemia and anemia (Venkatalakshmi & Thilagavathi, 2013). The average life span of RBCs is 100 to 120 days before being recycled by macrophages (Orriss, Key, Hajjawi, & Arnett, 2013). Hemoglobin (Hb) is found in RBCs, which are protected by a membrane made up of proteins and lipids (Dulińska et al., 2006). The RBCs need the iron-rich protein Hb to carry O_2 from the lungs to all areas of the body and give blood its red color (Ganguly & Alam, 2015).

2.2 Hemoglobin

Hemoglobin (Hb) is a hemeprotein, which carries O_2 from the lungs to peripheral tissues (Jorge et al., 2018b; Nagai, Mizusawa, Kitagawa, & Nagatomo, 2018). One heme group in the tetrahedral structure of Hb has an iron atom in its porphyrin ring, which has the ability to bind to O_2. As a result, one Hb molecule can bind to four molecules of O_2, and the globin comprises of two linked pairs of polypeptide chains (Basak & Sankaran, 2016).

When an O_2 molecule binds to deoxyhemoglobin in the lung, its O_2 affinity increases; when another binds, its O_2 affinity increases even more (Nagai et al., 2018). Increased blood volume and levels of Hb, as well as the level of O_2 transporter protein, contribute to increased internal O_2 storage (Coates & Decker, 2017). The concentration of Hb is frequently used to diagnose anemia. Low Hb levels could be due to hemodilution as a result of plasma volume (PV) expansion or by a true RBCs short fall as evidenced by a decreased in total RBCs volume (Flores-Montero et al., 2017). A dissociation curve is the curve that illustrates the relationship between the partial pressure of oxygen and saturation of the Hb with O_2, and this rightward shift depicts the decrease in the affinity, useful in distinguishing anemia and other hemoglobinopathies (Auer & Sutherland, 2002).

Adult hemoglobin (HbA) 1 (HbA1) and 2 (HbA2) and fetal hemoglobin (HbF) are the three types of Hb. The major HbA1 chain contains two α and two β chains accounts for approximately 96% of total Hb, minor HbA2 is made up of two α and two δ chains, while HbA2 and HbF accumulatively accounts for about 4% of total Hb (Ozcan & McLeod, 2016). In the late gestation, HbF start to convert to adult state, HbA1 and HbA2 and defects in this conversion by mean of genetic mutations which

leads to Hb disorders (Danjou et al., 2015). Hereditary Hb disorders are the most common monogenic diseases as an estimated 7% of human carry one of the mutations responsible for these disorders (Piel, 2016). As the Hb is tetramer or dimer of two chains, it requires the coordinated expression of these dimers for the formation of functional tetramers by the percentage of HbF (α2γ2), HbA2 (α2δ2), and HbA1 (α2β2) (Danjou et al., 2015) as shown in Table 2.1.

Table 2.1 Percentage of different Hb types during adult life

Types of Hb	Types of Globin Chain	Normal Range
HbA1	α2β2	~96%
HbA2	α2δ2	~3%
HbF	α2γ2	~1%

Heme production rises in the hemopoietic compartment during the development of erythroid progenitors and is closely regulated by iron uptake and globin gene expression (Jianbing & Hamza, 2019). Tetramer of all types of Hb have common dimer of α-globin, produced by two genes that are nearly identical and present on chromosome 16 (HbA1 and HbA2). When the second dimer has β chains it forms HbA. However, if the second dimer is delta it forms δ-globin chains encoded by the minor HbA2 complex which includes δ-globin. If the second dimer is of γ chains it encodes HbF which consists of Gγ1 and Gγ2 for γ-globin genes. On chromosome 11, there are β-globin genes that make up the main HbA (Figure 2.1) (Danjou et al., 2015).

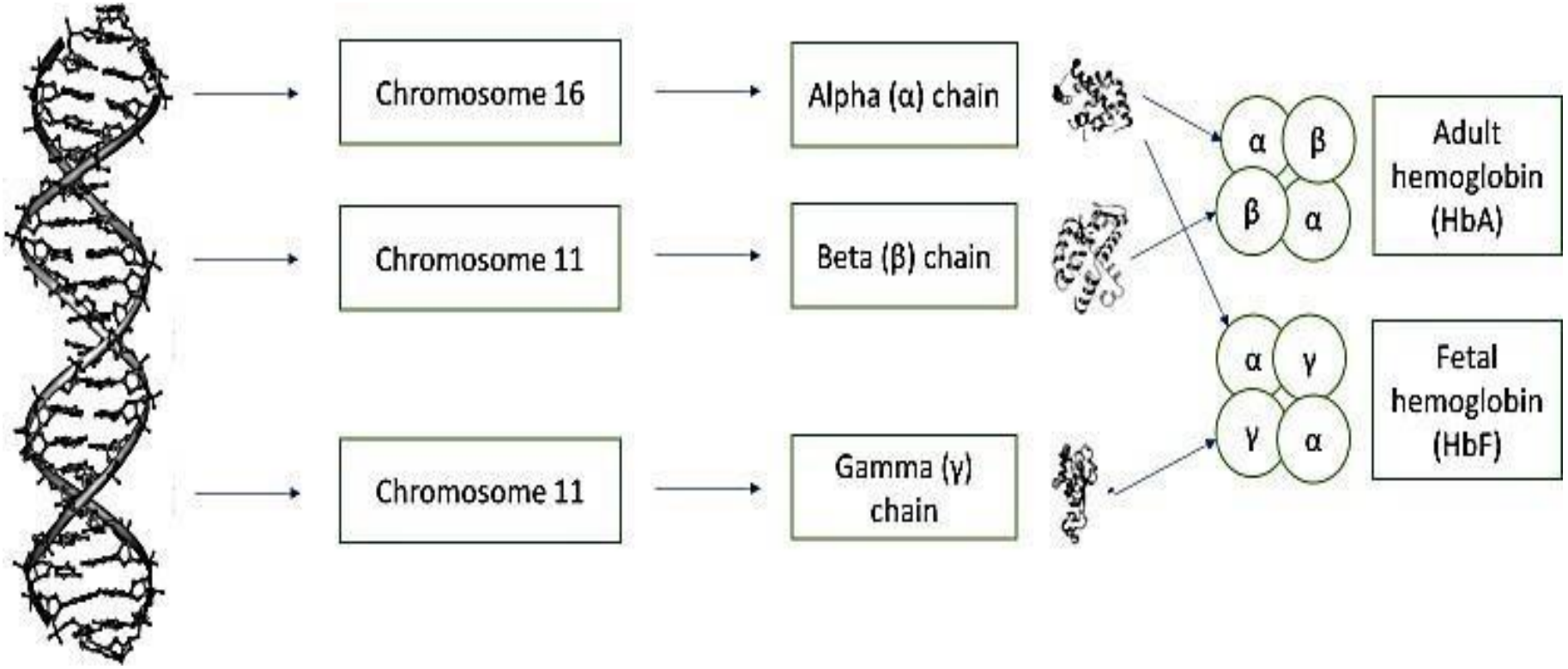

Figure 2.1 Chromosomal organization of the α, β, and γ globin gene clusters.

HbF is different from HbA in its structure and it has more O_2 binding capacity. After birth, the γ-globin chain that codes for HbF switch off and the β-globin chain that codes for adult hemoglobin switch on as some transcription factor (such as *MYB*, *KLF1*, and *BCL11A*) bind to regulatory DNA regions that control the expression of the globin gene results in the molecular switch between HbF and HbA (Danjou et al., 2015). Hence, HbA2 is used as one of the marker for identificationof thalassemia, as it rises as a result of the post-transcription mechanism caused by β-globin deficiency and increased availability of α-globin chains (Pivetta et al., 2021).

2.2.1 Adult hemoglobin (HbA)

HbA (tetramer: 2 α, 2 β\δ-globin), the most abundant protein in RBCs, each containing one redox iron heme group (Molaabasi, Hosseinkhani, Moosavi-Movahedi, & Shamsipur, 2015). HbA production increases after birth and makes up 95-98% of Hb in adults (Barrett, 2017). The function of Hb is determined by the expressionof β chains as well as their post-translational assembly (Russo & Nestler, 2013). HbAhas two forms, HbA1 (αβ-dimers) and HbA2 (αδ-dimers) and as fetus start HbA2 production at week 35 of gestation (Yordanova & Tcherkezov, 2018), its expression is low at birth but reaches normal level to 2.5-3.5% during first year of life (Villegas et al., 2017).

2.2.2 Fetal hemoglobin (HbF)

HbF consists of two α- and two γ-chains is the most prevalent Hb type in fetuses (Hansson, Libby, & Tabas, 2015). At about sixth week of pregnancy, HbF is being produced and it predominates while after delivery, its level falls gradually as γ-globin gene expression reduces and β-globin gene expression increases (Tayebi,

Shekari, & Heydari, 2017; Yin, He, Moumni & Sun, 2016). HbF binds O_2 with higher affinity compared to HbA, giving the developing fetus better access to O_2 from the mother (Ratanasopa, Cedervall & Bülow, 2016). Gradually HbF level decreases in the body as HbA replaces it and at the end of first year, it accounts for less than 1% in healthy humans. HbF levels are related to the amount of F cells since HbF synthesis is restricted to this fraction (Moumni, Eck, Wendt, Reininga & Mokkink, 2016). Furthermore, as the HbF is present at birth therefore any Hb related complications may be due to abnormality in HbF and it may cause severe clinical conditions as β-Hb diseases, β-thalassemia, and sickle cell anemia (SCA) (Liu, DeVel, Han, Zhang & Xiang, 2018; Ratanasopa et al., 2016).

Increased γ-globin expression balances β-globin chains, which contributes to the pathogenesis of anemia in β-thalassemia, a β-globin deficiency condition (Lichen, Liu & Corma 2018). Additionally, a number of pharmaceutical medicines, including hydroxyurea (HU), 5-azacytidine, arac, butyrate, and other short-chain fatty acids, are available as HbF inducers (Tayebi et al., 2017). The first approved drug for treating SCA was HU, but HbF response to this treatment varies and suboptimal, and therefore additional agents would be beneficial (Dai et al., 2017; Estepp et al., 2017).

2.3 Hemoglobinopathies

Hemoglobinopathies are hereditary diseases caused by variations in the normal HbA components of α-globin and/or β-globin (Sugiarto, Moore, Makani & Davis, 2018). Nonetheless, chronic anemia has been linked to major hemoglobinopathies caused by genetic defects, such as thalassemia and SCA (Alavi & Kirsner, 2015). Major hemoglobinopathies are rare inherited blood illnesses that affect about 330,000 newborns each year worldwide, with SCA accounting for 77% of cases (Pandarakutty,

Murali, Arulappan & Thomas, 2019). DNA mutations in the genes that produce polypeptide globin chains of Hb are the cause of hemoglobinopathies (Traeger-Synodinos & Harteveld, 2017). Hemoglobinopathies are thus classified into two main groups, thalassemia syndromes and variant Hb. In thalassemia syndromes, there is a change in the number of chains of tetramer which is reduced in most cases while variant Hb is due to structural abnormality in globin chain (Traeger-Synodinos & Harteveld, 2017). Severe hemoglobinopathies are distinguished with anemia by prolonged hemolysis, which results in decreased oxygen-carrying capacity and a rightward shift of the dissociation curve due to a flaw in the Hb molecule (Machogu & Machado, 2018). Thus, the diagnosis of thalassemia or hemoglobinopathy in anemic patients begins with family history and pertinent laboratory screening test such as Hb electrophoresis screening test (Ippolito, Dekker, Wang & Lee, 2014). *BCL11A* (B-cell lymphoma/leukemia 11A) is repressor of γ-globin gene in adults, andpolymorphisms in *BCL11A* influence HbF levels and clinical severity in patients with hemoglobinopathies (Pasricha & Drakesmith, 2018).

2.3.1 Thalassemia

A genetic disorder known as thalassemia is caused by mutation in α and β-globin chains which causes an imbalance in the number of the affected and unaffected globin chains (Lee et al., 2019; Santosh, 2017). Thalassemia patients have low Hb levels as well as poor Hb quality (Helmi, Bashir, Shireen & Ahmed, 2017). Thalassemia is the most prevalent disease across Asia, Middle East, Africa, and Mediterranean nations like Greece and Turkey, according to the Centers for Disease Control (CDC) (Vageriya & Sharma, 2017).

2.3.1(a) Alpha Thalassemia

Alpha (α)-thalassemia is caused by changes in the α-globin gene cluster on chromosome 16p13.3 (Huang et al., 2016). There are four α-globin genes on each chromosome 16, with two α-globin genes on each (HbA2 and HbA1) (Huang et al., 2016). Over 95% of α-thalassemia mutations are caused by deletions of either one(-α) or both (--α) α-globin genes, while the remaining mutations are due to non-deletional alterations (Huang et al., 2016). The prevalence of the α-thalassemiasilent carrier or trait varies greatly in different population, with some regional and ethnic groups having a frequency of gene mutations of 5% or less and others having frequencies of up to 80% (Fogel & Kvedar, 2018). The incidence of α- thalassemia has increased in North America as a result of migratory trends from higher risk of population (Kreger, Dougherty, Greene, Cerione & Antonyak, 2016).

α-thalassemia is also known as hemoglobin H (HbH) and it is caused by deletion of three α genes. A two-gene α deletion plus an extra non-deletional globin deficiency produces around 5% of HbH illness (Fogel, 2018). Homozygous mutations in α-globin gene in patients with α-thalassemia major produce a non-functional tetramer with chains and failure to form functional Hb (Kreger et al., 2016). α-thalassemia can be screened by using several methods such as Hb electrophoresis, a complete blood count, and the assessment of HbA2 levels with High Performance Liquid Chromatography (HPLC). Normal HbA2 level with abnormal or low mean corpuscular volume (MCV) and mean corpuscular hemoglobin (MCH) indicates α-thalassemia while HbA2 level of more than 3.4% indicate possible β-thalassemia (Tamaddoni, Naimi-Jamal, Rohlwing, Hussein & Abu-Zahra, 2019).

2.3.1(b) Beta thalassemia

β-thalassemia is characterized by deletion or mutation of β-globin gene on chromosome 11 that lead to an excess of α-globin chains and causing inherently recessive disorder (Mettananda et al., 2017; Zhao, Jing & Liu, 2018). β- thalassemia is the most frequently observed form of clinical thalassemia, with the presence of the β-thalassemia gene detected in almost 3% of the global population (Darvishi et al., 2016). The World Health Organization (WHO) has identified β- thalassemia as a worldwide health problem (Algiraigri & Kassam, 2017). The likelihood of having β-thalassemia is higher among people from the Mediterranean, Middle East, Central Asia, India, and Southern China regions (Algiraigri, Wright, Paolucci & Kassam, 2017). Depending on how severe the symptoms are, β- thalassemia is categorized into three phenotypes; β-thalassemia major, β-thalassemia intermedia, and β-thalassemia minor. Homozygous β-thalassemia major is known as Cooley's anemia (Graffeo, Link, Brown, Young & Pollock, 2018). Anemia,hemolysis, and inadequate erythropoiesis make up the diagnosis for β-thalassemia clinical presentations (Ansari, Saadatnia & Asghar, 2017). These extra α- chains precipitate on the inner membrane surface of RBCs, causing hemolysis and ineffective hematopoiesis (Akers, Howard & Ford, 2017). Anemia is the result of a series of events that are brought on by mutations in the β-globin gene, which result in inadequate β-chain production, an imbalance in α/β-globin chain synthesis, insufficient erythropoiesis, decreased RBC survival, and anemia (Liaska et al., 2016). Many β-thalassemia patients have severe anemia and major consequences such as liver damage, heart disease, and endocrine dysfunction.

β-thalassemia genotype is made up of more than 200 distinct β-globin gene variants (Dede et al., 2016). There are two clinically important β-thalassemia

phenotypes which are β-thalassemia major (β-TM) and β-thalassemia major intermediate. Both types are distinguished based on the Hb steady state and the need for blood transfusions (Algiraigri et al., 2017). Due to the mutation of both β-globin genes, the production of β-globin chains is substantially hampered in β-TM, a homozygous β-thalassemia) (Langhi et al., 2016). However, compared to other thalassemia subtypes, β-TM exhibited a higher rate of cardiac iron overload (Koohi, Kazemi & Miri-Moghaddam, 2019). The treatment of patients with thalassemia depends on sufficient blood transfusion and other therapeutic modalities (Liaska et al., 2016). The United Kingdomn (UK) has high proportion of thalassemia patients, and the current UK guidelines suggest for patient to undergo routine blood transfusions to maintain Hb levels above 10g/dL (Weidlich, Kefalas & Guest, 2016). Therefore, allogeneic hematopoietic stem cell transplantation, iron chelation, splenectomy, and supportive treatments are used to treat individuals with β-TM (Langhi et al., 2016).

2.3.2 Hereditary persistence of fetal hemoglobin (HPFH)

In addition to α and β-thalassemia, there is a third family of globin production defects known as hereditary persistence of fetal Hb (HPFH), in which the normal transition from HbF to HbA synthesis is disrupted and HbF production continues into adulthood (Thanh et al., 2018). HPFH have HbF homogenously distributed in RBCs due to some point mutations, the heterozygote genotypes have deletion in promoter region (Akinbami et al., 2016), and some non-deleted HPFH due to single nucleotide polymorphisms (SNPs) or small deletions in globin genes promoter region that interfere with the binding of regulating transcriptional factors (Hariharan et al., 2017). HPFH is a rare genotype of SCA with mild microcytosis and absence of sickle cell disease feature (Belisário, Sales, Silva, Velloso-Rodrigues & Viana, 2016).

2.3.3 Sickle cell anemia (SCA)

Sickle cell anemia (SCA) is a genetic disorder of Hb inherited in autosomal recessive pattern where RBCs become sickle shaped due to Hb mutation and less O_2 tension. These sickled cells RBCs are rigid in structure and they usually block the small blood vessels and cause several tissue damages. As the abnormal sickled RBCs break down, they cause anemia and severe complications as stroke, severe infections, lung damage, delayed growth and development (Owusu-Ofori & Remmington, 2017; Wilson & Nelson, 2015.). The sickle mutation on chromosome 11 of the β-globingene (HBB) results in the inheritance of faulty β-globin alleles, forming HbS which causes SCA (Ware, de Montalembert, Tshilolo & Abboud, 2017). If the mutation is inherited from both parents, it is termed as HbSS while if inherited from single parent it is HbS, in which a milder form of SCA (Anie & Green, 2015). According to various clinical studies, more than 95% of children with SCA are expected to live to adulthood, with the remaining 5% in mortality was due from acute chest syndrome, sepsis, splenic sequestration, stroke, and aplastic crises (Fitzhugh et al., 2010; Gardner et al., 2010). Some complications of SCA include avascular necrosis(Carroll et al., 2016). Moreover, mortality in SCA has been extremely high, with deathalways occurring before a diagnosis (Patrick et al., 2015). Hydroxyurea (HU) therapy, bone marrow transplantation (BMT), and continuous blood transfusion are the three disease- modifying treatments for SCA (Bakshi et al., 2017.). Treatment with HU has been shown to increase the level of HbF and decrease cellulardehydration in people with HbSS (Anie & Green, 2015). Transfusion therapy is used to reduce the HbS concentration to treat or prevent acute sickle cell-related complications (Anie & Green, 2015).

2.3.4 Anemia

Anemia is a condition when there is a reduction in the normal healthy RBCs or Hb which affects the ability to deliver O_2 to tissues (Derzon et al., 2019). Nonetheless, anemia is generally defined by low Hb levels, which can vary depending on a variety of factors, including age, gender, and ethnicity. For women, a normal Hb level is more than 12 g/dL, whereas for men, it should be more than 13 g/dL. At any stage of pregnancy, Hb level of less than 11 g/dL is regarded as abnormal and diagnosed as anemic (Abu-Ouf & Jan, 2015). Anemia is categorized into mild, moderate, or severe on the basis of Hb level or RBCs count (Hoepers, Menezes & Fröde, 2015). Anemia is a public health problem as Global Disease Burden (GBD) reports that 2.36 billion people are affected by anemia (Camaschella, 2015). According to WHO, 3.9% of men, 17.3% of women, and 38.5% of pregnant women were affected by anemia and increases the risk of adverse events like hospitalization, morbidity, and mortality (Assis, Macêdo, Oliveira, Rezende & Antunes, 2018; Derzon et al., 2019).

2.3.4(a) Risk factors of anemia

Several risk factors can cause anemia. Nutritional factors including iron, folate, and vitamin B12 are important risk factors along with infections such as human immunodeficiency virus (HIV), malaria, and hookworm infections can cause anemia. In addition, blood loss, chronic disease anemia secondary to malignancy and defects in the structure or production of Hb, including SCA and thalassemia are also major factors causing anemia (Fowler et al., 2015; Masukume et al., 2015).

Adolescent pregnancy, low educational level, poor socioeconomic situation, short inter-pregnancy time, and high parity are additional risk factors in the conceptual model of the determinants of anemia (Masukume et al., 2015). A type of anemia known as iron deficiency anemia (IDA) occurs when blood lacks healthy RBCs due to less iron (Johnson-Wimbley & Graham, 2011). Hence, iron supplementation for 60mg to 120mg per day is required to treat IDA. Pregnant women are recommended to consume 30mg to 60mg of iron each day, according to the WHO and CDC (Moretti et al., 2015). Blood iron levels in people with IDA will be low, or less than 10 mmol/L, in both men and women. The normal range is between 10 and 30mmol/L. Anemia will result in low ferritin levels, which is less than 10mg/L in both men and women (Clara, 2015).

2.3.4(b) Types of anemia

2.3.4(b)(i) Iron deficiency anemia (IDA)

In IDA, RBC levels become excessively low due to iron insufficiency (Anand & Gupta, 2018), reduced serum ferritin (<12µg/L), and Hb level <110g/L which can contribute to a significant burden of disease in infancy and childhood (McDonagh, Blazina, Dana, Cantor & Bougatsos, 2015). Iron deficiency caused by chronic illnesses or inflammation, poor iron absorption, and excessive iron loss are the most frequent causes of IDA (Wu & Tsai, 2016).

For a number of functions, including O_2 transport as part of the Hb molecule, enzymatic reactions as part of the cytochrome system, electron transport, and energy metabolism throughout the body, sufficient iron stores are required (Hempel & Bollard, 2016). A mismatch between iron intake and utilization causes decreased iron reserves (Jimenez, Kulnigg-Dabsch & Gasche, 2015). Healthy adults are encouraged

to consume approximately 10-15% of the iron in their diets, which may vary depending on body iron storage, iron content (heme vs non-heme), and other dietary factors (Wu & Tsai, 2016).

The iron in the human body, an important mineral that is frequently bound in Hb, is between 30 and 40mg per kilogram of body weight (Mücke, Mücke, Raine & Bettenworth, 2017; Taylor & Rampton, 2015). According to current IDA treatment recommendations, pregnant women, adults, and teenagers with IDA should take ferrous sulfate daily for at least three months, along with 60–120mg of elemental iron (Wu & Tsai, 2016). Serum ferritin values less than 15 to 100ng/mL and transferrin saturation less than 16 to 20% are typically considered as IDA indications depending on whether there is concurrent Inflammation (Stein, Connor, Virgin, Ong & Pereyra, 2016).

Low iron levels may result in anemia. IDA is the outcome of anemia with hypochromic microcytic RBCs and intestinal iron absorption that is larger than iron losses (McDonagh et al., 2015). This occurs in patients with low iron intake, low iron absorption, high iron demand, or recurrent iron loss (Taylor & Rampton, 2015). IDA and the resulting anemia are prevalent among women and children worldwide, especially in developing countries (Nazari, Mohammadnejad, Dalvand, & Ghanei Gheshlagh, 2019). Multiple nutrient deficiencies during pregnancy and infancy increase morbidity and mortality in mothers, babies, and children and prevent the surviving offspring from developing to their fullest potential (Cantor, Bougatsos, Dana, Blazina & McDonagh, 2015).

2.3.4(b)(ii) Vitamins deficiency anemia

Anemia can also be caused by deficiency of vitamin B12, vitamin D, and folate (Tran, 2018; Soofi et al., 2017). Vitamin B12, is crucial for DNA synthesis, normal erythrocyte development, and neurologic function and adequate B12 is highly recommended especially during the pregnancy (Yeruva, Manchandani & Oneal, 2016). Inadequate intake or absorption issues can lead to vitamin B12 deficiency leading to pernicious anemia (Hannibal et al., 2016). Vitamin B12 deficiency can result in osteoporosis, weakened muscles, and increased disease rates, particularly affecting children and women (Aydogmus et al., 2015; Girelli, Marchi & Camaschella, 2018; Liu et al., 2015).

2.3.4(b)(iii)Hemolytic anemia

There are two types of hemolytic anemia, autoimmune hemolytic anemia (AIHA) and non-immune hemolytic anemia. AIHA is an autoimmune syndrome when autoantibodies against RBC surface antigens attack RBCs causing early death of peripheral RBCs (Ungprasert, Tanratana, & Srivali, 2015). Pathophysiological classification of warm, mixed, and cold-reactive subtypes of AIHA is also based on the optimal RBC autoantibody temperature response (Ungprasert et al., 2015). Warm antibodies are the most prevalent subtype of AIHA, making up 75% of all AIHAs in adults. Meanwhile, non-immune hemolytic anemia is caused by intracorpuscular defects within the RBCs due to different variants or may be due to some extracorpuscular defects (Beris & Picard, 2015).

2.3.4(c) Anemia in pregnancy

Anemia is a prevalent issue for expecting mothers all around the world. WHO estimates that anemic conditions affect half of all pregnant women worldwide (Casanova, Sammel & Macones, 2005). Approximately 60% of pregnant women in Asia and 52% in Africa are anemic in which 80% of anemic pregnant women comes from South Asia (Shoboo, Delpisheh, Parizad & Sayehmiri, 2016). Anemia in pregnancy remains a major health concern in low- and middle-income countries. However, this continues to be one of the most serious health problems in developing nations due to a number of socio-cultural problems such as illiteracy, poverty, lack of awareness, cultural and religious taboos, poor dietary practices, and a high prevalence of parasitic infestation (Gebre & Mulugeta, 2015). Prevalence of anemia in least developed countries is also an issue where there is a high need to improve or take preventive measures to increase the access of early diagnosis, iron supplementation and treatment of hemoglobinopathies (Safiri et al., 2021). Anemia is prevalent when Hb levels are less than 10.5g/dL in the third trimester of pregnancy and less than 10.5g/dL in the first and second trimesters (Rahmati, Delpishe, Azami, Hafezi Ahmadi & Sayehmiri, 2017). Furthermore, there are three levels of anemia based on Hb level; mild (10.0 - 10.9g/dL), moderate (7.0 - 9.9g/dL), and severe (< 7.0g/dL) (Gebre & Mulugeta, 2015).

The prevalence of mild anemia is higher than that of moderate or severe anemia. In second and third trimester of pregnancy, anemia rates were higher than in the first trimester (Zhao et al., 2018). Reduced exercise tolerance, puerperal infection, thromboembolic issues, postpartum hemorrhage, pregnancy induced hypertension, placenta previa, heart failure, low birth weight, early delivery, and prenatal mortality are further signs of anemia during pregnancy (Rahman et al., 2016; Stephen et al., 2018; Tadesse et al., 2017; Taner et al., 2015; Wirth et al., 2017). Maternal mortality

has also been connected to Hb concentrations throughout pregnancy, with the chance of death falling by 25% for every 10g/L increase in Hb level (Wirth et al., 2017). Pregnancy at a young age, parasitic infections, smoking, a low socioeconomic status, insufficient space between births, poor diets and insufficient weight gain during pregnancy are some risk factors for pregnancy anemia (Figueiredo et al., 2018; Mardani et al., 2017).

Anemia during pregnancy is further divided into pathogenic and physiological types. Hemorrhagic anemia and anemia brought on by deficiencies, such as IDA and vitamins and protein deficiency anemia, are examples of pathogenic anemia. Due to the increased blood volume needed to support developing fetus, physiological anemia is frequent in pregnant women and is linked to a slight drop in Hb in levels (Cantor et al., 2015). Therefore, increased risk for anemia during pregnancy is brought on by the mother's increased need for iron as well as the placenta's and fetus's growing demands, which increase erythrocyte mass (Cantor et al., 2015).

Additionally, the etiology of anemia in pregnant women has been linked to infectious diseases such as malaria, hookworm, schistosomiasis and Helicobacter pylori infection as well as hereditary Hb apathies such as thalassemia (Rahmati et al., 2017; Taner et al., 2015), although the former conditions can not provoke hereditary Hb apathies. Severe hookworm infections have been linked to morbidity, with high-intensity infections having the highest morbidity among populations with insufficient iron stores (McClure et al., 2014). Although urogenital schistosomiasis can result in adverse health effects like anemia, its connection to maternal anemia is less certain. In regions where these parasitic infections are common, poor nutrition is reasonably common, which contributes to a lack of iron, folate, and other

micronutrients intake that might be a significant factor between infections and anemia (McClure et al., 2014).

Anemia during pregnancy is commonly linked to iron deficiency (ID) as the leading cause (Nguyen et al., 2016; Taner et al., 2015). Ferritin levels are still regarded as the gold standard test to identify ID during pregnancy, despite its limitations (Casanova et al., 2005). Serum ferritin level gradually decreased to 50% of the normal value during pregnancy as a result of the mobilization of iron from reserves to fulfill the demands of gestation (Casanova et al., 2005).

Hyperemesis gravidarum, vegetarian diet, irregular menstruation prior to pregnancy, being a minority, high parity (risk rises three fold in women with two to three children compared to nulliparous women), and low income are the most common risk factors for the development of IDA in pregnancy (Casanova et al., 2005). Expecting woman with IDA may clinically present with fatigue, weakness, pallor, tachycardia, and shortness of breath (Cantor et al., 2015). The first-line treatment for IDA during pregnancy is oral iron supplementation where WHO recommends 30 to 120mg of elemental iron and 0.4mg of folic acid per day for pregnant woman (Govindappagari & Burwick, 2019; Viteri, Casanueva, Tolentino, Díaz-Francés & Erazo, 2012). The restricted intestinal absorption of oral formulations can be treated with intravenous iron treatment, which may result in a faster rise in iron reserves (Govindappagari & Burwick, 2019). Health professionals have been instructed to screen for anemia in high-risk pregnant women during each trimester and at 4 to 6 weeks after delivery as a prophylactic step (Gupta & Gadipudi, 2018).

2.4 Genetic influence of fetal hemoglobin

After infancy, HbF level rises as a result of three non-pharmacologic processes. Firstly, definitive erythroid cells generated from bone marrow can exhibit aberrant γ-globin gene expression as a result of γ-globin gene abnormalities, which exclusively express β-globin. Secondly, mutations in hypothetical trans-acting regulatory locations are located far away. Finally, metabolic persistence of HbF after infancy can be connected to both hereditary and acquired diseases (Karkashon et al., 2015). HbF level is increased in anemic condition and pathophysiological conditions such as SCA which is highly dependent on polymerization of deoxy sickle hemoglobin that further increases HbF. This further results in recruitment of erythroid progenitor cells which further differentiate and terminate immaturely (Akinsheye et al., 2011).

While typical genetic variation has little effect on HbF levels, uncommon variants with single-base mutations or deletions in the β-globin gene cluster or microdeletions in the γ-globin gene promoters result in large elevations of 10% to 30% of total Hb (Liu et al., 2018). Additionally, some transcription factors, such as *TR2/TR4*, *MYB*, *BCL11A*, *GATA1*, and Kruppel-like factor 1 (*KLF1*), are crucial for the developmental silencing of γ-globin (Figure 2.2) (Pace, Liu, Li & Makala, 2015).