Deanship of Graduate Studies

Al-Quds University

Genotyping of Human Erythrocyte Antigens for Safe Blood Transfusion in Thalassemia Patients

Dorgam Muffed Ibraheem Yasin

M.Sc. Thesis

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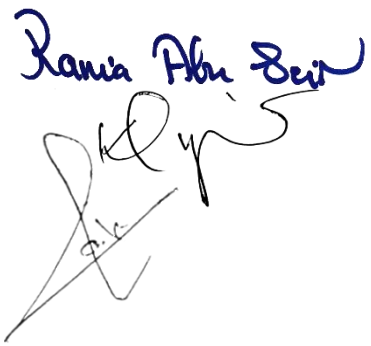

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Dedication

I dedicate this thesis to:

Firstly, to my all-powerful God, who has provided me with strength and knowledge throughout my life.

Special thanks to Dr. Rania, my friend, excellent instructor, and exceptional human being.

To my family (my mother, wife, and my sister), who have encouraged me to be strong in the face of adversity, as well as for their love and support.

I'd also want to express my gratitude to my beloved brother and buddy Mr. Basem Abu Al-Rub for his assistance with my research.

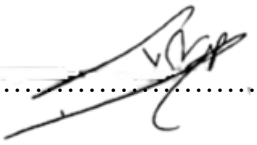
To all my friends who have aided, counseled, and supported me, thank you.

Dorgam Muffed Yasin

Declaration:

I certify that this thesis submitted for the degree of Master, is the result of my own research, except where otherwise acknowledged, and that this study (or any part of the same) has not been submitted for a higher degree to any other university or institution.

Signed ……..



Dorgam Muffed Ibraheem Yasin

Date: 23/05/2022

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Last but not least, I am thankful to my family, my friends, and my colleagues for the psychological and moral support in helping me finish my studies.

Genotyping of Human Erythrocyte Antigens for Safe Blood Transfusion in Thalassemia Patients

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Abstract

Background

Management of β-thalassemia is a major challenge, especially in low resource countries. Blood transfusion is the mainstay treatment of patients with β-thalassemia major. However, blood transfusion is associated with several side effects including hemolytic and allergic reactions, iron overload, and transfusion-transmitted diseases. In this study, we assessed the biochemical, hematological, and hormonal parameters, estimated the prevalence of complications, studied the frequency of red blood cell alloimmunization and autoimmunization, and determined the genotype frequencies of blood group systems among transfusion-dependent β-thalassemia patients in the West Bank.

Methods

This study was conducted using 100 frequently transfused thalassemia patients. The patients were recruited through Thalassemia Daycare Units in five governmental hospitals patients with the highest transfusion frequency were selected for this study. A questionnaire was used to collect data regarding the basic characteristics of the patients. In addition, medical records were used to collect data regarding the complications of thalassemia among the patients. Blood samples were collected from the patients to measure biochemical,

hematological, and hormonal parameters, in addition to screening and identification of antibodies and for DNA extraction. DNA samples were genotyped for Rhesus, Kell, Duffy, Kidd, MNS, Dombrock, Colton, Cartwright (Yt), Lutheran, Knops, Deigo, and Vel blood groups. Genotyping for blood groups was performed by sequence-specific primers (SSP)-PCR method.

Results

A total of 100 patients were included, 51% were males. The mean age among the patients was 21.9±10.9 years. The majority of the patients (60%) were recruited from Al Watani Hospital. The mean pre-transfusion hemoglobin level was found to be 7.89±0.99 g/dL and the mean serum ferritin level was 3670.42±3742.71 ng/dL. The results of liver function tests showed that 32%, 42%, and 34% had elevated ALT, ALP, and AST levels, respectively. Regarding the hormonal results,10% of the patients had subclinical hypothyroidism. The prevalence of growth hormone deficiency was 8%. Also, 8% of the patients had hypocalcemia and 70% had vitamin D deficiency. Elevated glucose levels were found among 15% of the patients. The most encountered complications were arthropathy (44%), hypogonadism (16%), and hepatic failure and delayed growth (7%). The genotyping results of the *RHD* blood group showed that 88% of the patients were *RHD-positive* whereas 7% were *RHD-negative* and 5% had no clear results. The allele frequencies of *RHCE* alleles were 0.440 and 0.560 for *RHCE*C* and *RHCE*c*, respectively, and 0.165 and 0.835 for *RHCE*E* and *RHCE*e*, respectively. Unexpectedly, for the Duffy blood group system, the null genotype (*FY*02N.01/02N.01*) was observed in 46% of the patients and the allele frequencies of *FY*01* and *FY*02* were 0.195 and 0.345, respectively. Furthermore, the allele frequencies of *GYPA*M* and *GYPA*N* were 0.585 and 0.405, respectively, and those of *GYPB*S* and *GYPB*s* were 0.275 and 0.725, respectively. The *KEL*02*, *KEL*04*, and *KEL*07* allele frequencies were high among the patients in this study (0.920, 0.985, and 0.980,

respectively). Furthermore, the allele frequencies of *YT*A, LU*02, CO*01, KN*01, DI*B, DI*02.04*, and *VEL*01* were 0.940, 0.990, 0.990, 1.000, 0.980, 1.000, and 0.990. In addition, 2% of the patients had the *Vel*01/-0.1* (*Vel/Vel*$_{null}$) genotype. The rate of alloimmunization among patients was 8% and the most common antibodies were anti-E, anti-K and anti-D, and anti-C, respectively. The rate of autoimmunization was 5%.

Conclusions

The management of thalassemia should be based on internationally established guidelines. Understanding the frequencies of the major blood group systems other than the ABO and Rh systems is essential to provide accurate information regarding the local population's requirements, reduce transfusion-related complications among frequently transfused patients, and facilitate the challenging task of providing antigen-negative blood for patients with multiple antibodies. Phenotyping of patients' RBCs could have prevented the development of alloantibodies against Rh antigens C, E, and K. Furthermore, accurate testing for weak RhD among donors could also prevent alloimmunization against RhD antigens. The genotype and the allele frequencies observed among the sample of this study revealed several interesting findings that prompt further research.

Keywords

Alloimmunization, red cell genotyping, iron overload, beta-thalassemia, frequently transfused.

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List of Abbreviations

AIHA	Autoimmune hemolytic anemia
ALP	Alkaline phosphate
ALT	Alanine aminotransferase
AMA	Anti mitochondrial antibodies
ANA	Antinuclear antibodies
ANCA	Antineutrophil cytoplasmic antibodies
ANOVA	One-way analysis of variance
AST	Asparatate aminotransferase
β-Thalassemia	Beta-thalassemia
BUN	Blood urea nitrogen
Ca	Calcium
CBC	Complete blood count
CCP	Cyclic citrullinated peptide antibody
DNA	Deoxyribonucleic acid
EDTA	Ethylene diamine tetra acetic acid
ELISA	Enzyme linked immunosorbent assay
FT4	Free thyroxin
GH	Growth hormone
Hb	Hemoglobin
HbA	Adult hemoglobin
HbF	fetal hemoglobin
HBs Ag	Hepatitis B virus antigen
HCV	Hepatitis C virus
HEA	Human erythrocyte antigens
HFA	High frequency antigen
HTR	Hemolytic transfusion reaction
IQR	Interquartile range
LKM-1	Liver-kidney microsomal type 1
MoH	Ministry of Health
NTDT	Non-transfusion dependent thalassemia
OD	Optical density
PCR	Polymerase chain reaction
PTH	Parathyroid hormone
RBC	Red blood cell
RBS	Random blood sugar
REC	Research Ethics Committee
RFU	Relative fluorescence unit
SD	Standard deviation
SF	Serum ferritin
SNP	Single nucleotide polymorphism
SNV	Single nucleotide variant
SSP	Sequence-specific primer

TDT	Transfusion dependent thalassemia
TIF	Thalassemia International Federation
TPFS	Thalassemia Patients' Friends Society
TSH	Thyroid stimulating hormone
TT3	Triiodothyronine
TTI	Transfusion-transmitted infection

Chapter One

Introduction

This is an opening chapter that provides the background of this study, problem statement, study justification, objectives, and expected outcomes.

1.1 Background

Thalassemia is defined by the Thalassemia International Federation (TIF) as a category of blood disorders distinguished by abridged or missing construction of normal globin chains (Viprakasit, Origa, & Fucharoen, 2014). It results from imbalance in the synthesis of the alpha and beta globin chains, resulting in anemia due to inefficient erythropoiesis and hemolysis (Shawkat & Jwaid, 2019).

Thalassemias are *"classified according to which globin chain(s) is/are formed in a decreased amount, which may result in an imbalance in globin chain synthesis, inefficient erythropoiesis, or hemolysis and eventually to a variable degree of anemia"* (Angastiniotis & Lobitz, 2019).

The two most common types of thalassemia are α and β-thalassemia, both of which originate from a deficiency in the synthesis of globin chains (Forget & Bunn, 2013; Olivieri, 1999). β-thalassemia is the most common type and causes severe anaemia in both homozygous and compound heterozygous people (Cao & Galanello, 2010).

Thalassemia is classified clinically into three categories based on severity (Cao & Galanello, 2010; Lulla et al., 2020; Shaheen, 2019)(Figure 1.1):

1. β-thalassemia major, which necessitates regular blood transfusions right through life, also called transfusion-dependent thalassemia (TDT).
2. β-thalassemia intermedia, which is characterized by anemia but not severe enough to necessitate regular blood transfusions also called non–transfusion-dependent thalassemia (NTDT).

3. β-thalassemia minor, which is a symptomless carrier state.

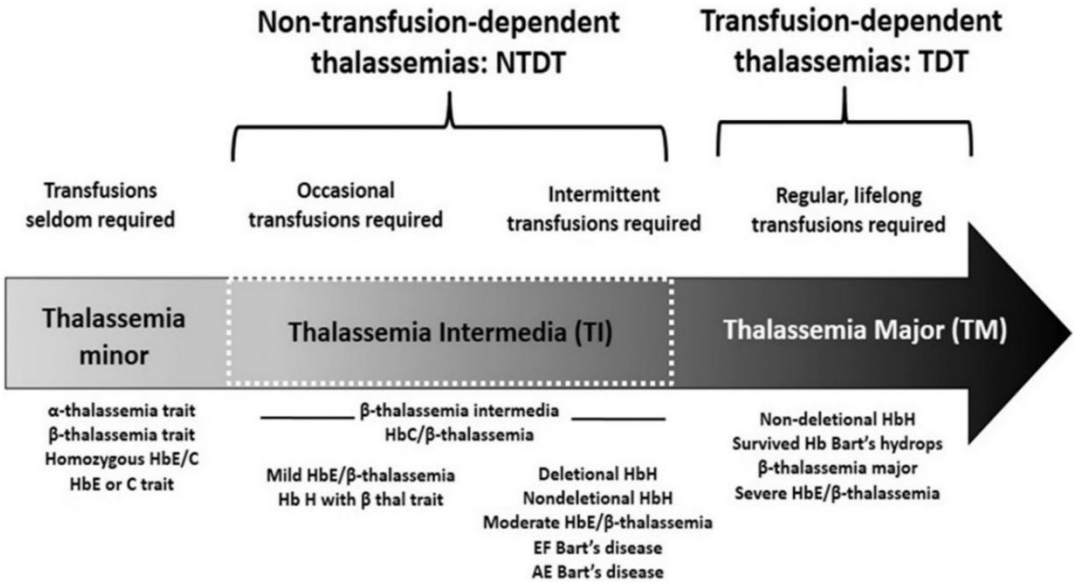

Figure (1.1): Thalassemia classification based on their severity (Musallam, Rivella, Vichinsky, & Rachmilewitz, 2013).

There are well-established links among the hematological-clinical phenotype and the type of mutation (Lulla et al., 2020). More than 400 distinct mutations in the globin gene have been discovered and known as being accountable for the development of thalassemia (Patrinos et al., 2004). The major types of β-thalassemia are due to point mutations, and large deletion mutations have been found in rare cases (Galanello & Origa, 2010; Lulla et al., 2020). As a result, identifying the mutation in patients is necessary for improved management (Lulla et al., 2020; Malik, Malik, Al-Shammaa, & Al-Rubaei, 2010).

β-Thalassemia genes mutations are found in numerous ethnic groups throughout a large geographic span stretching from the Mediterranean to the Middle East, the Indian subcontinent, and Southeast Asia (Surapon, 2011). In certain populations, the prevalence of thalassemia carriers is estimated to be between 3 and 10%. A little number of frequent mutations, as well as rarer ones, is identified in each group at risk (Jaing et al., 2021). In Palestine, the prevalence of thalassemia carriers was estimated to be 3.5% (Yunis, Abdeen, & Barghuthy, 1996). According to the Thalassemia Patients' Friends Society (TPFS), thalassemia prevalence was 17.4 per 100,000 population in 2018, with 847 symptomatic

thalassemia patients in West Bank and Gaza Strip (Aldwaik et al., 2021). Seventeen β-globin gene mutations were detected among thalassemia patients (Darwish, El-Khatib, & Ayesh, 2005).

β-Thalassemia major (β-TM) is the most severe form of thalassemia and refers to people who have no or extremely limited production of the β-globin chain. More than five decades ago, β-thalassemia major (β-TM) was fatal in the first decade of life; however, the improvement of diagnostic and therapeutic methods have significantly improved the prognosis of β-TM (Rivella & Rachmilewitz, 2009). Treatment of severe types of β-thalassemia include a combination of three different regimens; blood transfusions on a regular basis, chelating medicines such as Deferoxamine and Exjade are used to remove excess iron, and splenectomy when the rate of transfusion is increasing (Viprakasit et al., 2014).

Even though blood transfusion is a lifesaving treatment which is considered the mainstay of treatment for individuals with thalassemia, transfusion-related complications are considered a major source of morbidity in frequently transfused thalassemia patients and can be a serious challenge in the management of thalassemia (Shander, Cappellini, & Goodnough, 2009). The liver, parathyroid, heart, pituitary, thyroid, adrenal, pancreas, renal medulla, spleen, and bone marrow are all common sites for iron deposition. In severe cases, parenchymal iron overload is the leading reason of morbidity and death. Moreover, when the typical teenage growth spurt fails, hepatic, endocrine, and cardiac issues develop, resulting in diabetes, hypoparathyroidism, adrenal insufficiency, and liver failure, among other clinical problems. In addition, secondary sexual development is either delayed or absent (Webb & Krone, 2015).

The development of antibodies against red cell antigens (alloimmunization) is a significant threat to the long-term success of transfusion therapy (Molina-Aguilar, Gomez-Ruiz, Vela-Ojeda, Montiel-Cervantes, & Reyes-Maldonado, 2020). Alloimmunization is defined as the development of antibodies against specific RBC antigens leading to various complications including difficulties in RBC cross matching and shortened in vivo survival of donor blood (Dhawan et al., 2014). This could be due to blood group system antigenic difference between the donor and recipient. Over 300 inherited blood group antigens have been identified as reported by The International Society Blood Transfusion (ISBT). These antigens are

categorized interested in 36 blood group systems (International Society of Blood Transfusion "ISBT", 2021).

In transfusion-dependent patients with β-thalassemia major, alloimmunization occurs at the rate of 10–20%, mostly involving anti-Kell anti-E, and anti-C as the most common alloantibodies (F. T. Shah, Sayani, Trompeter, Drasar, & Piga, 2019). Exposure to the donor erythrocyte antigens that are absent in the recipient initiates the formation of antibodies against erythrocytes resulting in alloimmunization and transfusion reactions (Molina-Aguilar et al., 2020). Furthermore, alloimmunization alter the equilibrium of the immune system as alloimmunization requires depletion of CD4+ lymphocytes (Molina-Aguilar et al., 2020), complicates the selection of compatible donors making the process expensive and time-consuming (Paccapelo, 2018).

Serological phenotyping based on hemagglutination is the conventional method used to determine blood group antigens (Monteiro et al., 2011); however, the presence of donor's erythrocytes in the circulation from the preceding transfusion prevents precise blood group phenotyping (A. Belsito, Magnussen, & Napoli, 2017; Fasano & Chou, 2016). In addition, serological phenotyping has several other limitations (Matteocci & Pierelli, 2014).

Advancements in molecular diagnostics have made it possible to genotype human erythrocyte antigens (HEA). HEA genotyping can give necessary information that is hard or not possible to get with serological methods given that molecular assays are not influenced by the limitations of the traditional methods. RBC antigens even in recently transfused patients (Fasano & Chou, 2016; Osman et al., 2017). In addition, using molecular blood group genotyping can support accurate transfusion decisions and minimize alloimmunization. Furthermore, genotyping allows for testing of a wider diversity of variants, including rarer variants that are typically not detectible with custom serological reagents (Khan & Delaney, 2018; Kutner, Mota, Conti, & Castilho, 2014).

To reduce alloimmunization, thalassemia patients should be transfused with ABO, Rh (C, c, D, E, e), and Kell well-matched blood. Furthermore, antigen typing of already transfused patients can be performed using molecular rather than serological testing based on TIF's recommendations (Thalassemia International Federation (TIF), 2021).

1.2 Problem Statement

Beta-thalassemia remains to be one of the major health problems particularly in developing countries, where 80% of new cases occur each year (De Sanctis et al., 2017). Transfusion therapy is a key component of the comprehensive management of patients with thalassemia. Although blood transfusion prevents most of the serious complications of thalassemia, transfusion related complications remain a major source of morbidity among polytransfused thalassemia patients, particularly in low resource countries. Regular blood transfusions are required for patients with β -thalassemia-major throughout their life. Blood is normally given every 2–5 weeks to maintain hemoglobin levels between 9.5-10.5 g/dL (Thalassemia International Federation (TIF), 2021).

Although blood transfusions save lives in patients with β -thalassemia, they also burden the body with extra iron, which causes hemosiderosis and other comorbidities, as well as irreversible biological damage such as cirrhosis, liver fibrosis, heart disease, endocrine abnormalities, and, most importantly, heart failure. Moreover, one of the most serious side effects of chronic blood transfusion is alloimmunization to red cell antigens. It can complicate transfusion therapy by causing delayed transfusion reactions and making it more difficult to find compatible blood, leading to increased morbidity and mortality in transfusion-dependent patients (Abu Taha et al., 2019).

Transfusion therapy of frequently transfused patients is complicated by alloimmunization. The strategy of the Thalassemia International Federation (TIF) recommend that comprehensive red cell antigen typing that include at least A, B, O, C, c, D, E, e, and Kell should be performed before embarking on blood transfusion (Thalassemia International Federation (TIF), 2021). Serological phenotyping based on hemagglutination is the conventional method used to determine blood group antigens (Monteiro et al., 2011). However, the presence of donor's erythrocyte in the circulation from the preceding transfusion prevents precise blood group phenotyping (A. Belsito et al., 2017; Fasano & Chou, 2016). Therefore, it is recommended to perform antigen typing by molecular assays rather than serological testing (Thalassemia International Federation (TIF), 2021) to minimize the alloimmunization and other complications in frequently transfused patients.

1.3 Study Justification

The prevalence of thalassemia trait in the West Bank region was reported to be around 3.5%(Yunis et al., 1996). Thalassemia Patients' Friends Society (TPFS) reports show that there are around 847 symptomatic thalassemia patients in the West Bank and Gaza Strip. A previous study among thalassemia patients in the West Bank showed the lack of guidelines and protocols for the management of thalassemia (Aldwaik et al., 2021).

The purpose of blood transfusion in thalassemia is to deliver a safe and effective transfusion regimen. The probability of making one or more type of antibodies against the RBCs was estimated to be approximately 1% per unit of transfused blood (Samarah, Srour, Yaseen, & Dumaidi, 2018), but these odds are drastically higher in frequently transfused patients as the alloimmunization rates may reach 50% (Dhawan et al., 2014). A previous study conducted among transfusion dependent thalassemia patients in Palestine in 2019 reported that the rate of alloimmunization was 12.6% (Abu Taha et al., 2019). The most common involved antibodies were reported to be those against antigens from Rh and Kell blood group systems (Datta, Mukherjee, Talukder, Bhattacharya, & Mukherjee, 2015).

The formation of clinically significant alloantibodies may result in hemolytic transfusion reactions throughout the subsequent transfusions. In addition, it delays the process of finding compatible blood, shortens the survival of the transfused erythrocytes, and increases the frequency of transfusion requirement, which increases the burden of iron overload (F. T. Shah et al., 2019). Moreover, alloimmunization also triggers additional alloantibodies and autoantibody formation (Dinardo, 2018).

The understanding of blood group typing in Palestine is limited to the ABO and Rh blood groups. However, despite the need for extended phenotyping, serologic methods are not reliable in frequently transfused patients due to the presence of donors' RBCs in their circulation. Therefore, HEA genotyping can provide vital information that is difficult or impossible to get with serological methods as molecular assays are not influenced by the limitations of the conventional method and can be used to determine RBC antigens even in recently or frequently transfused patients (Fasano & Chou, 2016; Osman et al., 2017). In addition, using molecular blood group genotyping can support transfusion decisions and prevent alloimmunization by genetic matching of donor and recipient and allow for testing

of a wider range of variants including rarer variants that are usually not detectible with routine serological reagents (Khan & Delaney, 2018; Kutner et al., 2014).

1.4 Aims and Objectives

The aim of this study is to assess the overall health status of frequently transfused Palestinian β-thalassemia patients and provide data regarding blood group systems in order to enhance the safety of blood transfusion among multi transfused thalassemia patients. The specific objectives of the study are:

- To assess the biochemical, hormonal, and hematological parameters of frequently transfused thalassemia patients.
- To estimate the prevalence of the complications of iron overload in frequently transfused thalassemia patients.
- To determine the genotype and the allele frequencies of Rhesus, Duffy, Kell, Kidd, Colton, Knops, Lewis, Luth, Dombrock, MNS, Diego, Yt, and Vel blood group major antigens among frequently transfused thalassemia patients.

Chapter Two

Literature Review

An overview of the literature available on hemoglobin and hemoglobin disorders, the pathophysiology, classification, epidemiology, diagnosis, treatment, and management of thalassemia, and phenotyping and genotyping of red blood cell (RBC) antigens will be reviewed in this chapter.

2.1 Human Hemoglobin

Human hemoglobins (Hb) are heterogeneous proteins present inside the red blood cells. This heterogeneity is expressed at all stages of development, during which different forms of hemoglobin are synthesized (Marengo-Rowe, 2007).

2.1.1 Structure of Hemoglobin

All the human hemoglobins are tetrameric in structure containing protein that plays an essential role in O_2 transport (Gell, 2018) as shown in Figure 1 (Haddad, 2012a), made up of two different pairs of globin polypeptide chains (2α like, 2β like). Each globin chain is attached to one heme molecule (Marengo-Rowe, 2006). During the developmental phases of human life, however, multiple forms of hemoglobin are created.

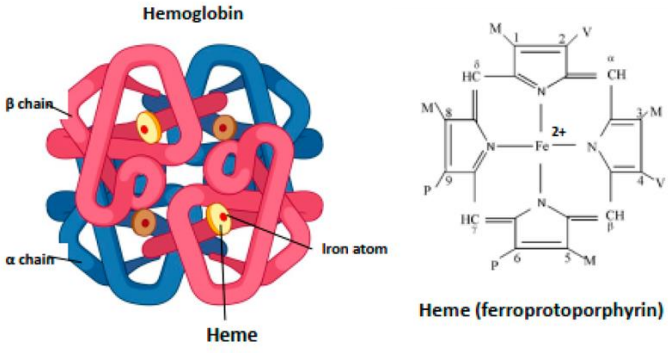

Figure (2.1): The three-dimensional structure of hemoglobin tetramer and the chemical structure of heme (Frimat, Boudhabhay, & Roumenina, 2019).

2.1.2 Ontogeny of Human Hemoglobins

The normal hemoglobin tetramer contains 2α-like (α or ζ) and 2 β-like (β, γ, δ, £) chains (Forget & Bunn, 2013; Galanello & Origa, 2010; Sankaran & Orkin, 2013). Hemoglobin production (Figure 2.2) begins in the yolk sac during the second month of pregnancy. Hb Gower1 (ζ2 £2) is the first embryonic hemoglobin tetramer, consisting of 2α-like (2ζ) and 2β-like (2ε) chains. Then, two more embryonic hemoglobins are produced; Hb Gower2 (α2 ε2) and Hb Portland (ζ2 γ2). During the first 10 to 11 weeks of pregnancy, erythropoiesis takes place in the liver and spleen, at which point embryonic Hb (Hb Gower1, Hb Gower2 and Hb Portland) begin to diminish and fetal hemoglobin (HbF: α2 γ2) eventually becomes the predominant throughout the fetal life (Table 2.1) (E. George & Ann, 2010; L. R. Manning et al., 2010; Lois R Manning et al., 2007; Schneider & Schechter, 1983).

Table (2.1): Hemoglobin types in the diverse developmental stages of human life

Hemoglobin Type	Structure	Developmental Stage
Hb Gower 1	ζ2 ε2	Embryo
Hb Gower 2	α2 ε2	Embryo
Hb Portland	ζ2 γ2	Embryo
HbF	α2 γ2	Fetal and adult
HbA2	α2 δ2	Adult
HbA	α2 β2	Adult

2.1.3 Fetal to Adult Hemoglobin Switch

The adult β and δ-globin chains progressively begin to replace the γ-globin chain after birth. This causes a substantial transition from HbF (α2 γ2) to adult hemoglobin HbA (α2 β2) production which starts around the time of birth and lasts for about 6 months (Figure 2.2). After switching from fetal to adult hemoglobin, HbA accounts for 97-98% of the hemoglobin, with HbA2 (α2 γ2) accounting for around 2%. Adult blood contains a little quantity of HbF (less than 1%)(Sankaran & Orkin, 2013; Thein, Menzel, Lathrop, & Garner, 2009).

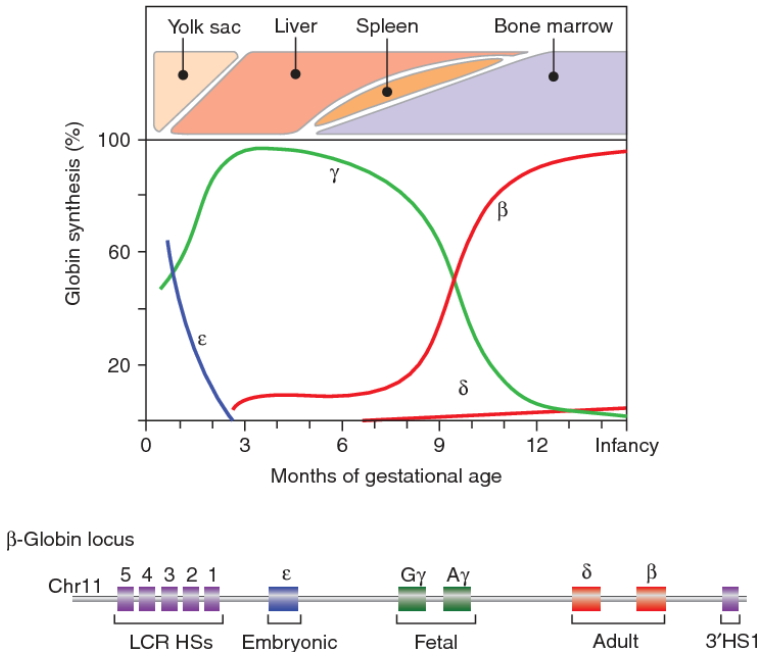

Figure (2.2): The development hemoglobin switch from fetal hemoglobin to adult hemoglobin (Sankaran & Orkin, 2013).

2.1.4 Genetic Control of Human Hemoglobin

Two gene clusters regulate the formation of different kinds of human globin chains: the α-like genes and the β-like genes cluster (Proudfoot, Shander, Manley, Gefter, & Maniatis, 1980).

2.1.4.1 α-Like Genes Cluster

The α-like globin genes are clustered in a 26-kilobase DNA sequence on the short arm of chromosome 16's distal portion. Three functional genes are found in the cluster (α_1, α_2, and ζ_2), three pseudo genes (evolutionary remains of genes that are not expressed due to inactivating mutations that impede the creation of a functioning globin protein) and one unidentified gene (a globin-like gene without inactivating mutations) (Figure 2.3) (Ribeiro & Sonati, 2008).

2.1.4.2 β-Like Genes Cluster

The β-like globin genes cluster is situated near the end of chromosome 11's short arm. The cluster spanned around 60 kilobytes, and contains five functional genes (β, δ, $^{G}\gamma$, $^{A}\gamma$, and ε) and one pseudo gene (Figure 2.3). The genes are all in the same 5′-3′ orientation inside each complex, and they are organized in the order in which they are expressed during development (Ribeiro & Sonati, 2008).

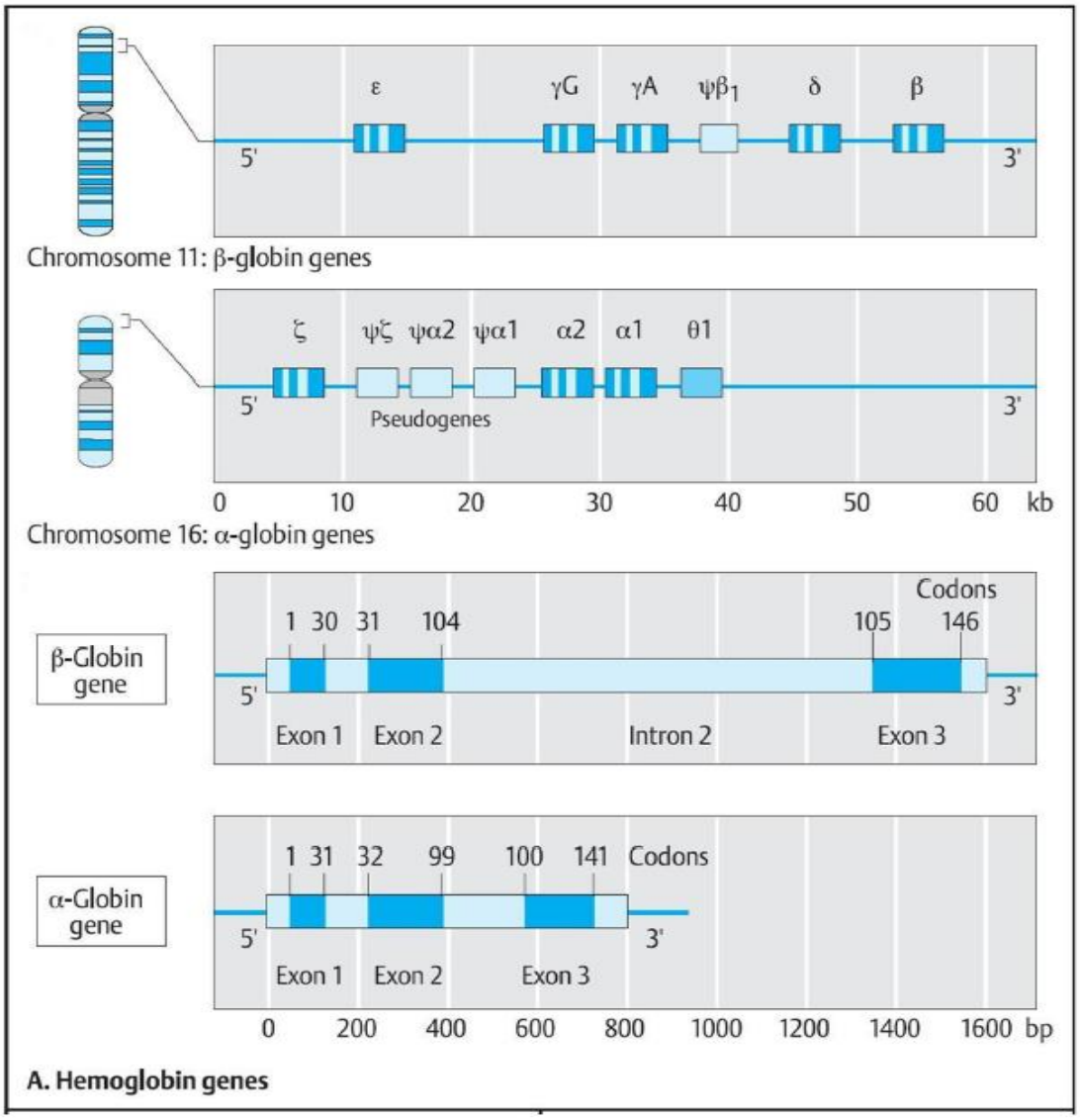

Figure (2.3): Structure of the α-globulin & β-globulin genes (Shawky & Kamal, 2012).

2.1.5 Hemoglobin Disorders

According to the World Health Organization (WHO), 7% of the world's population are hemoglobin disease carriers, and 300,000 to 500,000 infants are born each year with clinically severe hemoglobin abnormalities, the majority of whom are born in poor countries (World Health Organization & Thalassaemia International Federation, 2008).

2.1.5.1 Acquired Hemoglobin Disorders

Rather than being caused by a genetic defect in hemoglobin production or structure, acquired hemoglobin abnormalities are caused by other disease processes or external causes. Acquired disorders can be divided into two types: those characterized by defective globin chain synthesis (e.g. elevated HbF levels in states of erythroid stress and bone marrow dysplasia) and those characterized by toxins altering the structure of hemoglobin molecules (e.g. acquired Methemoglobinemia) (Forget & Bunn, 2013).

2.1.5.2 Inherited Hemoglobin Disorders

Hemoglobinopathies are hereditary hemoglobin abnormalities that represent a substantial public health concern in many regions of the world (Haddad, 2012a). Quantitative (defects in the rate of creation of one or more of the globin chains) or qualitative (production of distinct hemoglobin molecules) abnormalities can emerge from mutations in the genes governing the development of human hemoglobins. Thalassemias are the quantitative abnormalities, whereas structural hemoglobin variations are the qualitative abnormalities (Haddad, 2012a). The hereditary single-gene diseases alpha and beta thalassemia are the most prevalent in the world, with the highest frequency in places where malaria was or currently is endemic (Weatherall & Clegg, 2001).

2.2 Thalassemia

Thalassemia is a category of blood disorders caused by a hereditary deficiency in the rate of synthesis of one or more globin chains, resulting in an unbalanced globin chain production,

inefficient erythropoiesis, hemolysis, and varying degrees of anemia (Shawkat & Jwaid, 2019). Thalassemias are categorized α, β, γ, δ, δβ, or γδ- thalassemia.

Thalassemia is transmitted down from parent to child in an autosomal recessive inheritance pattern. This means that if both parents have a thalassemia gene, there is a 25% chance of having a child without thalassemia genes (a completely healthy child with normal hemoglobin), a 50% chance of having a child with only one gene affected, and a 25% chance of having an affected child with both thalassemia genes. To acquire the full-blown illness, it is required to inherit abnormal genes from both parents (Shawkat & Jwaid, 2019) (Figure 2.4).

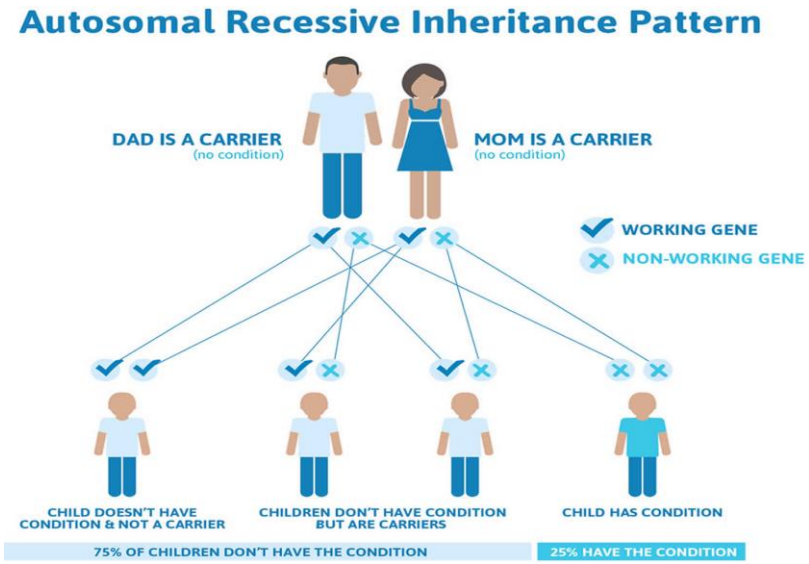

Figure (2.4): Autosomal recessive inheritance pattern (Source: https://healthjade.com/autosomal-recessive/).

Thalassemias are caused by a high number of mutations that cause aberrant globin gene expression, resulting in the complete absence or quantitative decrease of globin chain synthetization (Lulla et al., 2020; Marengo-Rowe, 2007; Muncie & Campbell, 2009). They are categorized into the following categories based on which globin chain is synthesized in lower amounts:

1. Reduced or absent α-globin chain: α-thalassemia
2. Reduced or absent β-globin chain: β-thalassemia

3. Reduced or absent δβ- globin chain: δβ -thalassemia
4. Reduced or absent γδβ globin chain: γδβ – thalassemia

Quantitative hemoglobin disorder refers to all forms of thalassemias. Only the α and β-thalassemia are prevalent enough to be of concern from a public health standpoint (Cao, Saba, Galanello, & Rosatelli, 1997; Galanello & Origa, 2010; Lulla et al., 2020).

Excess β-globin chains are generally caused by deletions within the α-globin gene cluster, which result in the loss of function of one or both α-globin genes at each locus. In most cases, α-thalassemia is a milder version of the illness. This is because there are four α-globin genes, each of which requires several mutations to cause a clinical effect. In addition, the unpaired β-globin chains are intrinsically less prone to precipitation as compared with unpaired α-globin chains in β-thalassemia (Lulla et al., 2020; Marengo-Rowe, 2007; Muncie & Campbell, 2009). On the other hand, because β-thalassemia is so frequent and generally causes severe anemia, it is the most significant of the thalassemia disorders (Cao & Galanello, 2010).

2.3 β-Thalassemia

β-thalassemia is caused by the lack of or absence of beta globin chain synthesis, resulting in an overabundance of alpha chains. β-thalassemia is a severe variant of the illness that causes severe anemia in both homozygous and compound heterozygous individuals (Cao & Galanello, 2010; Lulla et al., 2020; Muncie & Campbell, 2009; Saxena, Banerjee, & Aniyery, 2017). Beta thalassemia is one of the most common monogenic diseases, affecting over 150 million people in more than 60 nations (A. T. Taher, Weatherall, & Cappellini, 2018). β-thalassemia is so frequent and generally causes severe anemia, therefore, it is the most significant of the thalassemia disorders.

2.3.1 Epidemiology of β-Thalassemia

Thalassemia is considered the most common genetic disorder as 1.67% of the population area of Karachi, in Pakistan are heterozygous for thalassemia and 4.83% of the population area of Karachi, in Pakistan carry a globin defect (Rund & Rachmilewitz, 2005).

Thalassemia affects males and females evenly at equal rates and occurs in about 4.4% of every 10,000 live births (Muncie & Campbell, 2009). β-Thalassemia has been reported in almost every racial group on a periodic basis. However, 95% of patients are Asian, Indian, or from Eastern regions and 90% of them are in low- or middle- income countries (A. T. Taher et al., 2018).

The thalassemia gene affects 5 to 30% of people in various ethnic groups.In the Mediterranean basin countries, the carrier frequency for β-thalassemia ranges from 1% to 20%, and is rarely greater (World Health Assembly, 2019). Figure 2.5 shows geographical distribution of α-thalassemia around the world.

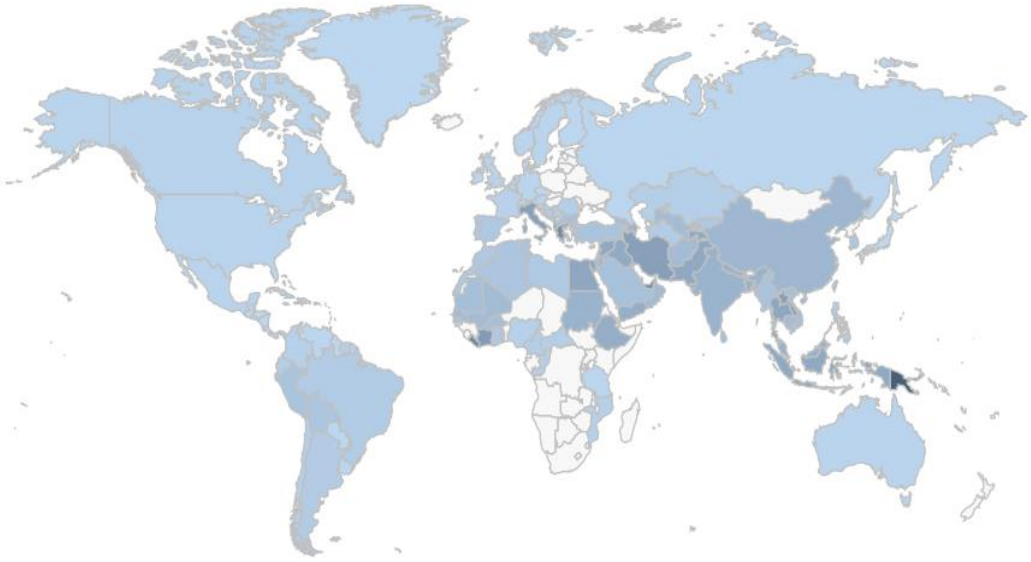

Figure (2.5): Geographical distribution of β-thalassemia around the world (Yazji & Mansour, 2011).

2.3.2 Thalassemia in Palestine

Despite the fact that Palestine is one of the Mediterranean basin nations where β-thalassemia major is common, little research on the disease's incidence and treatment has been conducted. There are more than 200,000 carriers of the thalassemia trait, which accounts for 4% of the population (Al Sabbah et al., 2017; Alaki & Bagher, 2013). The Palestinian Ministry of Health (PMOH) reported an increase in the life expectancy of Palestinian thalassemic patients from 7-8 years in 1996 to 22 years in 2017 (Palestinian Ministry of

Health, 2018). According to reports provided by the PMOH in 2018, there were 526 beta-thalassemia major patients in the West Bank and 309 patients in the Gaza Strip. The implementation of mandatory pre-marital testing law in 2000 was a successful strategy in lowering new thalassemia cases from 40 per year before 2004 to less than 10 per year since 2004. In addition, just a few cases of β-TM have been documented since 2013 (Palestinian Ministry of Health, 2018).

The Thalassemia Patients' Friends Society (TPFS) which is a non-profit organization established in 1996 in order to provide the care and support to Palestinian thalassemia patients reported that in 2020 75% of thalassemia patients were between 15-29 years old, 60% had a hemoglobin level equal to or less than 8 g/dL, and 77% had osteoporosis.

2.3.3 Clinical Classification of β-Thalassemia

Because the degree of imbalance between the alpha and non-alpha globin chains affects the clinical severity of β-thalassemia, it may be divided into three categories based on the severity of the symptoms:

2.3.3.1 β-Thalassemia Minor

The β-thalassemia carrier conditions clinically asymptomatic and is caused by heterozygosity for β-thalassemia and is characterized by certain hematological characteristics (Lahiry, Al-Attar, & Hegele, 2008).

2.3.3.2 β-Thalassemia Intermedia

It is defined as a clinically and genotypically diverse set of thalassemia-like illnesses that range in severity from asymptomatic carrier to severe transfusion-dependent type (TDT) (Lulla et al., 2020; Muncie & Campbell, 2009; Saxena et al., 2017).

2.3.3.3 β-Thalassemia Major

β-thalassemia major is also known as Cooley's anemia or Mediterranean anemia. It is a severe transfusion-dependent anemia caused by homozygous or compound heterozygous status for a recessive mendelian disease that affects people all around the world, not just in the Mediterranean Region (Galanello & Origa, 2010; Lulla et al., 2020; Muncie & Campbell, 2009). Patients with β-thalassemia major are generally normal hematologically at birth, with normal globin chain synthesis and enough HbF (α2γ2). As a result, when these babies need to replace their fetal RBC (fetal to adult Hb switch) with cells that contain primarily HbA (α2β2), a deficiency in β-globin production is visible. Most severe types of β-thalassemia manifest within the first year of infant life because the primary transition from HbF to HbA occurs during the first year of life (Sankaran, Xu, & Orkin, 2010)**.**

β-thalassemia major demand a lifelong regular blood transfusion (Muncie & Campbell, 2009). Untreated thalassemia major eventually leads to death usually by heart failure (Dharmesh Chandra, Anita, Purnima, Poonam, & Jyoti, 2017). Universally, β-Thalassemia can be classified into two categories; transfusion dependent thalassemia (TDT) and non-Transfusion dependent thalassemia (NTDT) depending on the mutation if it is in a homozygous or a heterozygous state (M. D. Cappellini & Motta, 2017).

2.3.4 Pathophysiology of β-Thalassemia

In β-thalassemia, the fundamental molecular flaw causes either no (βo) or diminished (β+) beta chain formation; nonetheless, chain synthesis continues at a normal pace. Diminished generation of adult hemoglobin (HbA: α2 β2) is the initial effect of reduced -chain production. The second effect is unbalanced globin chain synthesis, in which chain synthesis progresses at a normal pace, resulting in an excess of chain in the erythrocytes. Excess chains are unstable and precipitate in red cell precursors in the bone marrow, resulting in massive intracellular inclusions that obstruct red cell maturation, function, and survival (Figure 2.6) (E. George & Ann, 2010; Lulla et al., 2020).

As a result of these intracellular inclusions interfering with red-cell maturation, red-cell precursors are destroyed intramedullary, resulting in inefficient erythropoiesis. However, mature red cells that reach the circulation carry chain inclusions that obstruct their transit

through the microcirculation, notably in the spleen, and therefore extra medullary red cell death becomes the normal. As a result, anemia in β-thalassemia is caused by both inefficient erythropoiesis and a reduction in red cell survival (Ginzburg & Rivella, 2011).

The increased erythropoietin production from the kidney stimulates erythropoiesis. However, the severe inefficient erythropoiesis produces significant bone marrow enlargement and hyperplasia, resulting in not only major abnormalities of the skull and long bones, but also increased iron absorption and gradual accumulation of iron in tissues. Extra-medullary erythropoietin tissue, mainly in the thorax and Para-spinal area, may be stimulated by increased erythropoietin production (Galanello & Origa, 2010; Lulla et al., 2020; Muncie & Campbell, 2009). Furthermore, because the spleen is continually assaulted with aberrant RBC production in thalassemic individuals, it hypertrophies, and splenomegaly develops as a hallmark of the disease. The anemia is exacerbated and worsened by the increase in plasma volume caused by shunting through enlarged marrow and increasing splenomegaly (Ginzburg & Rivella, 2011; Lulla et al., 2020). There have been several reports of thalassemic erythrocyte membrane anomalies. Excess α-globin chains and their breakdown products inside the RBC membrane and its skeleton have been linked to anomalies in important red-cell membrane cytoskeleton proteins such as spectrin, band 3, and band 4.1 (Ginzburg & Rivella, 2011). Through the RBC-platelet interaction, these anomalies were found to impair not just RBC survival, but also platelet function (Vallés et al., 2002).

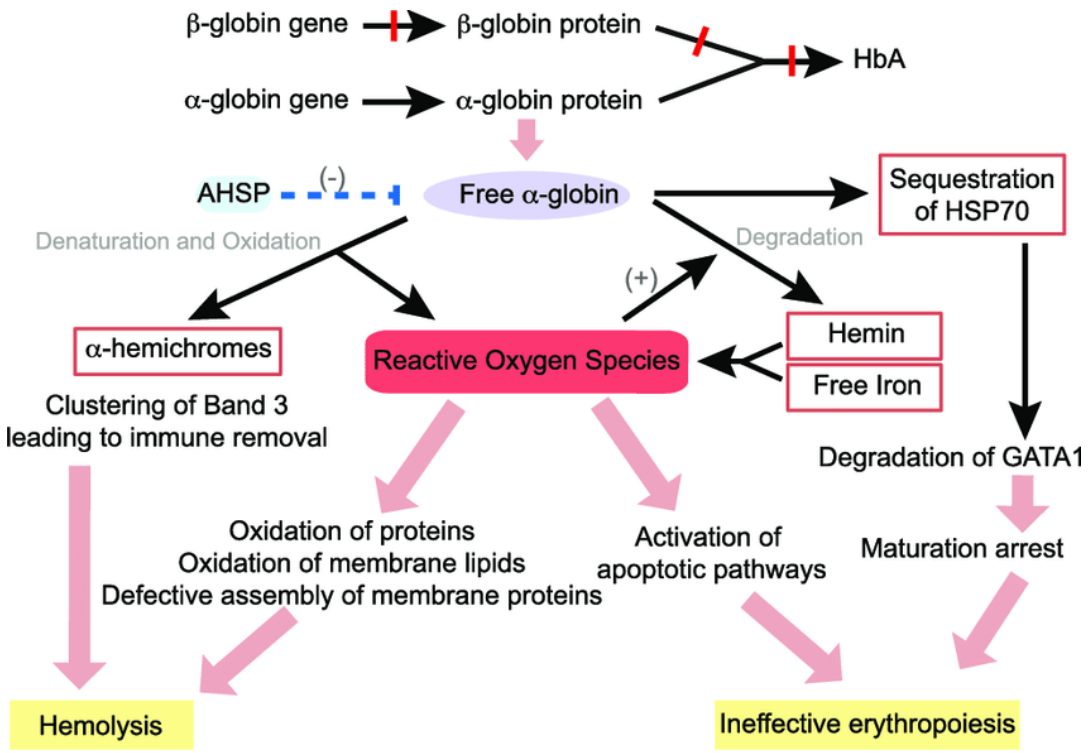

Figure (2.6): Pathophysiology of β-thalassemia (Fibach & Rachmilewitz, 2017).

2.3.5 Diagnosis of β-Thalassemia

2.3.5.1 Clinical Diagnosis

Thalassemia major is frequently suspected in a child under the age of two who has severe microcytic anemia, moderate jaundice, and hepatosplenomegaly. Thalassemia intermedia manifests later in life with comparable but milder clinical features. Carriers are normally asymptomatic; however, mild anemia might occur in certain cases (M. D. Cappellini & Motta, 2017; Lulla et al., 2020).

Clinically, β-TM appears between the ages of 6 and 24 months. A hemoglobin level of 6.0 g/dL or below cause afflicted infants to underperform and become pale. Feeding problems, diarrhea, irritability, recurrent bouts of fever, and abdominal enlargement owing to spleen and liver enlargement are all possible signs. Severe microcytic anaemia, mild jaundice, and hepato-splenomegaly have all been reported (Bajwa & Basit, 2021).

2.3.5.2 Hematologic Diagnosis

Reduced Hb level (7 g/dL), mean corpuscular volume (MCV) >50 and <70 fl, and mean corpuscular Hb (MCH) >12 and <20 pg describe this condition. RBC morphologic abnormalities (microcytosis, hypochromia, anisocytosis, poikilocytosis, spiculated tear-drop and elongated cells) and nucleated RBCs are seen in afflicted patients' peripheral blood smears (i.e., erythroblasts). The quantity of erythroblasts is proportional to the severity of anemia and is significantly enhanced following splenectomy (M. D. Cappellini & Motta, 2017; Galanello & Origa, 2010; Lulla et al., 2020).

2.3.5.3 Qualitative and Quantitative Hb Analysis

The amount and type of Hb present is determined using cellulose acetate electrophoresis and DE-52 micro chromatography or HPLC. The Hb pattern in β-thalassemia differs depending on the kind of β-thalassemia. HbA is nonexistent in beta0-thalassemia homozygotes, while HbF accounts for 92-95% of total Hb. HbA levels range from 10% to 30% in beta+-thalassemia homozygotes and beta+/beta0 genetic compounds, while HbF values range from 70% to 90%. In beta-thalassemia homozygotes, HbA2 is variable, and it is increased in beta thalassemia minor. Other hemoglobinopathies (S, C, E, O Arab, Lepore) that may interact with β-thalassemia can be detected using electrophoresis and HPLC (M. D. Cappellini & Motta, 2017; Galanello & Origa, 2010; Lulla et al., 2020).

2.3.5.4 Molecular Genetic Analysis

The presence of a limited number of mutations in each ethnic group has made molecular genetic testing easier. PCR-based techniques detect commonly occurring mutations in the beta globin gene. Reverse dot blot analysis or primer-specific amplification are the most prevalent approaches, which employ a set of probes or primers that are complementary to the most frequent mutations in the group from which the afflicted individual came. Beta globin gene sequence analysis can be utilized to find mutations in the beta globin gene if targeted mutation analysis fails to detect the mutation (M. D. Cappellini & Motta, 2017; Galanello & Origa, 2010; Lulla et al., 2020).

2.3.6 Management and Treatment of β-Thalassemia

Individuals with thalassemia major and many intermedia patients require blood transfusions as part of their treatment. Transfusion treatment has two major goals: providing normal erythrocytes to reduce anemia risk and reducing inefficient erythropoiesis, effectively managing the downstream pathophysiological pathways in thalassemia. Thalassemia patient treatment in many countries has improved survival rates well into adulthood, owing to effective transfusion and chelation techniques, as well as follow-up protocols aimed at detecting and preventing problems to key organs as early as feasible. The standard of transfusion services should be protected by following the directives of the European Union (EU), the World Health Organization (WHO), the American Association of Blood Banks (AABB), and other international organizations, as well as taking into account national needs, resources, and the prevalence of infectious agents. Patients with β-thalassemia major, severe HbE-thalassemia, transfusion dependent HbH disease or HbH hydrops, and surviving Hb Bart's hydrops are all transfusion dependent thalassemia (TDT) patients who require frequent blood supplies. According to current standards, blood transfusions should be given every 2–5 weeks to maintain a pretransfusion hemoglobin level of 9–10.5 g/dL. The current standard for mean target hemoglobin is 12 g/dL, with a post transfusion Hb of 14–15 g/dL. Lower post transfusion Hb levels demand shorter transfusion intervals, but higher post transfusion Hb concentrations raise the risk of hyper viscosity and stroke (Lulla et al., 2020).

2.3.6.1 Chelation Therapy

For thalassemia patients, iron excess is the major cause of mortality. Hemochromatosis develops in non-transfused individuals as a result of increased dietary iron absorption in the intestine. Iron deficiency plays a significant role in mortality and organ harm. Chelators are used to treat iron excess. The type of chelation used has the following goals: prevention treatment, curative treatment, treatment in an emergency, therapy dose modification, and adherence therapy. The goal of iron chelation treatment is to keep dangerous non-transferrin bound iron out of the bloodstream while also removing iron from the body. Chelation therapy's most essential purpose is presumably to detoxify excess iron. It has been established that the presence of a chelator in the plasma can ameliorate certain symptoms of

iron overload, such as cardiac arrhythmia and heart failure, much before local tissue levels of iron have diminished (Lulla et al., 2020).

Specialists commend that iron overload be preserved when serum ferritin levels beat 1000 Ïg/L, which will happen after 10 to 20 red cell transfusions (Angelucci et al., 2014). Chelation therapy is frequently started among five and eight years of age. Deferoxamine (Desferal), subcutaneously or intravenously, has been the treatment of choice. Suggested dosage be contingent on the individuals age and the serum ferritin concentration (M.-D. Cappellini, Cohen, Porter, Taher, & Viprakasit, 2014). While this therapy is comparatively nontoxic, it is unwieldy and expensive.

The U.S. Food and Drug Administration newly approved oral deferasirox (Exjade) as another treatment. Adverse properties of deferasirox were passing and gastrointestinal in wildlife, and no cases of a granulocytosis were described (Table 2.2).

Table (2.2): Differences between drug administrations used in chelation therapy.

Compound	Desferrioxamine (DFO)	Deferasirox (DFX)	Deferiprone (DFP)
Route	SC/IV 8-12hrs 5days a week	Once orally	Orally once
Iron log binding capacity	26.6	22.5	19.9
Lipid solubilty	Low	High	Intermediate
Half life	20-30 min	12-16 hrs	3-4 hrs
Max plasma level	7-10uM	80uM	90-450uM
Recommended dose(mg/kg/d)	30-60 5- 7 weeks	20-40 once daily	75-100 3 divided dose
Chelation efficacy	13	27	7
Therapy	First line for TM	If another chelator ineffective	First line for TM and NTDT

2.3.6.2 Bone Marrow Transplantation

The only cure for β-thalassemia major is bone marrow transplantation in infancy. Low-risk patients, defined as those with no hepatomegaly, no portal fibrosis on liver biopsy, and frequent chelation treatment, or at most two of these anomalies, had a favourable prognosis

with hematopoietic stem cell transplantation (E. George & Ann, 2010; Muncie & Campbell, 2009).

2.3.6.3 Splenectomy

Can be explored if hypersplenism results in a significant rise in transfusion needs. It should be postponed as long as possible to avoid life-threatening infections, pulmonary hypertension, and thrombo-embolic consequences. Currently, interventions such as fetal haemoglobin induction, antioxidants, and stem cell gene therapy are being studied (E. George & Ann, 2010).

2.3.7 RBC Phenotyping

The assessment of antigen expression on RBCs surface using serology is called Phenotyping, it is used to establish the donor's or recipient's blood group antigens (Hendrickson & Tormey, 2016), this method depends on hemagglutination (Swati Kulkarni & Maru, 2020), by binding anti-sera to specific antigens (Quirino, Colli, Macedo, Sell, & Visentainer, 2019). Antibody-based agglutination is the conventional method of phenotyping red blood cell (RBC) antigens. There are two major disadvantages to this serologic technique. The first is the antigen testing's limited opportunity. Blood donation qualification laboratories in the French Blood Service, the Establishment Francais du Sang (EFS), tested all blood donations for ABO, Rhesus (RH1), and KEL (KEL1), but only a small percentage of donations are tested for other clinically significant antigens, such as FY1, FY2, JK1, JK2, MNS3 (S), and MNS4 (s). The extended process time is the second disadvantage of antibody-based agglutination. For these reasons, traditional hemagglutination is unsuitable for high-throughput phenotyping of blood groups (Paris et al., 2014).

Although serologic blood transfusions are typically safe, alloimmunization is a dreaded complication that can create issues ranging from a delayed haemolytic transfusion response to difficulty getting matched RBCs. When several antigen-negative RBC products are required for patients with alloantibodies or sickle cell disease who require long-term transfusion, the risk of alloimmunization is highest (Paris et al., 2014). In such cases, standard procedure is to do thorough RBC phenotyping, however utilizing traditional

serologic donor screening methods, obtaining a significant amount of extensively typed blood units would never be possible (Paris et al., 2014).

Kutner et. al. (2014) conducted a review that summarized the evidence regarding the role of blood genotyping in improving clinical and long-term outcomes in chronically transfused patients, blood transfusions. The review showed how certain problems, such as alloimmunization against RBCs might complicate patient care and how the use of phenotype-matched blood units for transfusion and routine phenotyping of blood recipients has both been shown to reduce the incidence of red cell alloantibodies in chronically transfused patients. However, the review showed that thorough phenotyping is costly, time-consuming, and in some cases impossible. This review summarized significant findings on red cell alloimmunization, the current and possible future advantages of blood group genotyping, and how molecular typing is being integrated into the blood bank's routine to enhance clinical and long-term outcomes in chronically transfused patients (Kutner et al., 2014).

Owaidah, Naffaa, Alumran, and Alzahrani (2020) conducted a study that focused on the frequency of major blood group and phenotype in Eastern region of Saudi Arabia and compare to anther population. They followed by collecting samples from voluntary donors and analyzed the samples using the gel microtube technique. The study reported that for the Rh blood system antigens, the e antigen was identified in 97% of donors, followed by c (86%). On the other hand, the Kell system's k antigen was identified in all donors, while the Kell (K) antigen was only found in 8% of them. Furthermore, the study reported that the K+k- phenotype was not found. Surprisingly, the null phenotype FY(a-b-) was discovered in 61% of Duffy blood group donors. Furthermore, M+N-S+s+ was the most frequent phenotype in the MNS blood group system, accounting for 24% of all cases. Due to the heterogenous ethnic backgrounds of individuals who live in the Eastern area of Saudi Arabia, the frequency of blood group phenotypes varied from that of other groups. The findings of this study could be used to establish a local donor registry to assist in the provision of antigen-negative blood for patients with unexpected antibodies, or to develop an in-house antibody identification panel to supplement the commercial panel for confirming antibody identification results (Owaidah, Naffaa, Alumran, & Alzahrani, 2020).

Abdelrazik et al. (2016) A study performed in Fayoum University Hospital analyzed 188 samples from multi transfused patients by using DiaMed-ID micro typing system for

alloantibody identification. The study reported that 7.98% of patients had alloimmunization. Anti-D was the most prevalent alloantibody identified in eight of the 188 patients (4.25%), followed by anti-C in two patients (1.1%), anti-E in two patients (1.1%), anti-c in two patients (1.1%), anti-Fy^a in two patients (1.1%), anti-K in one patient (0.53%), and an unknown antibody in one patient (0.53%). The study also reported that female patients, patients with β-thalassemia intermedia, splenectomized patients, RhD-negative patients, and patients who began blood transfusion after the age of three were all shown to have higher rates of alloimmunization. The study underlined the need of developing countries having a cost-effective thalassemia transfusion plan and that antigen typing of red blood cells prior to transfusion and the provision of antigen-matched or antigen-negative blood can be provided to all immunized multiple transfused patients. The study also concluded that another way to reduce alloimmunization could be to start transfusion treatment as soon as possible following a diagnosis (Abdelrazik et al., 2016).

2.3.8 Genotyping of RBC antigens

Since the 1990s, specialized facilities have used in-house PCR-based tests to genotype blood groups for a single or small number of single nucleotide variants (SNVs). PCR-restriction fragment length polymorphism (RFLP), sequence-specific primer (SSP)-PCR, single-nucleotide primer extension, and, more recently, real-time (RT)-PCR and high-resolution melt (HRM) studies are examples of these approaches (McBean, Hyland, & Flower, 2014). Determination of antigens using deoxyribonucleic acid (DNA) with high accuracy can make a great difference and lower the rate of alloimmunization, even though the serological phenotyping depending on agglutination has been used for a long time it faces several limitations (Gholamrezazade, Amirizadeh, & Oodi, 2021), and that is why genotyping has been proven to be efficient and advantageous in comparison to phenotyping in some cases (Swati Kulkarni & Maru, 2020), as it overcomes the limitations that make phenotyping not a much reliable method. This method can be used in recently transfused patients, patients with interfering alloantibodies or autoantibodies, discrepant serologic typing results or when anti-sera for certain rare antigens are not available. The method is also efficient in identifying RBCs units with rare or uncommon antigen phenotypes and provide antigen negative units. Despite their accuracy, these assays are limited by their poor throughput, limited multiplex capacity, and the amount of human processes required (McBean et al., 2014).

Lately, there have been many methods for RBCs genotyping and they vary on multi levels like their complexity and single nucleotide polymorphisms (SNPs) can be determined by a variety of genotyping methods. The most modern technique for DNA analysis is the microarray, a method for large-scale erythrocyte genotyping (Majid Naderi et al., 2013).

Bakanay et. al. (2013) in Turkey performed a cross-sectional study in 39 multi-transfused patients using DNA samples for testing blood group genotype. The study found nineteen out of the 37 patient (51%) had discrepancies between phenotype and genotype and the differences in 12 patients showed the potential to cause alloimmunization. The study found that blood group genotyping is critical in the treatment of chronically transfused patients, especially if they phenotypes were not determined before transfusion (Bakanay et al., 2013).

Lilian Castilho (2002) in Brazil a cross-sectional study selected 10 patients from Homocentric (Unicamp, Campinas, Brazil) who received RBC units matched for antigens in the Rh, Kell, Duffy, and Kidd blood group systems. Anti-K was present in one patient, anti-E was present in two patients, anti-c was present in three patients, anti-Fy^a was present in one patient, and anti-Jk^a was present in three patients. These individuals had been transfused at least three times, and blood samples from each transfusion had been phenotyped. Each patient's most recent blood sample was additionally genotyped for *RH, KEL, JK,* and *FY*. DNA samples were prepared and tested by multiplex-PCR. They found that nine of the ten samples had different phenotypes and genotypes. The study concluded that genotyping proved critical in establishing the real blood groups of many polytransfused β-thalassemia patients, as well as in identifying suspected alloantibodies and selecting antigen-negative RBCs for transfusion (L. Castilho, Rios, Pellegrino, S, & F, 2002).

Paris et. al. (2014) Standard hemagglutination-based test methodologies limited appropriateness for large-scale automated screening of red blood cell antigens significantly inhibits blood banks' capacity to offer comprehensively phenotype-matched blood. With a greater knowledge of the molecular basis of blood antigens, single-nucleotide polymorphisms in genomic DNA may now be used to predict blood group phenotypic. Blood banks will be able to give ideally matched donations thanks to the development of DNA-typing techniques for antigen screening in blood donation qualifying laboratories. We developed an automated genotyping method for blood donor screening that uses 96-well DNA microarrays and a first panel of eight single-nucleotide polymorphisms to detect 16 alleles in four blood group systems (Kell, Kidd, Duffy, and MNS). The goal of this study

was to test the system on 960 blood donor samples with known phenotypes. Between anticipated and serologic phenotypes, the study found a high concordance rate (99.92%; 95% CI, 99.77-99.97%). The results of the study showed that the utilized test can accurately predict phenotype at the DNA level at a cheap cost. Other blood group indicators, blood units for IH panels, or antigens from other systems might simply be added to this system to identify donors with rare blood types or antigens from other systems (Paris et al., 2014).

A review on Emerging strategies of blood group genotyping for patients with hemoglobinopathies conducted by **Belsito, Magnussen, and Napoli (2017)** reported that traditional serologic antigen typing has been extended or replaced by a number of high-throughput DNA tests and that DNA-based typing techniques are simple to automate and multiplex, and they give accurate patient information. Furthermore, the authors concluded that molecular genotyping promises to be less expensive because it is not dependent on serologic immunoglobulin reagents and that extended genomic typing may be beneficial to patients with hemoglobinopathies depending on minor antigenic variations between donors and patients, which might reduce post-transfusion problems. The review also reported that compatibility between the patient and the donor may have gone beyond the Rh/Kell phenotype (A. Belsito et al., 2017).

Belsito et. al. (2015) chose 225 blood donors and 50 transfusion-dependent patients from the Second University of Naples' Division of Immuno-hematology. Blood samples were genotyped for 38 red blood cell antigens with HEA Bead Chip TM kit and phenotypic variations using the NEO Immucor automated system for traditional phenotype analysis. The comparison was done for RhCE and Kell antigens, which may be typed using both techniques. They found that for donors, there were an excellent correlation between serological and molecular techniques, with 99.5% concordance and 0.5% discordance. However, only 46.0% of patients were concordant, whereas 54.0% (27/50) were discordant; disparities were 46.0% and 8.0% for the RhCE and Kell systems, respectively. Polymorphisms in the RhCE, Kell, Duffy, Colton, Lutheran, and Scianna loci were also discovered in donors and patients using molecular genotyping. The study concluded that polytransfused individuals can benefit from blood group genotyping and that molecular analysis verifies and extends the results of serological tests, allowing to get a better match. The authors also concluded that the molecular test might be utilized to avoid alloimmunization in patients who rely on blood transfusions (Angela Belsito et al., 2015).

Kulkarni et. al. (2017) demonstrated in their study that phenotyping depending on hemagglutination in thalassemia patients faces several limitations that genotyping can overcome. The study included 200 thalassemia patients, RBC antigens of these patients were tested using serological phenotyping method and using genotyping through PCR for the common antigens (C, c, D, E, e, Fy^a, Fy^b, Jk^a, K, k, M, N, S, and s). The results showed that most discrepancies between phenotyping and genotyping were in Rh, Duffy, Kell and Kidd blood group systems (S. Kulkarni et al., 2018).

Gholamrezazade et. al. (2021) genotyping of the MNS blood group system was conducted on 104 patients. Comparison using agglutination phenotyping method and genotyping using PCR for M, N, and S antigens revealed some discrepancies between phenotyping and genotyping (Gholamrezazade et al., 2021).

Castilho et. al. (2000) showed that genotyping is more accurate than phenotyping for determining blood group antigens in polytransfused patients. The study included 50 β-thalassemia patients. First these patients were phenotyped using agglutination, and after that DNA was prepared and tested for Kell, Kidd, and Duffy blood group systems by PCR. The genotyping was performed without any previous information about the phenotyping results. The study showed phenotyping – genotyping discrepancies in 5 cases (Lilian Castilho et al., 2000).

Bakanay et. al. (2013) collected 39 DNA samples and used them for RBC antigens genotyping. The results of genotyping were compared to previously obtained results of phenotyping by hemagglutination. Discrepancies between genotyping and phenotyping were seen in 51% of the study subjects and 12 patients had the potential of alloimmunization (Bakanay et al., 2013).

2.3.9 Microarray

Microarray-based genotyping technologies were created in the twenty-first century to overcome these limitations. The Blood Chip Reference from Progenika (Grifols) and the Bead Chip from Bio Array Solutions (Immucor), which were among the first to be created and get regulatory approval, are discussed here. Furthermore, SNV typing platforms based on technologies capable of even greater throughput, such as Luminex xMAP and single

nucleotide primer extension followed by matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), have just become commercially accessible (McBean et al., 2014).

Microarray genotyping depends on the detection of a colour that is generated as a result of contacting the target DNA with fluorescently labelled oligonucleotide probes (M Naderi et al., 2013) as it is hybridized by base pair matching to its cognate recognition probe, put on a plate or a DNA chip which is an arrangement of chemically bounded DNA molecules to fin gird of surface (Wang et al., 2015). The results are read by an automated system which provides the results in graphs or tables (Gholamrezazade et al., 2021). One of the advantages of Microarray is its ability to cover much sequence space with little spots (Hoheisel, 2006). This method is the one used in our study.

2.3.10 Complications of β-Thalassemia

Most living forms, as well as normal human physiology, require iron. Iron intake for children is 8 mg per day, 11 mg per day for adult males, and 18 mg per day for adult females (Food Nutrition Board, 2001). In industrialized countries, the majority of well-nourished persons have 4 to 5 g of iron in their bodies. About 2.5 g of this amount is in hemoglobin, with the remainder being stored as ferritin (Johnson-Wimbley & Graham, 2011).

The majority of iron absorbed from digested food or supplements is absorbed in the duodenum by duodenal lining enterocytes (Johnson-Wimbley & Graham, 2011). Iron is immediately combined in the blood plasma with a beta globulin, apo transferrin, to generate transferrin, which is then carried in the plasma once it is absorbed from the duodenum (Johnson-Wimbley & Graham, 2011). Because the iron in transferrin is loosely bonded, it may be released to any tissue cell at any location in the body. Ferritin, the primary iron storage protein complex found largely in the liver, reticuloendothelial cells, and erythroid precursors of the bone marrow, stores excess iron in the blood (Haddad, 2012b).

The human body's ability to eliminate excess iron is restricted. Because there is no natural channel for excreting excess iron, patients who consume more iron are at risk of harmful and increasing accumulation of bodily iron reserves, which leads to an abnormally high level of iron in tissues and, as a result, deadly tissue damage. Iron is accumulated in the liver, heart,

and a subset of endocrine organs' parenchymal cells (Ali T Taher & Saliba, 2017). Iron overload is caused by accelerated erythrocyte catabolism, which occurs in individuals who get frequent blood transfusions, such as those with thalassemia major or sickle cell disease. In terms of iron content, one unit of packed RBCs utilized in the transfusion regimen contains around 200 mg. Thus, a 6-year-old thalassemic child getting 60-75 units of packed RBCs is projected to collect 12-15 grams of extra iron, compared to 3-4 grams in normal non-transfused individuals, with regular blood transfusion. Iron accumulates initially in the reticuloendothelial macrophages, then in the parenchymal cells (Brittenham et al., 1994; Kushner, Porter, & Olivieri, 2001). This causes tissue damage and fibrosis, which eventually leads to organ damage (Nancy C. Andrews, 1999; N. C. Andrews, 2000; Waldmeier et al., 2010).

Iron overload problems in transfused thalassemic individuals are a major cause of morbidity. Growth retardation and failure or delays of sexual maturation are two complications of iron overload in children. Later, iron overload problems include heart (dilated myocardiopathy or, less occasionally, arrythmias), liver (fibrosis and cirrhosis), and endocrine gland involvement (diabetes mellitus, hypogonadism, and insufficiency of the parathyroid, thyroid, pituitary, and, less commonly, adrenal glands) (Forget & Bunn, 2013; Galanello & Origa, 2010; Haddad, 2012b; Marengo-Rowe, 2007). Hypersplenism, chronic hepatitis (caused by infection with viruses that cause hepatitis B and/or C), HIV infection, venous thrombosis, and osteoporosis are some of the other consequences (Sayani & Kwiatkowski, 2015). Patients with a fatty liver, viral infection, or iron excess are more likely to develop hepatocellular carcinoma. Individuals who have not been consistently transfused generally die before reaching their third decade; however, those who have been regularly transfused and treated with adequate chelation can live into the age of 40. The most serious life-threatening consequence of iron overload in β-thalassemia is cardiac illness induced by myocardial siderosis. In fact, cardiac problems are the leading cause of mortality in 71% of β-thalassemia major patients (Eshragi, Tamaddoni, Zarifi, Mohammadhasani, & Aminzadeh, 2011; Galanello & Origa, 2010).

Even though the most common endocrine disorder in thalassemia patients is hypothyroidism (Asad, Ghazanfari, Naleini, Sabagh, & Kooti, 2016) which could be due to the deposition of iron on the thyroid gland (Abdulzahra, Al-Hakeim, & Ridha, 2011), hyperthyroidism could

also be seen (Ragab, Hamdy, Shaheen, & Yassin, 2013). Hypothyroidism degree depends on different variables including age and rate of blood transfusion (Asad et al., 2016).

Growth hormone is commonly reduced in thalassemia patients and that is thought to be due to neurosecretory dysfunction because of iron over load (Asad et al., 2016). Exogenous administration of GH is used in thalassemia patients (Mahachoklertwattana, Yimsumruay, Poomthavorn, Chuansumrit, & Khlairit, 2011).

ALT and AST are enzymes that are produced in the liver and indicates liver damage. Also, ALP is an enzyme that represents several tissues including the liver, bone and the kidneys. It was reported from previous studies that ALT, AST, and ALP levels are elevated in multi blood transfused thalassemia patients (Al-Moshary et al., 2020).

The two tests that asses the kidney function are a two body waste products (Mahachoklertwattana et al., 2011), Creatinine and urea (Majid Naderi et al., 2013). Some previous studies have showed that thalassemia patients have low Creatinine level because it depends on the body mass index which is low in thalassemia patients. And urea is thought to be increased because of the deposition of iron in the kidneys (Jafari, Lahsaeizadeh, Jafari, & Karimi, 2008).

Thalassemia patients suffer from vitamin D deficiency despite getting adequate amount of sunshine or vitamin D complements (Soliman & Kalra, 2013). Due to the hypothyroidism that is commonly seem in thalassemia patients they suffer from abnormal calcium haemostasis `(Golub & Boesze-Battaglia, 2007).

PTH is an 84 amino acid protein which its main role is to regulate calcium homeostasis (Wojda & Donahue, 2018). Due to the iron overload in thalassemia patients, hypoparathyroidism is induced and so low levels of PTH (Goyal, Abrol, & Lal, 2010).

Laksmitawati et al. (2003) demonstrated significant increase in serum iron ferritin, AST, ALT, and bilirubin. Non-transfused thalassemia intermedia patients show minor indicators of oxidative stress and increased hemoglobin degradation, but no major tissue or cell damage, according to the findings. This image is very different from that of transfusion-dependent thalassemia major patients, who have a large drop in antioxidants and thiols, as well as considerable iron overload and cell damage. Long-term transfused patients have an even worse situation. In Indonesia, iron chelation after transfusion is insufficient since it is

usually performed just once with transfusion (with a few exceptions). As a result, frequent transfusions (on average one per month) and insufficient chelation (one therapy per month) appear to be a primary factor in the poor health of transfusion-dependent thalassemia patients in Indonesia (Laksmitawati et al., 2003).

2.3.10.1 Alloimmunization

In the absence of stem cell transplantation, β-thalassemia is managed with lifelong red blood cell (RBC) transfusions to maintain a hemoglobin (Hb) level of 9.5-10.5 g/dL (Thalassemia International Federation (TIF), 2021). Despite being a life-saving procedure, blood transfusion carries the danger of alloimmunization to red cell antigens. Alloimmunization of red blood cells (RBCs) occurs when the antigens of donor and recipient red blood cells differ genetically. The formation of alloantibodies and autoantibodies against RBC antigens complicates RBC cross-matching in the lab, reduces the in vivo survival of transfused red cells, delays safe transfusions, and may hasten iron overloading. Globally, alloimmunization rates between transfusion-dependent thalassemia patients ranged between 2.9-37% (Franchini et al., 2019).

Genetic factors, factors that modulate the immune responses like infections, patients' immune status, number of the previously transfused bags, and age at which the patient started getting transfused blood are all factors that can affect the rates of alloimmunization (El-Beshlawy, Salama, El-Masry, El Husseiny, & Abdelhameed, 2020; Gehrie & Tormey, 2014). Even though alloimmunization is common between adults, it is rare between infants from 1 to less than 12 months (El-Beshlawy et al., 2020; Molina-Aguilar et al., 2020; Tamai, Ohto, Takahashi, Kitazawa, & Consortium, 2021) . As a preceding study by Flocs established that regardless the exposure to several red blood antigens infants do not produce alloantibodies (Dean & Dean, 2005). Several studies followed demonstrating that patients who had the transfusion therapy onset at an early age (<1-3 years) in comparison to patients who did not start the transfusion therapy at an early age have shown low rates of alloimmunization (El-Beshlawy et al., 2020). This could be attributable to some immune tolerance against alloimmunization (Singer et al., 2000). Furthermore, based on previous research, the most alloantibodies were directed against Kell and Rh antigens (El-Beshlawy et al., 2020), and in infants, anti-M is more common than in adults (El-Beshlawy et al., 2020).

Al-Riyami and Daar (2019) In the Eastern Mediterranean Region, a review was conducted on available studies on alloimmunization rates and risk factors in transfusion dependent and non-transfusion dependent-thalassemia. There was a total of 17 publications discovered. Alloimmunization rates among transfusion-dependent β-thalassemia patients varied from 2.87 to 30%, whereas rates among TI patients ranged from 6.8 to 19.5%. RBCs that are ABO and RhD matched are used in the majority of centers. Anti-K and anti-E antibodies were the most commonly reported antibodies. Age at the time of transfusion, gender, history of Splenectomy, length of transfusion, and quantity of units transfused were all included as risk factors. The percentage of those who developed autoantibodies ranged from 0.1 to 45%. The review showed that in thalassemia patients, varying alloimmunization rates and risk variables were found. Furthermore, data regarding TI patients were insufficient, therefore, the study concluded that more research is needed to determine the incidence of alloimmunization, cross-match requirements, and the impact of genotyping. In addition, the review reveled that availability of blood bank facilities and specialist knowledge is required for the transfusion assistance of patients with thalassemia (Arwa Z. Al-Riyami & Daar, 2019).

Gholami et. al. (2021) included 1147 alloimmunized β-thalassemia major patients. First antibodies screening and identification tests were performed in addition to phenotyping and genotyping for the Rh, Kell, Kidd and Duffy blood groups. The study showed that the most common alloantibodies were directed at the Rh (48.5%) and at the Kell (23.7%) while only (8.5%) of the study subjects formed alloantibodies directed against minor blood group antigens (Gholami, Shahidi, Tabibian, Naderi, & Dorgalaleh, 2021).

2.3.10.2 Transfusion-Transmitted Infections

Transfusion-transmitted infections (TTIs), which include viral, bacterial, and parasitic infections, represent a significant danger of regular blood transfusion. All given blood should be tested for HIV, HBV, HCV, and syphilis, according to the WHO and the TIF. Screening for other local infectious illnesses such as human T lymphotropic virus (HTLV1 and 2) and Chagas disease is also recommended in some countries. In certain locations, emerging infections such as the Zika virus, variant Creutzfeldt-Jakob disease, and hepatitis E have led to further screening recommendations. Over the last few decades, strict donor blood

screening and quality-control methods have considerably improved blood safety, resulting in a very low risk of TTIs in nations with well-regulated blood supplies. TTI risk among donors in the United States is less than one in one million for HIV, HCV, and HTLV, and one in 300,000 for HBV. In recent decades, the prevalence of transfusion-transmitted syphilis infections in the United States and Australia has been so low that it has raised controversy about the necessity to test blood donors for syphilis.

2.3.10.3 Other Blood Transfusion-Related Complications

Acute complications of blood transfusions include febrile non-hemolytic transfusion reactions (which occur in 1% of transfusions), allergic reactions (which occur in 1% of transfusions), transfusion-related acute lung injury (which occurs in 0.01% of transfusions), and intravascular acute hemolytic transfusion reactions (which occur in 0.004% of transfusions).

Extra vascular delayed hemolytic transfusion responses (which occur in 0.4% of transfusions) and transfusion-associated graft-versus-host disease (which is extremely rare) are examples of delayed adverse reactions to blood transfusions (Ogedegbe, 2002). There have also been reports of bone disease and osteoporosis, which have a multifactorial origin (F. T. Shah et al., 2019). Although autoantibodies against RBCs are uncommon, they can cause clinical haemolysis and make cross-matching more challenging. Patients with autoantibodies are more likely to require blood transfusions and are more likely to require immunosuppressive medicines or splenectomy. In the context of alloantibodies, the term "clinically significant" can refer to either an antibody that causes an overt clinical haemolytic transfusion reaction (fever, chills, hemoglobinuria, etc.) or an antibody that causes no overt clinical symptoms but is associated with laboratory signs of hemolysis (increased bilirubin, decreased haptoglobin…etc.) or an antibody that isn't linked to any clinical or laboratory symptoms of hemolysis, but causes RBCs to have shorter lives than normal (Mukherjee & Bhattacharya, 2011).

Chapter Three

Study Framework

This chapter describes the framework of this study and provides definitions of study variables.

3.1 Conceptual Framework

Severe thalassemia can cause bone deformities, spleen enlargement, delayed growth, and heart problems. Regular blood transfusion and administration of iron chelation therapy are the mainstays of treatment for transfusion-dependent thalassemia patients. However, blood transfusion is associated with several complications such as iron overload, blood-borne infections, allergic reactions, and hemolytic and non-hemolytic transfusion reactions (Higgins, 2000; F. T. Shah et al., 2019).

This descriptive study aims to determine the prevalence of transfusion related complications, assess the hematological, biochemical, and hormonal parameters, determine the rates of allo- and autoimmunization, and determine the genotypes of blood group system antigens among frequently transfused thalassemia patients using molecular genotyping methods. Figure 3.1 shows the conceptual framework for this study.

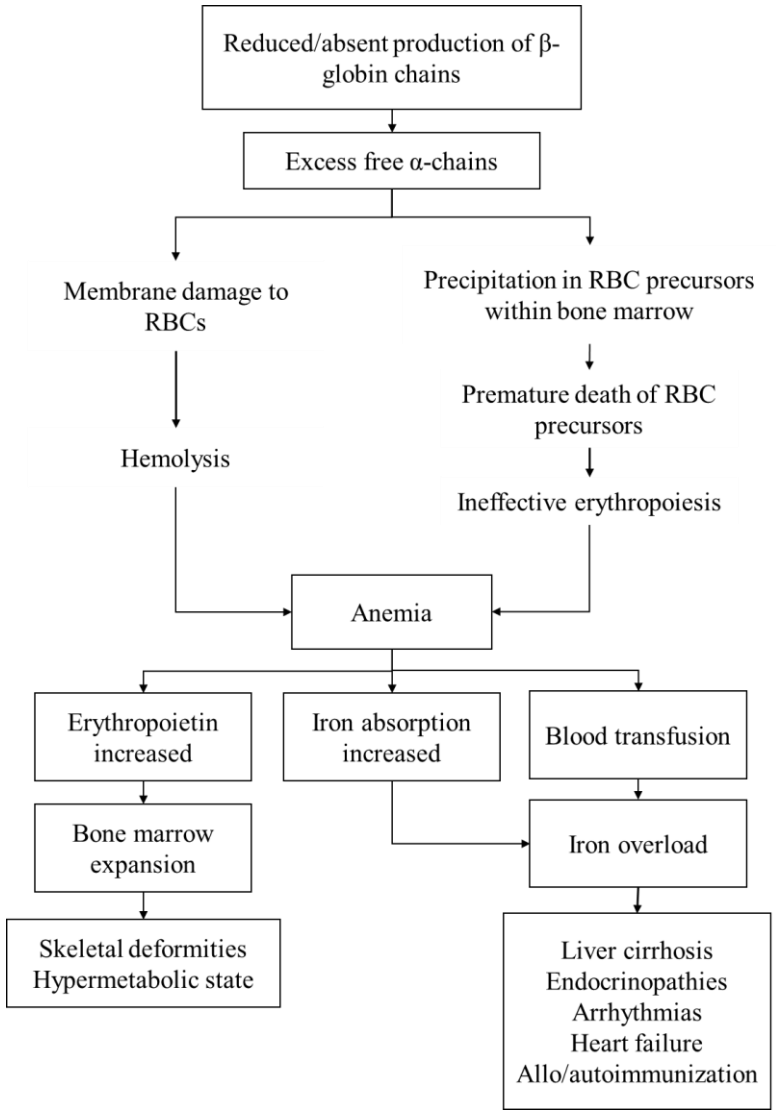

Figure 3.1: Pathophysiology and treatment of β-thalassemia.

3.2 Study Variables & Definitions

- Demographic variables: including gender, age, treatment center.
- Medical characteristics: Diagnosis, Blood Type, Age at first blood transfusion (months), Frequency of blood transfusion, Bone marrow transplantation, Complications.
- Laboratory parameters: blood urea nitrogen (BUN), creatinine, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), glucose, calcium, serum ferritin, hemoglobin, 25-hydroxy vitamin D,

parathyroid hormone (PTH), growth hormone (GH), total triiodothyronine (TT3), free thyroxine (FT4), and thyroid stimulating hormone (TSH).

- Alloimmunization and autoimmunization: antibody screen and antibody type.
- Molecular genotyping: allele frequency and genotype frequency.

Table (3.1): Laboratory tests normal ranges.

Laboratory Test	Normal Range	Unit
Blood urea nitrogen (BUN)	8 – 26	mg/dL
Creatinine	• Adults: – Male :0.7-1.3 – Female :0.6-1.1 • Children: – 4 days -2 years :0.2-0.4 – 2-12 years: 0.3-0.7 – 12-16 years: 0.5-1.0	mg/dL
Serum Calcium	8.8 – 10.2	mg/dL
Aspartate transaminase (AST)	• Male :0 – 50 • Female: 0 – 37	U/L
Alanine transaminase (ALT)	• Male: 0 – 41 • Female: 0 – 33	U/L
Alkaline phosphatase (ALP)	• Adults: – Male: 40 – 129 – Female: 35-140 • Children: – 1-10 years ▪ Male: 142-335 ▪ Female: 142-335 – 10-13 years ▪ Male: 129-417 ▪ Female: 129-417 – 13-15 years ▪ Male: 116-468 ▪ Female: 57-254 – 15-17 years ▪ Male: 82-331 ▪ Female: 50-117 – 17-19 years ▪ Male: 55-149 ▪ Female: 45-87	U/L
Fasting blood sugar	75 – 110	mg/dL
Tri-iodothyronine (TT3)	0.58 – 1.59	ng/mL
Free thyroxin (FT4)	0.7 – 1.48	ng/mL
Thyroid-stimulating hormone (TSH)	0.35 – 4.94	mIu/mL
Parathyroid hormone (PTH)	15 – 68	pg/mL
Vitamin D	20 – 40	ng/mL
Serum Ferritin	• Male: 21.8 – 274	ng/mL

	• Female: 4.06 – 204	
Hemoglobin	• Adults: − Male :13-17 − Female:12.1-15.1 • Children: − 6 month -2years 10.5-13.5 − 2-6 years :11.5-13.5 − 6-12 years :11.5-15.5	g/dL
Growth hormone (GH)	Adults: <5.0 Children: • 1-3 years: − Male 0.43-2.4 − Female 0.50-3.5 • 4-6 years: − Male 0.09-2.5 − Female 0.10-2.2 • 7-8 years: − Male 0.15-3.2 − Female 0.16-5.4 • 9-10 years: − Male 0.09-1.95 − Female 0.08-3.1 • 11 years: − Male 0.08-4.7 − Female 0.12-6.9 • 12 years: − Male 0.12-8.9 − Female 0.14-11.2 • 13 years: − Male 0.1-7.9 − Female 0.21-17.8 • 14 years: − Male 0.09-7.1 − Female 0.14-9.9 • 15 years: − Male 0.1-7.8 − Female 0.24-10.0 • 16 years: − Male 0.08-11.4 − Female 0.26-11.7 • 17 years: − Male 0.17-12.2 − Female 0.3-10.8 • 18-19 years: − Male 0.97-4.7 − Female 0.24-4.3	ng/mL

Chapter Four

Methodology

This chapter provides a comprehensive description of our study methods including the study design, setting, subjects, sample size, data collection methods, laboratory methods, data analysis, and ethical considerations.

In this study, we investigated the prevalence of transfusion-related complications and assessed the biochemical, hematological, and hormonal parameters of a sample of multi-transfused thalassemia patients from the West Bank. Furthermore, we described the frequencies of the main blood group systems by genotyping human erythrocyte antigens. This is crucial to improve the safety of blood transfusion and facilitate the exigent task of providing antigen-negative blood for patients with multiple antibodies.

4.1 Study Design

This descriptive cross-sectional study was conducted among frequently transfused thalassemia patients during 2021. The study included 100 patients recruited from Governmental hospitals by the help of the treating hematologists.

4.2 Study Setting

The study included patients treated in five governmental hospitals in the West Bank that has thalassemia daycare units. The participating hospitals included Al Watani Hospital in Nablus, Palestine Medical Complex in Ramallah, Khaleel Sulaiman Hospital in Jenin, Darweesh Nazal Hospital in Qalqilia, and Thabit Thabit Hospital in Tulkarem. Thalassemia daycare units manage patients under the direction of the medical staff for the stipulation of regular, screened blood transfusions, viral markers, screening, baseline investigations, iron chelation therapy, and other medications.

4.3 Study Sample

The study population included frequently transfused thalassemia patients treated in governmental hospitals in the West Bank in 2021. The sample size was determined based on the budget. Furthermore, to obtain the maximum benefits from genotyping results, we purposely selected patients who had the highest frequency of blood transfusion in the participating hospitals. All the study subjects are diagnosed using hemoglobin electrophoresis. Patients who were not regularly transfused during 2021 and those who did not agree to participate in the study were excluded.

4.4 Data Collection

Study participants were recruited during their visit to the thalassemia daycare units in the five participating hospitals. Patients were approached before blood transfusion. Upon consenting, a sample of plain blood and a sample of EDTA blood were collected from the patients. The blood samples were used for antibody screening and identification, hematological, biochemical, and hormonal analysis which included blood urea nitrogen (BUN), Creatinine, Asparatate Aminotransferase (AST), Alanine Aminotransferase (ALT), Alkaline Phosphatase (ALP), Calcium, Serum Ferritin, hemoglobin, 25-hydroxy vitamin D, growth hormone (GH), parathyroid hormone (PTH), Total Triiodothyronine (TT3), Free Thyroxine (FT4), fasting blood sugar, and Thyroid Stimulating Hormone (TSH). Furthermore, the EDTA blood was used for the extraction of DNA from the buffy coat for genotyping of blood group antigens.

In addition, each patient was asked to complete a brief questionnaire. The questionnaire consisted of two sections. The first section collected demographic information including name, ID number, date of birth, gender, contact number, and the place where the patient got his last blood transfusion. The second part consisted of a medical history including blood type, history of bone marrow transplantation, history of splenectomy, date of diagnosis, age at first blood transfusion, frequency of the blood transfusion, and history of transfusion reactions. The questionnaire was developed in English and then translated to Arabic (Appendix 1). The translation was validated by the forward-backward method and by a panel of experts. Furthermore, data regarding current medical status were retrieved from patients' medical files after obtaining their consent.

4.5 Laboratory Testing

4.5.1 Antibody Screening and Identification

Antibody screening was performed for all the patients using the plain blood sample. Three cell antigen panels were used for antibody screening. In the case of positive antibody screening, antibody identification including the most common antigens such as D, C, c, E, e, M, N, S, s, K, k, Le^a, Le^b, Fy^a, Fy^b, Jk^b and P was performed by solid phase method using NEO Iris (Immucor, USA).

Autoantibodies including liver kidney microsomal type 1 antibodies (LKM-1), anti-mitochondrial antibodies (AMA), antinuclear antibodies (ANA), antineutrophil cytoplasmic antibodies (ANCA), and cyclic citrullinated peptide antibodies (CCP) were detected by enzyme linked immunosorbent assay (ELISA) using (Algeria, Germany) based on manufacturer instructions under the instrument instructions, only anti-CCP was performed by Abbott ARCHITECTT 1000 under the instrument instructions by Chemiluminescent immunoassays (CLIA) method.

4.5.2 Biochemical Analysis

The plain blood sample was used for biochemical analysis which included BUN, Creatinine, Ca, AST, ALT, and ALP. Biochemical measurements were performed by the photometric enzymatic method. All tests were performed following manufacturers' instructions. All measurements were performed using Roche kits and Cobas c 311 analyzer (Roche, Switzerland).

4.5.3 Hormonal Analysis

Hormone tests including GH, TT3, FT4, TSH and PTH, in addition to Vitamin D were measured using Abbott ARCHITECTT 1000 under the instrument instructions, using Abbot Kits.

4.5.4 Hematological Analysis

Patients' hemoglobin levels were measured using Nihon Kohden MEK-6510 and ferritin levels were measured using Abbott ARCHITECTT 1000.

4.5.5 Serological testing for infectious diseases

The serological testing for Hepatitis B (HBs) and Hepatitis C (HCV) were performed using the plain tube. The test was performed by Abbott ARCHITECTT 1000 under the instrument instructions.

4.5.6 DNA Purification and Quantification

Genomic DNA was obtained from peripheral venous blood samples drawn into EDTA blood tubes. The DNA was extracted using Promega Wizard Genomic DNA Purification Kit (Promega, USA) according to manufacturer instructions. The DNA concentration and purity of each sample were determined by the measurement of optical density (OD) at 260 and 280 nm using Nanodrop. All samples had purity indices (OD260/OD280 and OD260/OD230) of between 1.5- 2.0 and >1.8, respectively. For each sample, a total of 9 μL of 10–30 ng/μL DNA were sent to the University of Gothenburg for genotyping.

4.5.7 Molecular Genotyping

ERY Q Kits (BAG Diagnostics, Germany) were used for the determination of blood groups. The kits cover all clinically relevant alleles. The molecular genetics typing is carried out using the sequence-specific primers (SSP)-PCR technique and real-time PCR (RT-PCR).

In this assay, the DNA is amplified in a PCR with sequence-specific primers (SSP) that were specially developed for the selective amplification of segments of specific alleles or allele groups.

The amplicons are detected with likewise gene locus specific fluorescence dye-labeled hydrolysis probes (TaqMan® probes). If amplicons are present, the probes are hydrolyzed by the Taq polymerase and a fluorescence signal is generated which increases proportionally to the amount of the PCR product. The fluorescence signals are measured by the optical detection unit of the RT-PCR cycler. An internal amplification control (human HGH gene) is included in the multiplex PCR reaction which is detected in a different color channel than the specific reactions. The evaluation was done with the Plex Typer software.

4.5.7.1 RT-PCR

The CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad) was used for RT-PCR. The reaction volume for each RT-PCR-preparation was 10 µL per well. For one well, 2 µL Plex Mix, 1 µL DNA specimen, and 7 µL Aqua dest were pipetted into the reaction tube.

For each specimen (8 well strips) a pre-mix that contained 18 µL Plex Mix, 9 µL Specimen DNA, and 63 µL Aqua dest, was created. From this pre-mix 10 µL were dispensed into each of the 8 wells. Positive and negative controls were used with each run. The PCR reaction was performed using the program (Table 4.1).

Table (4.1): PCR reaction program

Program Step	Time [s]	Temperature [°C]	Plate read	Number of cycles
Initial Activation	120	96	-	1
Denaturation	5	98	-	13
Annealing +Extension	25	68	-	
Denaturation	5	98	-	37
Annealing +Extension	25	68	Yes	

4.5.7.2 Evaluation and interpretation of genotyping results

For the evaluation and interpretation of the data, the Plex Typer software (BAG Diagnostics, Germany) was used in conjunction with Plex Typer kit specific data files. The Plex Typer software determines the positive and negative reactions from which the molecular genetics

blood group type based on the Cq values, Relative Fluorescence Units (RFUs), and the shape of the amplification curve.

4.6 Ethical Considerations

This study was approved by the Research Ethics Committee (REC) at Al Quds University and the Ministry of Health. In addition, patients were asked to sign an informed consent form (Appendix 3) that describes the purpose of the study and ensure confidentiality and anonymity. Moreover, participation in this study was voluntary and participants were assured that they have the right to withdraw from the study at any time without any consequences.

4.7 Statistical Analysis

Data entry, cleaning and analysis were performed using IBM SPSS 24.0 (SPSS, Chicago, IL, USA). Results were reported as mean (±standard deviations), ranges, and median (interquartile range) for continuous variables, and frequencies and percentages for categorical variables. Laboratory parameters were categorized into low, normal, and high based on the normal values listed in Table 3.1.

Discrete variables were compared with the chi-square test and continuous variables with independent sample t-test or one-way analysis of variance (ANOVA) as appropriate. Correlation coefficients for biochemical and hormonal parameters and serum ferritin were obtained using Pearson correlations. A p value < 0.05 was considered significant and all tests were two tailed.

Chapter Five

Results

This chapter shows the findings of this descriptive study that aimed to evaluate the overall health status of frequently transfused β-thalassemia patients in the West Bank.

The levels of hemoglobin (Hb), serum ferritin (SF), blood urea nitrogen (BUN), creatinine, alanine transaminase (ALT), alkaline phosphatase (ALP), aspartate transaminase (AST), serum calcium, vitamin D, tri-iodothyronine (TT3), free thyroxin (FT4), thyroid-stimulating hormone (TSH), parathyroid hormone (PTH), and growth hormone (GH) using blood samples collected from a sample of frequently transfused thalassemia patients. In addition, based on data from medical files we determined the prevalence of reported complications among frequently transfused β-thalassemia patients from the West Bank. Furthermore, antibody screening and identification were performed for each patient in order to detect allo- and auto-antibodies. Finally, genotyping of human erythrocyte antigens (HEA) was performed using DNA extracted from peripheral blood samples.

5.1 Baseline Characteristics of Study Subjects

Patient characteristics for the total group are given in Table 5.1. A total of 100 thalassemia patients were included in this study. The group consisted of 51 (51.0%) males and 49 (49.0%) females. The mean age (±standard deviation) of the patients was 21.9±10.9 years while the median (interquartile rang (IQR)) was 22 (15-28) years and the ages ranged between 2-75 years. Among the patients, 13 (13.0%) were <10 years old, 30 (30%) were between 10-19 years old, 52 (52%) were between 20-39 years old, and 5 (5%) were ≥40 years old.

The majority of the patients (60%) were recruited from Al Watani Hospital in Nablus and 22% were recruited from Palestine Medical Complex (PMC) in Ramallah. The other patients were recruited from Khaleel Sulaiman Hospital in Jenin (6%), Thabit Thabit Hospital in Tulakrem (10%), and Darweesh Nazal Hospital in Qalqiliya (2%).

Table 5.1 also shows the clinical characteristics of study subjects. Eighty-nine patients (89%) had β-thalassemia major and 11 patients (11%) had sickle cell thalassemia. The most common ABO-RhD blood types among the patients were A+ (39%), followed by O+ (36%), and B+ (14%). Furthermore, medical records showed that forty-four patients (44%) had their spleen removed (Table 5.1). The mean age at the first blood transfusion was 17.0±21.6 months ranging between 1-120 months with a median (IQR) of 6 (5-18) months. The majority of the patients (59%) received blood transfusion at a frequency of once every 2-3 weeks while 38 (38%) received blood transfusion every 4-6 weeks, and only three patients (3%) received blood transfusion at lower frequencies. Only two patients (2%) had undergone bone marrow transplantation. Moreover, 89% of the patients received iron chelation therapy. Deferasirox (DFX) was the most commonly used iron-chelating agent (92.1%) followed by Deferoxamine (DFO)(7.9%). More than half of patient (57%) had a history of transfusion reaction.

Table (5.1): Baseline characteristics of study subjects (n=100).

Characteristic	Category	Frequency (%)
Gender	Male	51 (51.0)
	Female	49 (49.0)
Age (years)	Mean±SD	21.9±10.9
	Median (IQR)	22 (15-28)
	Range	(2-75)
Age (years)	0-9	13 (13.0)
	10-19	30 (30.0)
	20-29	35 (35.0)
	30-39	17 (17.0)
	40+	5 (5.0)
Study Center	Al Watani Hospital (Nablus)	60 (60.0)
	Khaleel Sulaiman Hospital (Jenin)	6 (6.0)
	Palestine Medical Complex (Ramallah)	22 (22.0)
	Darweesh Nazal Hospital (Qalqiliya)	2 (2.0)
	Thabit Thabit Hospital (Tulkarem)	10 (10.0)
Diagnosis	β-Thalassemia	89 (89.0)
	Sickle cell thalassemia	11 (11.0)
Blood Type	O-	5 (5.0)
	O+	36 (36.0)

	A-	1 (1.0)
	A+	39 (39.0)
	B-	3 (3.0)
	B+	14 (14.0)
	AB+	2 (2.0)
Age at 1st blood transfusion (months)	Mean ±SD	17.0±21.6
	Median (IQR)	6 (5-18)
	Range	(1-120)
Frequency of blood transfusion (days between transfusions)	Mean ±SD	24.6±8.7
	Median (IQR)	21 (21-30)
	Range	13-60
Frequency of blood transfusion	2-3 weeks	59 (59.0)
	4-6 weeks	38 (38.0)
	>6 weeks	3 (3.0)
Bone marrow transplantation	No	98 (98.0)
	Yes	2 (2.0)
Splenectomy	No	56 (56.0)
	Yes	44 (44.0)
Chelation Therapy	No	11 (11.0)
	Yes	89 (89.0)
Type of chelation	Deferasirox (DFX)	82 (92.1)
	Deferoxamine (DFO)	7 (7.9)
History of transfusion reactions	No	43 (43.0)
	Yes	57 (57.0)

5.2 Biochemical, Hematological, and Hormonal Parameters Among Frequently Transfused Thalassemia Patients

As shown in table 5.2, the patients in this study had a mean pre-transfusion hemoglobin of 7.89±0.99 g/dL ranging between 5.60-11.80 g/dL with a median (IQR) of 7.90 (7.25-8.60) g/dL and a mean serum ferritin of 3670.42±3742.71 ng/mL ranging from 107.08-19374.37 ng/mL with a median (IQR) of 2538.20 (131.16-4833.02) ng/mL. Among the patients, 51% had serum ferritin levels of at least 2500 ng/mL.

Alanine transaminase (ALT), Alkaline phosphatase (ALP) and Aspartate transaminase (AST) were used to assess liver functions. The results of liver function tests showed that the patients in this study had mean ALT, ALP, and AST levels of 36.80±31.06 U/L, 143.11±63.34, and 45.68±30.48 U/L, respectively, ranging between 4.60-211.00 U/L, 23.00-406.00 U/L, and 9.80-200.00 U/L, respectively, with medians (IQRs) of 25.70 (16.80-46.55),

134.50 (99.00-172.00), and 38.10 (25.95-54.35) U/L, respectively. Among the patients, 32% had high ALT levels, 34% had high ALP levels, and 42% had high AST levels (Table 5.2). Furthermore, both ALT and AST levels were positively correlated with serum ferritin levels (r=0.541 and 0.543, respectively), and the p-value was <0.0001 for both correlations.

Both blood urea nitrogen (BUN) and serum creatinine were used to evaluate kidney function among the patients in this study. The mean BUN level among the patients was 11.82±4.19 mg/dL ranging from 4.10-27.00 mg/dL with a median (IQR) of 11.50 (9.10-14.00) mg/dL. High levels of BUN were found among 3% of the patients. Moreover, the mean of creatinine among the patients was 0.48±0.16 mg/dL, ranging from 0.25-1.23 mg/dL with a median (IQR) of 0.45 (0.37-0.55) mg/dL. All patients had low to normal serum creatinine levels (Table 5.2).

Tri-iodothyronine (TT3), free thyroxin (FT4), and thyroid-stimulating hormone (TSH) levels were assessed in order to evaluate thyroid function. All patients had normal TT3 levels with a mean of 1.07±0.17 ng/mL, a range between 0.52-1.57 ng/mL, and a median (IQR) of 1.05 (0.98-1.15) ng/mL. On the other hand, one patient had low FT4 level. The mean FT4 level among the patients was 0.97±0.13 ng/mL ranging between 0.67-1.40 ng/mL with a median (IQR) of 0.95 (0.87-1.03) ng/mL. Furthermore, ten patients (10%) had high TSH levels indicating subclinical primary hypothyroidism. The mean TSH among the patients was 3.00±3.23 mIu/mL ranging between 0.81-31.56 mIu/mL with a median (IQR) of 2.40 (1.70-3.51) mIu/mL (Table 5.2).

Hypoparathyroidism (decreased parathyroid hormone (PTH) level) was found among 8% of the patients (n=8) while 17% had hyperparathyroidism (increased level of PTH). The mean level among the patients was 46.54±29.66 pg/mL ranging between 4.70-190.10 pg/mL with a median (IQR) of 39.40 (26.80-58.20) pg/mL. PTH is key to regulating and maintaining a balance of two minerals in the body: calcium and phosphorus.

Serum calcium (Ca), which is usually utilized for screening or monitoring bone diseases or calcium-regulation disorders (diseases of the parathyroid gland or kidneys), showed that 8% of the patients had low calcium levels (n=8) while 37% had high levels (n=37). The mean level of calcium among the patients was 9.80±0.83 mg/dL, ranging from 5.72-11.45 mg/dL with a median (IQR) of 9.93 (9.50-10.29) mg/dL. Moreover, Vitamin D is essential for Ca homeostasis and skeleton mineralization, particularly during periods of fast development.

Vitamin D deficiency causes rickets and osteomalacia. 70% of the patients (n=70) had vitamin D deficiency. The mean level of vitamin D among the patients was 16.21±8.66 mg/dL ranging between 3.70-58.70 mg/dL with a median (IQR) of 13.95 (9.75-20.95) mg/dL.

Growth hormone (GH) levels were low among 8% of the patients (n=8) and increased among 2% (n=2). The mean of GH among the patients was 1.46±1.65 pg/mL ranging from 0.01-11.00 pg/mL with a median (IQR) of 0.91 (0.341-2.15) pg/mL.

The results of the fasting blood sugar showed that 3% of the patients (n=3) had hypoglycemia and 15% (n=15) had hyperglycemia, both of which might indicate significant problems. The mean glucose level among the patients was 101.04±30.05 mg/dL, ranging from 36.80-312.00 mg/dL with a median (IQR) of 95.15 (88.55-105.60) mg/dL.

Table (5.2): Assessment of biochemical, hematological and hormonal parameters among frequently transfused thalassemia patients in the West Bank (n=100).

Laboratory Parameter	Category	Frequency (%)	Mean±Standard Deviation	Median (IQR)	Range	Unit
Hemoglobin	Low	100 (100)	7.89±0.99	7.90 (7.25-8.60)	5.60-11.80	g/dL
Serum Ferritin	<1000	21 (21.0)	3670.42±3742.71	2538.20 (1310.16-4833.02)	107.08-19374.37	ng/mL
	1000-<2500	28 (28.0)				
	2500-<5000	27 (27.0)				
	≥5000	24 (24.0)				
BUN	Low	19 (19.0)	11.82±4.19	11.50 (9.10-14.00)	4.10-27.00	mg/dL
	Normal	78 (78.0)				
	High	3 (3.0)				
Creatinine	Low	73 (73.0)	0.48±0.16	0.45 (0.37- 0.55)	0.25-1.23	mg/dL
	Normal	27 (27.0)				
Glucose	Low	3 (3.0)	101.04±30.05	95.15 (88.55-105.60)	36.80-312.00	mg/dL
	Normal	82 (82.0)				
	High	15 (15.0)				
ALT	Normal	68 (68.0)	36.80±31.06	25.70 (16.80-46.55)	4.60-211.00	U/L
	High	32 (32.0)				
AST	Normal	58 (58.0)	45.68±30.48	38.10 (25.95-54.35)	9.80-200.00	U/L
	High	42 (42.0)				
ALP	Low	1 (1.0)	143.11±63.34	134.50 (99.00-172.00)	23.00-406.00	U/L
	Normal	65 (65.0)				
	High	34 (34.0)				
Ca	Low	8 (8.0)	9.80±0.83	9.93 (9.50-10.29)	5.72-11.45	mg/dL
	Normal	55 (55.0)				
	High	37 (37.0)				
Vitamin D	Low	70 (70.0)	16.21±8.66	13.95 (9.75-20.95)	3.70-58.70	ng/mL
	Normal	29 (29.0)				
	High	1 (1.0)				
TT3	Normal	100 (100.0)	1.07±0.17	1.05 (0.98-1.15)	0.52-1.57	ng/mL
FT4	Low	1 (1.0)	0.97±0.13	0.95 (0.87-1.03)	0.67-1.40	ng/mL

	Normal	99 (99.0)				
TSH	Normal	90 (90.0)	3.00±3.23	2.40 (1.70-3.51)	0.81-31.56	mIu/mL
	High	10 (10.0)				
PTH	Low	8 (8.0)	46.54±29.66	39.40 (26.80-58.20)	4.70-190.10	pg/mL
	Normal	75 (75.0)				
	High	17 (17.0)				
GH	Low	8 (8.0)	1.46±1.65	0.91 (0.34-2.15)	0.01-11.00	pg/mL
	Normal	90 (90.0)				
	High	2 (2.0)				

5.3 Prevalence of Complications Among Frequently Transfused Thalassemia Patients

Table 5.3 shows the prevalence of disease and treatment-associated complications which included arthropathy, diabetes mellitus, hepatic failure, hypogonadism, conduction defects, cardiomyopathy, heart failure, hyperparathyroidism, delayed growth, hypothyroidism, hepatitis C (HCV), renal impairment, hearing defects, and metabolic acidosis with frequencies of 44.0%, 4.0%, 7.0%, 16.0%, 5.0%, 4.0%, 1.0%, 7.0%, 3.0%, 3.0%, 1.0%, 1.0%, and 1.0%, respectively. The most commonly reported complications were arthropathy followed by hypogonadism, while the least reported were hyperparathyroidism, renal impairment, hearing defect, and metabolic acidosis.

Table 5.3: Prevalence of complications among frequently transfused thalassemia patients.

Complication	Category	Frequency (%)
Arthropathy	No	56 (56.0)
	Yes	44 (44.0)
Diabetes mellitus	No	96 (96.0)
	Yes	4 (4.0)
Hepatic Failure	No	93 (93.0)
	Yes	7 (7.0)
Hypogonadism	No	84 (84.0)
	Yes	16 (16.0)
Conduction Defects	No	95 (95.0)
	Yes	5 (5.0)
Cardiomyopathy	No	95 (95.0)
	Yes	5 (5.0)
Heart Failure	No	96 (96.0)
	Yes	4 (4.0)
Hyperparathyroidism	No	99 (99.0)
	Yes	1 (1.0)
Delayed Growth	No	93 (93.0)
	Yes	7 (7.0)

Hypothyroidism	No	97 (97.0)
	Yes	3 (3.0)
HCV	No	97 (97.0)
	Yes	3 (3.0)
Renal Impairment	No	99 (99.0)
	Yes	1 (1.0)
Hearing Defect	No	99 (99.0)
	Yes	1 (1.0)
Metabolic Acidosis	No	99 (99.0)
	Yes	1 (1.0)

5.4 Molecular Genotyping of Blood Group Antigens among Frequently Transfused Thalassemia Patients

Red blood cell (RBC) antigen allele and genotype frequencies of the Rhesus (*RH*), Kell (*KEL*), Duffy (*FY*), MNS (*GYP*), Kidd (*JK*), Cartwright (*Yt*), Dombrock (*DO*), Lutheran (*LU*), Colton (*CO*), Knops (*KN*), Vel (*VEL*), and Diego *(DI* and *WR*) are shown in Table 5.4. Five "no clear results" were observed for the *RHD* genotype, one was observed for the *DI* alleles, one for the *GYPA* alleles, and one for the *KEL*03* and *KEL*04* alleles (Kp antigens).

The genotyping results of the *RHD* blood group showed that 88% of the patients were *RHD-positive* whereas 7% were *RHD-negative*. Furthermore, agreement between phenotype and genotype for *RHD* was observed in 94% of the patients. Of these 94 samples, 87 were both phenotyped and genotyped as *RHD-positive* and seven were phenotyped and genotyped as *RHD-negative*. One sample was phenotyped as RHD-negative but genotyped as *RHD-positive*, and the remaining five samples had weak or partial types that showed no clear results in the genotyping analysis with the utilized kit.

The genotyping results of the *RHCE* blood group were as follows: *RHCE*C/C*: (n: 23, 23%), *RHCE*C/c*: (n: 42, 42%), *RHCE*c/c*: (n: 35, 35%); *RHCE*E/E*: (n: 4, 4%), *RHCE*E/e*: (n:25, 25%), and *RHCE*e/e:* (n: 71, 71%).

The genotyping results of the Duffy blood group showed that 46% off the patients had the *FY*02N.01/02N.01* genotype, which was the most common. The allele frequency of *FY*02N.01* was 0.460.

The prevalence of *GYPA* alleles was identified as follow: *GYPA*M/N* (37%), *GYPA*M/M* (40%) and *GYPA*N/N* (22%), and for *S/s* alleles: *GYPB*S/s* (35%), *GYPB*s/s* (55%), and *GYPB*S /S* (10%).

The *KEL*02*, *KEL*04*, and *KEL*07* allele frequencies were high among the patients in this study (0.920, 0.985, and 0.980, respectively). 87% of the patients were homozygous for the *KEL*02* allele and 3% were homozygous for *KEL*01*. In addition, 98% of the patients were homozygous for the *KEL*04* and 96% were homozygous for *KEL*07*.

In addition, the most common genotype for Kidd antigens was the *JK*A/B* genotype (50%), *YT*A/A* had the highest frequency for Cartwright (Yt) (88%), the heterozygous genotype *DO*01/02* had the highest frequency for the Dombrock antigens (47%), the homozygous *LU*02/02* genotype had the highest frequency for the Lutheran blood group (98%), *CO*01/01* had the highest frequency for the Colton blood group (98%), and *DI*B/B* had the highest frequency for the Diego Di antigens (97%). Moreover, 2% of the patients had the *Vel*01/-0.1* (Vel/Vel_{null}) genotype. In addition, we detected two rare alleles *RHCE*C*^{w} (*RH8*) and *RHD*08N.01* (encoding antigen Psi/Ψ) at a frequency of 1% each.

Tale 5.4: Genotype and allele frequencies by blood group among frequently transfused thalassemia patients in the West Bank (n=100).

Blood group	Total (n)	Genotype	Genotype Frequency (%)	Allele	Allele Frequency
Rh	100	*RHD-*	7 (7.0)	*RHD-*	
		RHD+	88 (88.0)	*RHD+*	
		No clear result	5 (5.0)		
Rh	100	*RHCE*C/c*	42 (42.0)	*RHCE*C*	0.440
		*RHCE*C/C*	23 (23.0)	*RHCE*c*	0.560
		*RHCE*c/c*	35 (35.0)		
Rh	100	*RHCE*E/e*	25 (25.0)	*RHCE*E*	0.165
		*RHCE*E/E*	4 (4.0)	*RHCE*e*	0.835
		*RHCE*e/e*	71 (71.0)		
Duffy	100	*FY*01/02*	21 (21.0)	*FY*01*	0.195
		*FY*01/01*	9 (9.0)	*FY*02*	0.345
		*FY*02/02*	24 (24.0)	*FY*02N.01*	0.460
		*FY*02N.01/02N.01*	46 (46.0)		
MNS	100	*GYPA*M/N*	37 (37.0)	*GYPA*M*	0.585
		*GYPA*M/M*	40 (40.0)	*GYPA*N*	0.405
		*GYPA*N/N*	22 (22.0)		
		No clear result	1 (1.0)		
	100	*GYPB*S/s*	35 (35.0)	*GYPB*S*	0.275

		*GYPB*S /S*	10 (10.0)	*GYPB*s*	0.725
		*GYPB*s/s*	55 (55.0)		
Kell	100	*KEL*01/02*	10 (10.0)	*KEL*01*	0.080
		*KEL*01/01*	3 (3.0)	*KEL*02*	0.920
		*KEL*02/02*	87 (87.0)		
Kell	100	*KEL*03/04*	1 (1.0)	*KEL*03*	0.005
		*KEL*04/04*	98 (98.0)	*KEL*04*	0.985
		No clear result	1 (1.0)		
Kell	100	*KEL*06/07*	4 (4.0)	*KEL*06*	0.020
		*KEL*07/07*	96 (96.0)	*KEL*07*	0.980
Kidd	100	*JK*A/B*	50 (50.0)	*JK*A*	0.570
		*JK*A/A*	32 (32.0)	*JK*B*	0.430
		*JK*B/B*	18 (18.0)		
Cartwright (Yt)	100	*YT*A/B*	12 (12.0)	*YT*A*	0.940
		*YT*A/A*	88 (88.0)	*YT*B*	0.060
Dombrock	100	*DO*01/02*	47 (47.0)	*DO*01*	0.425
		*DO*01/01*	19 (19.0)	*DO*02*	0.575
		*DO*02/02*	34 (34.0)		
Lutheran	100	*LU*01/02*	2 (2.0)	*LU*01*	0.010
		*LU*02/02*	98 (98.0)	*LU*02*	0.990
Colton	100	*CO*01/02*	2 (2.0)	*CO*01*	0.990
		*CO*01/01*	98 (98.0)	*CO*02*	0.010
Knops	100	*KN*01/01*	100 (100.0)	*KN*01*	1.000
Diego	100	*DI*A/B*	2 (2.0)	*DI*A*	0.010
		*DI*B/B*	97 (97.0)	*DI*B*	0.980
		No clear result	1 (1.0)		
Diego	100	*DI*02.04/02.04*	100 (100.0)	*DI*02.04*	1.000
Vel	100	*VEL*01/01*	98 (98.0)	*VEL*01*	0.990
		*Vel*01/-0.1*	2 (2.0)	*VEL*-01/VEL$_{null}$*	0.010

5.5 Alloimmuniztion and Autoimmunization among Frequently Transfused Thalassemia Patients

We studied the frequency of red blood cell alloimmunization and autoimmunization among thalassemia patients in this study. As shown in Table (5.5), (8%; n=8) of the patients developed alloantibodies. Different types of alloantibodies were identified in our patients. The majority of patients (75%; n=6) had a single alloantibody, whereas two (25%) of them had multiple antibodies. The most common alloantibody was anti-E (62.5%; n=5), followed by anti-K and anti-D (25%; n=2), and anti-C (12.5%; n=1) (Figure 5.1).

Table 5.5: Prevalence of allo- and autoantibodies

	Frequency (%)
Autoantibodies screening	5 (5.0)
Anti-CCP	2 (2.0)
Alloantibodies screening	8 (8.0)

Of the 100 patients, (5%; n=5) developed autoantibodies. Two of the five patients had anti-CCP autoantibodies.

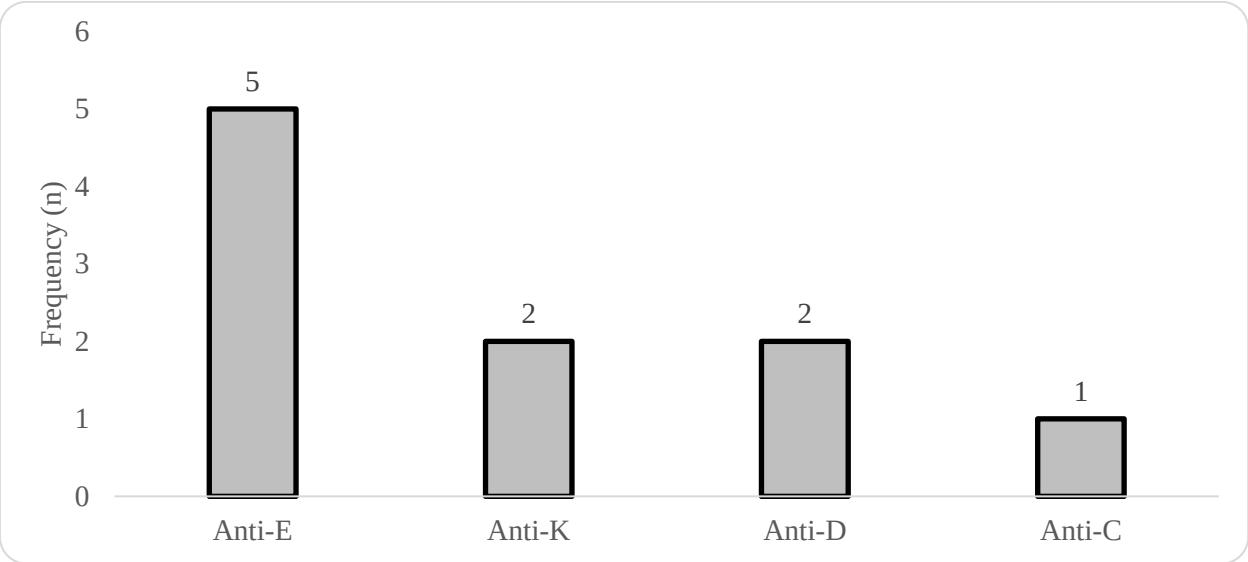

Figure (5.1): Frequency of antibodies among alloimmunized patients.

The characteristics of alloimmunized β-thalassemia patients are shown in Table 5.6. Five of the alloimmunized patients (62.5%) were females and (62.5%; n=5) were under 18 years old. Regarding the age at first transfusion, four patients (50%) received the first transfusion during the first six months of their lives, two (25%) during the second year of their life, and two (25%) at older ages (4 years and 10 years). Five patients (62.5%) received blood every 30 days, two (25%) received blood every 21 days and one (12.5%) received blood every two weeks. Two patients (25%) were splenoctomized, and out of the eight alloimmunized patients, seven (87.5%) reported suffering from hemolytic reactions. Furthermore, one (12.5%) of the alloimmunized patients also had anti-CCP autoantibodies.

Five patients (62.5%) had O blood type and (25%; n=2) had B blood type. Regarding the RhD phenotype of alloimmunized patients, (25%; n=2) had RhD-negative phenotype, both of which developed anti-D antibodies; however, there were no discrepancies between RhD phenotyping and genotyping results among these patients. Moreover, all the patients (n=5) who developed anti-E antibodies had *RHCE*e/e* genotype, both patients who developed

anti-K antibodies had the *KEL*02/02* genotype, and the one patient that developed anti-C antibodies had *RHCE*c/c* genotype.

Table 5.6: Medical and genetic characteristics of alloimmunized β-thalassemia patients

Patient No.		**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Gender		Male	Female	Female	Female	Male	Female	Female	Male
Age (years)		15	17	29	23	14	15	15	54
Diagnosis		β-thalassemia	β-thalassemia	β-thalassemia	β-thalassemia	β-thalassemia	β-thalassemia	β-thalassemia	Sickle cell thalassemia
RBC Antibody Specificity		Anti-E	Anti-D	Anti-D	Anti-E	Anti-C	Anti-K,E	Anti-K,E	Anti-E
Complications		No Reported Complications	Arthropathy, Hypogonadism	No Reported Complications	Conduction Defects, Arthropathy	Arthropathy	Arthropathy, Delayed Growth, Hypogonadism	Arthropathy, HCV positive	Heart Failure, Renal Impairment
Age at 1st BT (months)		5	18	5	6	3	48	14	120
Frequency of BT (Days)		30	21	21	30	30	14	30	30
ABO/RhD blood phenotype		A+	B-	O-	B+	O+	O+	O+	O+
Splenectomy		No	No	Yes	Yes	No	No	No	No
Iron Chelation Drug		DFX	DFX	DFO	DFX	DFX	DFX	DFX	DFX
Autoantibodies		Negative	Negative	Positive (Anti-CCP)	Negative	Negative	Negative	Negative	Negative
Hemolytic Reaction		Yes	Yes	Yes	Yes	Yes	Yes	Yes	No
Genotypes	***RHD***	RHD+	RHD-	RHD-	RHD+	RHD+	RHD+	RHD+	RHD+
	RHCE (C/c)	*RHCE*C/c*	*RHCE*c/c*	*RHCE*c/c*	*RHCE*C/c*	*RHCE*c/c*	*RHCE*C/c*	*RHCE*C/C*	*RHCE*C/C*
	RHCE (E/e)	*RHCE*e/e*	*RHCE*e/e*	*RHCE*e/e*	*RHCE*e/e*	*RHCE*E/e*	*RHCE*e/e*	*RHCE*e/e*	*RHCE*e/e*
	Duffy	*FY*A/B*	*FY*A/B*	*FY*A/B*	*FY*A/A*	*FY*02N.01/02N.01*	*FY*A/A*	*FY*B/B*	*FY*02N.01/02N.01*
	GYPA	*GYPA*M/M*	*GYPA*M/N*	*GYPA*M/N*	*GYPA*M/N*	*GYPA*M/M*	*GYPA*N/N*	*GYPA*M/M*	*GYPA*M/M*
	KEL	*KEL*01/02*	*KEL*01/01*	*KEL*01/02*	*KEL*01/02*	*KEL*02/02*	*KEL*02/02*	*KEL*02/02*	*KEL*02/02*

Chapter Six

Discussion, Conclusions, Limitations and Recommendations

This study was conducted to assess the overall health status of frequently transfused Palestinian β-thalassemia patients in the West Bank and provide data regarding blood group systems in order to improve the safety of blood transfusion among multi-transfused thalassemia patients. This chapter discusses our major findings, recommendations, and limitations of the study.

6.1 Discussion

6.1.1 Baseline Characteristics of Study Subjects

Thalassemias are inherited blood disorders characterized by decreased hemoglobin production. Management of β-thalassemia syndrome poses a major challenge to the healthcare systems in low-resource countries, where the highest burden of thalassemia is. It is estimated that 4% of the population in Palestine are thalassemia carriers (Al Sabbah et al., 2017). According to the Thalassemia Patients' Friends Society (TPFS), there were 847 patients in the West Bank and Gaza Strip in 2018 (Aldwaik et al., 2021). Based on the data from the TPFS, the majority of thalassemia patients were between 10 and 30 years old and most patients were from the northern region of the West Bank (Aldwaik et al., 2021). Comparing the characteristics of patients in the sample of our study to those of the general characteristics of thalassemia patients in Palestine, we find that the sample used in this study reflects the characteristics of the population of thalassemia patients, but Gaza Strip and the southern governorates of the West Bank were not included in this study.

The new treatment approaches along with the availability of blood transfusion and iron chelation therapy has improved the life expectancy of thalassemia patients significantly (Pinto & Forni, 2020). In this study, the age of the patients ranged between 2-75 years with an average age of 21.9±10.9 years. Similarly, the mean age of Irani patients was reported to be 22.5±9.5 years (Ghasemi, Abbasian, Ghaffari, & Salmanpour, 2016), Indonesian patients

were reported to have a median age of 21.5±7.2 years but ranging between 15-32 years (Fianza et al., 2021), and Malaysian patients had an average age of 23.1±5.9 years in one study (Ong, Lim, Tan, Ong, & Goh, 2008) and 28.8±6.9 years in another (Tat, Lin, & Sim, 2020). Furthermore, in France the mean age among thalassemia patients was reported to be 19.3 years ranging between 5-52 years (Thuret et al., 2010) while in north America, the mean of age of thalassemia patients was 23±2 years ranging from 6.1-75.4 years (Vogiatzi et al., 2009) and in Turkey, the mean age was 18.66±6.48 years, ranging between 10-34 years (Kurtoglu, Kurtoglu, & Temizkan, 2012). On the other hand, a study in India reported the mean age among patients to be lower (15.18 years)(Dhawan et al., 2014) and another study in Sri Lanka reported a median age of 17.70±2.36 years among thalassemia patients (Karunaratna, Ranasingha, & Mudiyanse, 2020).

Even before the establishment of the Thalassemia Friends Patients' Society (TPFS) in Palestine in 1996, prevention of thalassemia has been a primary research priority. A study conducted in 1994 in the West Bank among undergraduate students in higher educational institutions estimated the prevalence of β-thalassemia trait to be 3-4% (Yunis et al., 1996). The Palestinian Avenir Foundation, which is a non-governmental organization that was active in the West Bank at the time and was later dissolved in 2008, was the first to introduce the idea of premarital thalassemia screening in Palestine. In 2000, obligatory premarital thalassemia screening program was established. At the time, couples carrying the thalassemia trait were only advised against marriage and offered genetic counseling. The incidence of thalassemia had declined significantly since then. However, since 2010, the obligatory premarital testing took effect as a law and marriage between two thalassemia carriers has been against the law. Yet, we found that 13% of the patients in this study were less than ten years old, which shows that new cases of thalassemia continue to emerge despite the law. Although some of these children could be from old marriages. The records of the TPFS shows that this only explains a small part of the emerging cases. The high rate of consanguineous marriages in the Middle Eastern population in general, and in Palestine in particular could partly explain the high prevalence of thalassemia among the population (Ebrahim et al., 2019; Hamamy, 2012; Ishaq, Abid, Kokab, Akhtar, & Mahmood, 2012; Kumar, Arya, & Agarwal, 2015; Tadmouri et al., 2009; Williams & Weatherall, 2012).

In this study, our target was transfusion dependent patients, who would benefit most from the findings of this study. Therefore, we targeted patients with the highest transfusion

frequencies. Furthermore, the sample in this study included not only patients with β-thalassemia major, but also sickle cell thalassemia patients. However, sickle cell thalassemia patients were the minority (11%).

Regarding the ABO blood type of patients in this study, the majority of patients had O (41%) and A (40%) blood types, and only 2% had AB blood type. Furthermore, 91% had RhD-positive blood phenotype and 9% had RhD-negative phenotype. A previous study among Palestinian thalassemia patients showed that the most frequent blood type was A (87; 40.5%) followed by O (81; 37.7%), B (35; 16.3%), and AB (12; 5.6%) (Abu Taha et al., 2019). Regarding the distribution of the ABO and RhD blood group in the general Palestinian population, a study conducted in Gaza reported that A (39.3%) and O (32.9%) were respectively the most common blood types while the majority (97.3%) had RhD negative blood type (Skaik & El-Zyan, 2006).

In the current study, the mean age at first blood transfusion was 17.0±21.6 months ranging from 1-120 months. The previously mentioned study conducted among Palestinian patient by Abu Taha et. al. reported that the mean age of first blood transfusion was 16.55±27 months ranging from 1-300 months (Abu Taha et al., 2019). In comparison, a study in Iran showed that the mean age at the first blood transfusion was 5±3.6 months among patients with thalassemia major (Ghasemi et al., 2016). Another study in Penang reported the mean age at onset of blood transfusion to be 55.9±40.3 months (Ong et al., 2008). Higher age at first blood transfusion had been reported to be associated with increased rate of alloimmunization among multi-transfused patients in India (23.28 months vs 14.43 months) (Dhawan et al., 2014). On the other hand, the mean age of first blood transfusion was reported to have significant negative association with serum ferritin levels among Indonesian patients; which means, the earlier in life transfusion began, the highest serum ferritin levels were (Fianza et al., 2021). Furthermore, a report released by the Thalassemia International Federation (TIF) showed that the initiation of regular transfusion therapy for severe thalassemia usually occurs in the first two years of life (M. D. Cappellini et al., 2008).

Most of the patients 59 (59%) in this study were reported to receive transfusion at an interval of once per 2-3 weeks, 38 (38%) were transfused at an interval of once per 4-6 weeks and the remaining 3 (3%) patients received transfusion at a frequency of less than once per more than 6 weeks. A study in Indonesia reported the mean interval between blood transfusions among thalassemia patients to be 5.6±6.3 weeks (Fianza et al., 2021) while another study in

Bangladesh reported the mean transfusion interval to be 12 days (Karim, Ismail, Hasan, & Shekhar, 2016) and another in Pakistan reported the mean interval between transfusions in alloimmunized patients to be 23±8.81 days and in those without alloimmunization to be 31.8±16 days (Amoudi et al., 2014). Furthermore, a study conducted in Western Saudi Arabia reported that most patients (84.8%) received blood transfusion regularly every 3 weeks. Based on the recommendations of the TIF, treatment for thalassemia major involves lifelong regular treatment with blood transfusion that is administered every two to five weeks to maintain pre-transfusion hemoglobin level above 9–10.5 g/dL (M. D. Cappellini et al., 2008). On the other hand, decreased intervals between transfusion (higher frequency of blood transfusion) increases the susceptibility to iron overload, transfusion-transmitted infections, and alloimmunization. Therefore, in order to maximize the benefits of blood transfusion and lower the risk of transfusion-associated complications, extended blood typing of the patients should be done before the first transfusion and donor's blood should be cross-matched with that of the patients'. However, if the patient has been previously transfused, it is recommended that blood typing should be performed by molecular methods (F. T. Shah et al., 2019).

Hematopoietic stem cell transplantation is the only well-established curative treatment of thalassemia major and shows excellent long-term results, but allogenic bone marrow transplantation (BMT) is limited in Palestine due to the lack of BM Biobank or suitable donors and the high cost. Our results showed that only 2 (2%) had undergone BMT. However, both BMT operations were not successful as evident by the fact that these patients are currently on blood transfusion. In Palestine, there are no centers specialized in bone marrow transplantation for thalassemia patients. The history of those two cases date back to 2004/2005, when a group of 30 Palestinian patients were referred to be transplanted abroad in Italy. The availability of BMT in Palestine is limited by the high cost and the lack of donors or a bone marrow Biobank. Furthermore, other factors might have contributed to the failure of both BMT operations including the lack of experience in dealing with cases after transplantation, and the lack of specialized centers to follow up on them.

The spleen plays a crucial role in the cellular and humoral immune responses in addition to its activity as a filter, catabolic, and reservoir, where damaged red blood cells are disposed (Schwartz, 1996). Data form medical files showed that 44 patients (44%) underwent splenectomy. Splenectomy is highly common for patients with hematological diseases. The

hallmark of thalassemia is the production of defective red blood cells that are removed by the spleen, which results in an enlarged hyper functioning spleen (splenomegaly). The removal of the spleen may prolong red blood cell survival by reducing the amount of red blood cells removed from circulation and may ultimately result in the reduced need for blood transfusions (Sharma, Easow Mathew, & Puri, 2019). Therefore, until recently, the procedure was very frequently performed in patients with thalassemia major (Thuret et al., 2010). However, splenectomy reduces the removal of damaged red cells, which may expose patients to new antigens and increase the risk of alloimmunization (Evers et al., 2017; Jansuwan, Tangvarasittichai, & Tangvarasittichai, 2015).

The study conducted by Abu Taha et.al. in 2019 among Palestinian thalassemia patients reported that the percentage of splenectomized patients was 47.4%, and among alloimmunized patients 40.7% underwent splenectomy (Abu Taha et al., 2019). A study conducted in France reported the percentage of patients who had splenectomy was 48.9% (Thuret et al., 2010). Another study conducted in Pakistan showed that only 5.5% of the patients underwent splenectomy, and only one patient (7%) had undergone splenectomy and developed a red cell alloantibody after the removal of the spleen (Zaidi et al., 2015).

Together with blood transfusion, iron chelation therapy (ICT) has improved the survival of thalassemia patients significantly. In this study, 89% of the patients received iron chelation therapy, 92.1% of which received Exjade (Deferasirox) (DFX) and the remaining 7.9% received Desferal (Deferoxamine)(DFO). DFO was and is still one of the most frequently used chelators, however, due to the associated pain during the administration and dose related toxicity, the drug has low compliance rate (Haddad, 2012b; E Vichinsky & Levine, 2015). On the other hand, DFX is an oral chelator that was introduced in 2005. In comparison to DFO, DFX has higher compliance rates and thus has progressively replaced DFO (N. Shah, Mishra, Chauhan, Vora, & Shah, 2010). Iron chelation treatment in general is costly, therefore, neither the patients nor the healthcare system can afford this cost. As a result, iron chelation drugs are usually provided as donations from other governments. However, shortage in donations can affect drug availability. Furthermore, compliance with iron chelation therapy is one of the major factors affecting the efficacy of the treatment regimen, therefore, data regarding compliance should be provided in order to fully understand the factors contributing to the overall health status of thalassemia patients. Moreover, experimental studies provided evidence that a combination of DFX with DFO chelation

results in additive iron excretion, especially with high overload patients, without any significant complication. This approach can be useful especially in poor countries with limited resources (Kurtoglu et al., 2012; Rachmilewitz & Giardina, 2011).

The cornerstones of thalassemia care are blood transfusion treatment coupled with iron chelation. Although blood transfusion reduces the complications of severe anemia and increases survival time, it has many side effects that could compromise patient care such as hemosiderosis, transfusion reactions, alloimmunization, and infections (E. Vichinsky et al., 2014). As a result, monitoring the complications of blood transfusion is crucial in the treatment of thalassemic patients. Our results showed that 57% of the patients had history of reactions to blood transfusion. Compared to a study conducted in Egypt that reported the prevalence of history of previous allergic reaction to be 72%, the prevalence among the patients in this study is much lower. However, compared to a prevalence of 48% reported by the Center for Disease Control (CDC) (E. Vichinsky et al., 2014), transfusion reactions were more common among the patients in this study. Another study in Iraq reported that 49.5% of all patients had a positive history of transfusion reactions (Abdulqader, Mohammed, & Mohammed, 2020). Transfusion reactions could result from febrile non-immune reactions; however, they could also be more serious and life threatening. In this study, we did not investigate the type and severity of these reactions.

6.1.2 Prevalence of Complications and Assessment of Biochemical, Hematological and Hormonal Parameters Among Frequently Transfused Thalassemia Patients

The majority of thalassemia-related mortality and morbidity is caused by iron excess. Endocrine dysfunction, hypothyroidism, hyperparathyroidism, hypogonadism, cardiomyopathy, arrhythmia, progressive liver failure, and aberrant kidney function have all been observed in people who have received chronic transfusions (Peters, Heijboer, Smiers, & Giordano, 2012). The use of an iron-chelating drug has been shown in numerous studies to improve survival in transfusion-dependent thalassemic (TDT) patients. As a result, regular blood transfusions and iron chelation therapy should be utilized properly in the management of thalassemia (Peters et al., 2012).

Iron excess in the anterior pituitary causes hormone secretion to be disrupted, resulting in hypogonadism, short stature, acquired hypothyroidism, and hypoparathyroidism. Primary

hypothyroidism and hypoparathyroidism are common in the second decade of life, are caused by iron excess, and can be treated with rigorous chelation at an early stage. Moreover, iron overload, chronic liver disease, and genetic susceptibility are all common causes of glucose intolerance in youth and diabetes mellitus later in life, which are also frequent in thalassemic individuals. Furthermore, during early adolescence, there is a decrease in development velocity and a reduced or nonexistent pubertal growth spurt, resulting in short adult height. Hypogonadism and delayed or missing puberty can lead to reproductive problems, which can have a significant impact on thalassemics' lives. Additionally, osteopenia and osteoporosis, which have a convoluted pathophysiology, are common causes of morbidity in young adults with thalassemia, both males and females (Lulla et al., 2020; Toumba & Skordis, 2010).

In the present study, the most prevalent complications among frequently transfused thalassemia patients were arthropathy (44%; n=44), followed by hypogonadism (16%; n=7), hepatic failure (7%; n=7), and delayed growth (7%; n=7). Other complications included conduction defects, cardiomyopathy, diabetes mellitus, heart failure, hypothyroidism, HCV, hyperparathyroidism, renal impairment, hearing defect, and metabolic acidosis.

The improvement of the quality of life and psychological prognosis of individuals suffering from thalassemia and its complications requires early detection and avoidance of endocrine problems with early and frequent chelation treatment (Fianza et al., 2021). Therefore, in this study, we assessed hematological, biochemical, and hormonal parameters among the patients using blood samples.

6.1.2.1 Hemoglobin Level

In the current study, the mean and SD of pre-transfusion hemoglobin level was found to be 7.89±0.99 g/dL with a range of pre-transfusion hemoglobin level between 5.6-11.8 g/dL. The TIF recommends maintaining pre-transfusion hemoglobin level of at least 9.5 g/dL among transfused patients (Thalassemia International Federation (TIF), 2021). Therefore, the findings of this study reflect severe anemia among thalassemia patients. A previous retrospective cohort study conducted among thalassemia patients in the West Bank over a two years period reported similar findings in this regard (Aldwaik et al., 2021). These low hemoglobin levels reflect an improper blood transfusion policy, which could be explained

in part by insufficient administration of transfused blood, low patient adherence to the transfusion regimen, and/or limited supply of blood for some rare blood groups. Furthermore, another explanation could be that the administration of insufficient amount of blood; the standard dose of packed RBCs received is the same for all patients regardless of their body mass index (BMI) (1 unit=450 mL), a practice that contradicts globally approved standards and recommendations, which based on TIF's guidelines should take into consideration the target increase in hemoglobin level, hematocrit of donor cells, and body weight (Thalassemia International Federation (TIF), 2021). As a result of this, the patients' anemia could likely worsen, and there may be further consequences. On the contrary, following international guidelines and maintaining the recommended pre-transfusion Hb levels could likely enhance survival and improve quality of life of thalassemia patients. Evidence for such outcomes could be observed through studies conducted among patients in developed countries (Bayanzay & Alzoebie, 2016).

Similarly, other countries in the region reported similar results. For example, a study conducted in Bangladesh reported a mean hemoglobin level among thalassemia patients of 7.2±1.5 g/dL (Karim et al., 2016). Another study conducted in Pakistan reported the average hemoglobin level to be 7.2±1.8 g/dL (Al-Naama, Mea'adKadhum Hassan, & Karim, 2020), and among Egyptian patients the mean hemoglobin level was 5.7±1.16 g/dL (Ragab et al., 2013).

6.1.2.2 Serum Ferritin

In the current study, the average serum ferritin (SF) level among the patients was 3670.42±3742.71 ng/dL ranging between 107.08-19374.37 ng/dL. Only two (2%) patients had normal SF levels. Furthermore, more than 50% of the patients had SF levels of 2500 ng/dL and above and another 28% of the patients had levels between 1000-<2500 ng/dL. The cutoff point recommended for blood transfusion-dependent patients is 1000 ng/dL (Borgna-Pignatti et al., 2004). It is widely recognized that an increase in SF levels put transfused patients at a high risk of having cardiac injuries (Cario, Stahnke, & Kohne, 1999; Mohammad & Al-Doski, 2012), and it is highly recommended that SF levels should be maintained below the aforementioned cut-off point according to the TIF guidelines (Thalassemia International Federation (TIF), 2021).

High SF level in thalassemic patients likely indicates irregular or insufficient chelation therapy (Bayanzay & Alzoebie, 2016). Patients have variable response to chelation therapy. In addition, drug availability and compliance to chelation regimen could largely influence the efficacy of iron chelation therapy (Angulo et al., 2008; Bayanzay & Alzoebie, 2016). Together, these factors might largely explain the abnormally high serum ferritin levels reported among patient in this study.

Similarly, high serum ferritin levels were previously reported among Palestinian patients in the West Bank (Aldwaik et al., 2021) and Gaza Strip (Ayyash & Sirdah, 2018). This is also the case in most developing countries (Fianza et al., 2021; Mishra & Tiwari, 2013; Sadullah, Atroshi, & Al-Allawi, 2020). Uncontrolled ferritin levels put the patients at increased risk of complications caused by iron toxicity. As a result, affecting the overall survival and quality of life of thalassemia patients (Mishra & Tiwari, 2013; Porter, Viprakasit, & Kattamis, 2014).

In this study, given that our budget allowed the genotyping of 100 patients, we selected patients with the highest transfusion frequencies to optimize the benefits of genotyping. Therefore, the indicators of the sample of our study might not be reflective of all thalassemia patients.

On another level, although in this study we used SF to estimate iron loads in the body, SF is not an accurate measure of iron load (Bayanzay & Alzoebie, 2016). Based on TIF's guidelines, liver biopsy or MRI-based measurements provide the most reliable estimates (Thalassemia International Federation (TIF), 2021). However, liver biopsy is highly invasive and MRI-based iron measurement methods are not provided for Palestinian patients (Aldwaik et al., 2021).

6.1.2.3 Liver Function Tests

In transfusion-dependent β-thalassemia patients, the liver is the first affected organ by the toxic effect of excess iron on hepatocytes. The high ferritin levels and the frequency of transfusions appear to be related to abnormal liver function in thalassemic individuals. Furthermore, viral infections frequently worsen iron-induced liver damage. Despite iron

chelation treatment, hepatic hemosiderosis, portal fibrosis, and cirrhosis can occur in frequently transfused patients (N. Shah et al., 2010).

Alanine aminotransferase (ALT) is a transaminase enzyme that catalyzes the transfer of amino groups between L-alanine and glutamate and is mostly generated in the liver. Elevated levels of ALT have been linked to liver damage even before the onset of hepatic disorders (Mohammad & Al-Doski, 2012). Therefore, elevated ALT level is commonly used as an indicator of liver injury and dysfunction (Telfer et al., 2000). Furthermore, aspartate aminotransaminase (AST) is another transaminase enzyme that is found primarily in the liver and sometimes in the heart (Tienboon, Sanguansermsri, & Fuchs, 1996), which makes it a highly specific indicator for liver damage (Mohammad & Al-Doski, 2012).

The prevalence of impaired liver function is estimated at 40.5%. In the present study, 32% of the patients had elevated ALT levels while 42% had elevated AST levels. Furthermore, 26 patients (26%) had elevated levels of both enzymes. Both ALT and AST levels had significant positive correlation with serum ferritin levels (r=0.541 and 0.543, respectively).

Moreover, alkaline phosphatase (ALP) is an enzyme found in a variety of tissues and organs, including the liver, bone, intestine, placenta, kidney, and leukocytes growth (Shams et al., 2010; Wiwanitkit, 2001). Serum ALP levels have been shown to be elevated in a variety of bone disorders, and it acts as a marker for proper bone tissue growth (Shams et al., 2010; Wiwanitkit, 2001). High levels of ALP have been linked to osteomalacia and might have a role in the morphological aspects of bone abnormalities reported in thalassemia patients (Salama, Al-Tonbary, Shahin, & Eldeen, 2006). In our study, elevated ALP levels were found among 34 (34%) of the patients.

6.1.2.4 Kidney Function Tests

Renal impairment is common in transfusion-dependent thalassemia patients, but its pathophysiology has not been yet understood. However, it is suggested that renal impairment could be caused by iron overload, persistent anemia, and/or chelation treatment (Kassab-Chekir et al., 2003). The determination of biochemical markers of renal function may aid in the prevention of substantial kidney damage prior to the appearance of any clinical complaint (Kassab-Chekir et al., 2003).

Creatinine and urea are waste products of protein digestion that are used to remove nitrogen from the body. Normally, these substances are filtered from the blood and eliminated in the urine (Majid Naderi et al., 2013). In clinical practice, estimating the concentrations of creatinine and urea in serum are extensively used and acknowledged as markers of renal function.

In this study, low creatine levels were found among 73 patients (73%). In addition, blood urea nitrogen (BUN) levels were found to be elevated among 3 patients (3%).

Decreased level of creatinine might reflect low body mass index and could be related to growth retardation and lower muscle mass usually encountered in β-thalassemia patients (Hosen, Hasan, Azim, Sarder, & Uddin, 2015; Modell, 1974), in addition to the side effects of iron chelation therapy, mainly DFX (Kassab-Chekir et al., 2003; Rachmilewitz & Giardina, 2011). On the other hand, evaluating kidney function in our patients necessitates additional research at the anatomical level of renal tissue (Majid Naderi et al., 2013). A previous study conducted in Bangladesh reported that serum creatinine levels in β-TM were shown to be significantly higher (Hosen et al., 2015). It was also shown that the severity of renal abnormalities was related to the degree of anemia, regardless of creatinine levels (Hosen et al., 2015). Another study conducted in Jordan reported that higher creatinine and urea levels were related with iron disposition in the kidneys, suggesting that thalassemic patients' physiological abnormalities in the kidneys are caused by decreased red cell life span and increased iron (Hosen et al., 2015).

Moreover, higher frequency of blood transfusion and hypercalciuria were also previously linked to renal impairment in thalassemic patients (Sadeghi-Bojd, Hashemi, & Karimi, 2008). This might explain why hypercalcemia was seen in 37% of the patients. This highlights the necessity of routine calcium testing in thalassemic patients being a risk factor for kidney dysfunction (Sadeghi-Bojd et al., 2008).

6.1.2.5 Endocrinopathies

Multiple transfusions are required in thalassemic individuals due to severe anemia. Transfusion therapy improves these patients' life, but it comes with several side effects. With time, people who have many blood transfusions develop a variety of endocrinopathies

(Yousaf, Sarfraz, & Hussain, 2018). Growth hormone deficiency, diabetes, and hypothyroidism are examples of endocrinopathies that could be caused by iron overload (Yousaf et al., 2018).

The prevalence of hypothyroidism in individuals getting numerous transfusions varies widely, from as low as 13.5% to as high as 16-35% (Yousaf et al., 2018). Many theories have been proposed to explain the wide range of hypothyroidism prevalence in thalassemic patients, including differences in thyroid function testing methods, age group of thalassemic patients, and medications used by patients (Yousaf et al., 2018). Clinical hypothyroidism is defined as decrease in free T4 and increase in TSH while subclinical hypothyroidism is indicated when only elevated TSH levels are observed.

In the current study, tri-iodothyronine (TT3) was found to be normal in all the patients while free T4 (FT4) was low in one patient (1%), and thyroid stimulating hormone (TSH) was elevated (subclinical hypothyroidism) in 10 patients (10%).

Compared to our study, a previous study conducted in Egypt reported that 19.3% of the patients had subclinical hypothyroidism and none of the cases had overt hypothyroidism (Abdel-Razek, Abdel-Salam, El-Sonbaty, & Youness, 2013). Another study conducted in Egypt reported that 25% of β-TM patients had impaired thyroid function; primary 2.5%, subclinical 12.5%, and central (secondary) hypothyroidism 10%. The severity of endocrine disease was associated with increased ferritin levels (Fayed, Mawgood, & Qubaisy, 2018). Furthermore, a study conducted in Western Iraq reported thyroid dysfunction among 13.3% of the cases; overt hypothyroidism among 5%, and subclinical hypothyroidism among 8.3% (Kadhum, 2018) and the prevalence of hypothyroidism among Turkish patients was reported to be 12.8% (Fayed et al., 2018).

Parathyroid hormone (PTH) is produced by four parathyroid glands placed behind the thyroid gland in the neck. PTH controls calcium levels in the blood, mostly by raising them when they are too low. This is accomplished by affecting the kidneys, bones, and intestinal absorption (Goyal et al., 2010). Hyperparathyroidism is the condition in which one or more of the parathyroid glands become overactive and release PTH. This causes the levels of calcium in the blood to rise, a condition known as hypercalcemia (Goyal et al., 2010).

In the current study, decreased PTH levels were observed among eight patients (8%) and elevated levels were observed among 17 patients (17%). Furthermore, elevated PTH and

calcium levels were observed in 10 patients (10%) while low PTH levels and calcium levels were observed among 5 patients (5%). In comparison to our study, a study conducted in Turkey reported that 2.8% of TDT patients had hypoparathyroidism and 2.8% had hyperparathyroidism (Angulo et al., 2008). Furthermore, a study conducted in Sri Lanka showed that 2.5% of the patients had hypoparathyroidism (Angulo et al., 2008). Another study reported that 11.1% of patients in Saudi Arabia, 11.6% of patients in Italy, 2% of patients in Taiwan, 8.7% of patients in Iran, and 10.5% of patients in Dubai had hypoparathyroidism, while in Turkey, hyperparathyroidism was observed in 29% of the patients (Haliloğlu, Tüysüz Kintrup, & Tayfun, 2017).

Growth hormone (GH), commonly known as somatotropin or human growth hormone, is a peptide hormone released by the anterior lobe of the pituitary gland. It promotes the development of all bodily tissues including bones. Somatotrophs, which are anterior pituitary cells that synthesize and produce GH, release one to two milligrams of the hormone every day. GH is necessary for children's normal physical growth. PTH levels build gradually throughout infancy and peak during puberty's growth spurt. Growth retardation is common among poly-transfused β-thalassemia patients (Al-Naama et al., 2020). Although the specific mechanism of short stature in children with thalassemia major is unknown, it is thought to be multi-factorial (Arab-Zozani, Kheyrandish, Rastgar, & Miri-Moghaddam, 2021). In our study, decreased GH levels were found in 8% of the patients (n=8) and elevated GH levels were observed in 2% of the patients (n=2). Compared to our study, in Iran, the prevalence of growth hormone deficiency (GHD) was 26.6% while in Iraq, the prevalence of growth hormone deficiency (GHD) was 53%.

6.1.3.6 Calcium and Vitamin D Levels

Calcium is an essential element that serves a variety of biological processes in the human body, the most important of which is skeletal mineralization. Calcium, inorganic phosphorus, and alkaline phosphatase are considered important biomaterials in the development of human bones and teeth (Vannucci et al., 2018). Hypoparathyroidism, vitamin D deficiency, bone marrow expansion, or chronic liver involvement can all cause calcium homeostasis to be disrupted in thalassemic patients (Saboor, Qudsia, Qamar, & Moinuddin, 2014). Few of the patients in this study (8%; n=8) had calcium levels that were

below the reference normal range, a condition known as hypocalcemia. Hypoparathyroidism, which was observed among 8% of the patients in our study, appears to be linked to hypocalcemia (Mula-Abed, Al Hashmi, Al Muslahi, Al Muslahi, & Al Lamki, 2008).

On the other hand, our results revealed that 37 patients (37%) had elevated calcium levels, a clinical condition called hypercalcemia. According to Sanctis et. al., mild hypercalcemia is defined as calcium level of 10.5-12 mg/dL does not require any interventions, and only patients with a serum calcium level of 14 mg/dL require treatment (De Sanctis, Fiscina, & Ciccone, 2010). In our study, all cases had calcium levels below 12 mg/dL and thus were classified to have mild hypercalcemia.

Unexplained increase in calcium levels had been previously reported. Elevated calcium levels in thalassemic patients could be caused by an excessive consumption of vitamin D or active analogues, which results in increased calcium absorption from the stomach and mobilization of calcium from the bone (Soliman, De Sanctis, & Yassin, 2013). A previous study in Bangladesh showed the mean of calcium level to be 7.9±0.6 mg/dL (Arab-Zozani et al., 2021) while a study in the West Bank showed the mean calcium level to be 2.3±0.3 mmol/L ranging between 1.4-2.6 mmol/L (Aldwaik et al., 2021).

Vitamin D (VD) is essential for calcium homeostasis and bone mineralization. Therefore, vitamin alpha D3 is regarded the major support of hypoparathyroidism (HPT) therapy (Ismail et al., 2022). Despite the presence of excellent sunshine in our region, VD deficiency and insufficiency are known to be common among thalassemic patients (Soliman & Kalra, 2013). A previous study among thalassemia patients in the West Bank showed that VD is not regularly monitored (Aldwaik et al., 2021). In this study, vitamin D deficiency was observed in 70% of the patients. In the USA, 69.8% of the patients had low VD levels (Soliman et al., 2013). On the other hand, in Tehran, 37.2% of 220 thalassemic patients had VD deficiency (Soliman et al., 2013). In addition, a report from North India and another from Thailand reported the prevalence of VD deficiency to be 80% and 90%, respectively (Soliman et al., 2013).

6.1.2.7 Fasting Blood Sugar

Transfusion treatment can cause glucose intolerance and diabetes mellitus, which can lead to toxic hemosiderosis in the liver in β-TM patients (Najafipour et al., 2008). The actual mechanism of iron-induced diabetes, however, remains unknown (Najafipour et al., 2008). Several factors that contribute to the onset of diabetes mellitus have been previously reported, the most likely of which is iron overload in pancreatic beta-cells which leads to pancreatic malfunction. Insulin resistance, liver dysfunction, genetic susceptibility, and a family history of diabetes are all contributing factors (Daraghmeh, 2016). In our study, elevated glucose levels were reported among 15% of the patients (n=15). Patients with high glucose levels have increased risk of becoming diabetic. Therefore, more medical care is necessary, and the diagnosis must be validated by continuous monitoring and measurement of hemoglobin A1C levels. Furthermore, 4% of the patients have been reported to have diabetes mellites.

In comparison to our findings, high prevalence of diabetes mellitus as a secondary complication of transfusion therapy was widely reported. In Morocco, the prevalence was 7%, in China the prevalence was 21.7%, and in France and North America the prevalence of diabetes mellitus was 6% and 10%, respectively (Arfaoui, Quyou, & Khattab, 2010; Kurtoglu et al., 2012; Li et al., 2002).

6.1.3 Molecular Genotyping of Blood Group Antigens Among Frequently Transfused Thalassemia Patients

The main purpose of transfusion is to supply each patient with compatible blood products (Thalassemia International Federation (TIF), 2021). The International Society of Blood Transfusion (ISBT) currently recognizes 43 blood groups containing 345 RBC antigens (International Society of Blood Transfusion "ISBT", 2021). Providing compatible blood for patients who have alloantibodies and patients negative for a high-frequency blood group antigen (HFA) is one of the challenges in transfusion medicine (Wilkinson, 2016).

One of the challenges in transfusion medicine is to provide compatible blood for patients negative for a high-frequency blood group antigen (HFA). Studies have shown that

transfusing patients with blood products matched for Rh and Kell system antigens can significantly reduce the risk of alloimmunization (Tormey & Hendrickson, 2019).

The frequency and distribution of blood groups differ broadly by race and ethnicity, even in the same region. Understanding the frequencies of the major blood group systems other than the ABO and Rh systems is essential for all blood banks in order to improve the management of blood supplies and predict the availability of the required units. Therefore, extended red cell typing is crucial for facilitating the challenging task of providing antigen-negative blood, especially for patients with multiple antibodies. However, in regularly transfused patients who have been phenotyped only for ABO and RhD before the first transfusion, accurate determination of other RBC antigens by serologic methods can be challenging if not impossible due to the presence of donor's RBCs in the blood stream (A. Belsito et al., 2017; Fasano & Chou, 2016).

Although large scale studies are available from European and other countries, only a few studies are available from the Middle Eastern region and Arab populations. In Palestine, the understanding of blood group phenotypes is limited to the ABO and RhD antigens. Furthermore, there are no data regarding the frequency of the major blood group systems other than the ABO and Rh, and even for those blood groups, there is a lack of population-based data. Moreover, frequently transfused patients, who have the highest requirements for extended typing, are already on transfusion; as a result, RBC phenotyping utilizing serological assays might not be feasible and based on TIF's guidelines, it is highly recommended to use molecular assays in this case (Thalassemia International Federation (TIF), 2021).

In this study, we utilized molecular assays for typing of the major blood group systems in order to determine the frequencies of blood group systems' alleles and genotypes among multi-transfused thalassemia patients in Palestine.

6.1.3.1 The Rh and Kell Blood Group Systems

The Rh blood group is second only to ABO in clinical importance and include more than 50 antigens including C, c, E, e, and D. Rh antigens, particularly D, are highly immunogenic and antibodies can result in delayed hemolytic transfusion reactions (HTRs) and hemolytic

disease of the fetus and newborn (HDFN). Furthermore, the Rh blood group is one of the most complex and polymorphic blood group systems. More than 500 RHD alleles and 150 RHCE alleles have been documented. *RHD* and *RHCE* are the two genes that make up the RH locus. Many of the Rh antigens are not identified by serologic reagents, but RH genotyping using DNA techniques can detect them. Antibodies with a wide range of Rh specificities might make it challenging to obtain acceptable red blood cell products. RH genotyping improves transfusion management. This is especially important for patients with thalassemia and other hemoglobinopathies who require several blood transfusions (Costa et al., 2016). The frequencies of the Rh antigens among Caucasians are reported to be as the following: RhD-positive (85%), C (70%), c (80%), E (30%), and e (98%), while among African Americans they were 92%, 34%, 97%, 21%, and 99%, respectively, and among Asians 99%, 93%, 47%, 39%, and 96%, respectively (Bethesda, 2005). However, in our study, 88% were *RHD*-positive, and 65% had the *RHCE*C* allele, 77% had the *RHCE*c* allele, 29% had the *RHCE*E* allele, and 96% had the *RHCE*e* allele.

The utilized molecular assay is carried out using sequence-specific primers (SSPs), which means the primers were specially developed for the selective amplification of segments of specific alleles or allele groups. Unclear results are obtained due to unknown alleles which are not detected with the existing primers and probes. In this study, 5% of the patients had weak *RHD* genotypes that showed no clear results. *RHD* genotyping for weak D phenotype could potentially prevent unnecessary transfusion with Rh-negative RBC units and injections of RhIG injections. Furthermore, the *RHCE*C*^{w} (*RH8*) allele which encodes the low incidence antigen C^{w}, which is antithetical to the high-incidence antigen MAR, was detected in one patient. The C^{w} is found in around 2% of Caucasians 1% of Blacks, 4% in Finns, and 9% in Latvians (Marion E Reid, Lomas-Francis, & Olsson, 2012). In addition, the *RHD*08N.01* allele which is a partial D allele resulting in the Psi phenotype (Ψ) was also detected among one patient.

The Kell system is one of the most complex and polymorphic blood group systems and contains several immunogenic antigens. These antigens are the third most effective at inducing an immunological response, after those of the ABO and Rh blood groups. Kell antigen-specific antibodies can cause transfusion reactions and HDFN. However, although HDFN caused by anti-Kell is usually moderate, the HDFN caused by anti-Rh may usually be avoided. Furthermore, because maternal anti-Kell antibodies target fetal red blood cell

(RBC) precursors, limiting fetal RBC synthesis, the few occurrences of HDFN caused by Kell immunization usually result in severe fetal anemia (L. Dean, 2005). The Kell blood group system has 36 antigens carried on a type II transmembrane glycoprotein. The antithetical antigens *KEL*01* (K) and *KEL*02* (k), *KEL*03* (Kp^a) and *KEL*04* (Kp^b), and *KEL*06* (Js^a) and *KEL*07* (Js^b) are the most relevant in transfusion medicine. Kell antigen phenotyping is limited due to technical restrictions, even though it is essential in some instances, therefore, molecular methods could be an easy and efficient strategy (Arnoni et al., 2013). In the current study, the frequencies of HFAs *KEL*02*, *KEL*04*, and *KEL*07* were 0.920, 0.985, and 0.980, respectively.

Compared to our study, a previous one conducted among 1158 university students in Gaza Strip investigated the frequencies of ABO and RhD blood types and reported that 97.3% had RhD-positive blood type (Skaik & El-Zyan, 2006). Another study conducted among 232 school aged students in Gaza Strip, Palestine, reported that the frequencies of the D, C, c, E, and e antigens were 92%, 69%, 81%, 38%, and 97%, respectively (El-Wahhab Skaik, 2011).

Few studies described the frequencies of RBC blood systems in Arab populations. A study conducted among blood donors in Riyadh, Saudi Arabia determined the frequencies of the Rh antigens (D, C, c, E, and e) and the K antigen of the Kell blood group using phenotyping assays and estimated the allele frequencies for the six antigens to be similar to the results of our study. The frequency of the D antigen was reported to be 86.4% and the allelic frequencies of C, c, E, e, and K antigens calculated using the Hardy-Weinberg formula were 0.44, 0.56, 0.14, 0.86, and 0.08, respectively (Alalshaikh et al., 2021). Another study reported the distribution of D, C, E, c, and e antigens among Saudi blood donors to be 84.8%, 62.3%, 23.5%, 74.3%, and 95% (Elsayid et al., 2017). On the other hand, a study among Omani blood donors reported that the frequencies of RhD-positive, c, C, e, and E antigens were 89.3%, 77.3%, 74.3%, 98.3%, and 28.2%, respectively, and no RhD variants were found. Furthermore, the prevalence of the Kell antigens K, k, Kp^a, and Kp^b were 4.5%, 99.4%, 2.7%, and 100%, respectively (A. Z. Al-Riyami et al., 2019).

Furthermore, a study conducted in Tunis to characterize the RHD variants in 2000 blood donors. The study showed the frequencies of the RhD-positive and the RhD-negative phenotypes were 88.85% and 11.15%, respectively. Furthermore, 15 donors (0.75%) had aberrant *RHD* alleles (Ouchari et al., 2013). Another molecular study conducted among Arabian and Irani blood donors in Germany using the German rare donor panel included 800

donors from Syria, 147 from Iran, 123 from the Arabian Peninsula, and 41 from north African countries. The study reported that the *RHCE*C*^{w} allele was detected among 0.8% of the sample, and the frequencies of the Kell genotypes were similar to our findings (Flesch et al., 2020). Moreover, a molecular study conducted among 180 Omani blood donors reported that the genotype frequency of *KEL*07/07* was 95.8% (A. Z. Al-Riyami et al., 2022). Another molecular study among 917 Arab Kuwaiti blood donors reported that 92.2% were *RHD*-positive, 1.63% had variant *RHD* alleles including the *RHD*08N.01 (RHD*Ψ)* allele, and 15.26% had *RHCE* variant alleles detected. In addition, the study reported the allele frequencies of *RHCE*C*, *RHCE*E*, *RHCE*c*, *RHCE*e*, *KEL*01* and *KEL*02* to be 0.712, 0.246, 0.760, 0.966, 0.089 and 0.983, respectively (Reem Ameen, Al Shemmari, Harris, Teramura, & Delaney, 2020). Moreover, a study conducted among 200 alloimmunized multi-transfused Irani thalassemia major patients reported that 80% of the patients *RHD*-positive genotype and the genotype frequencies of *RHCE*C/C, RHCE*c/c,* and *RHCE*C/c* were 28.9%, 26.1%, and 45%, respectively, and the genotype frequencies of *RHCE*E/E, RHCE*e/e,* and *RHCE*E/e* were 2.4%, 75.5%, and 22.2%, respectively. In addition, the genotype frequencies of the Kell blood group system were 96% and 4% for *KEL*02/02* and *KEL*01/02*, respectively, and 98.5% of the samples had the *KEL*04/04* genotype. Furthermore, discrepancies between phenotyping and genotyping of the Rh and Kell blood group systems were observed in 47 and 10 cases (Sarihi et al., 2021).

On the other hand, the *KEL*01/01* genotype was detected among 68 out of 37253 blood donors in Switzerland (Gassner et al., 2018). Furthermore, among Koreans, the frequencies of *RHCE*C, RHCE*E, RHCE*c, RHCE*e, RHCE*C*^{w}*, KEL*01, KEL*02, KEL*03, KEL*04, KEL*06,* and *KEL*07* were reported to be 86.1%, 52.0%, 61.9%, 88.1%, 0.0%, 0.0%, 100.0%, 0.0%, 100.0%, 0.0%, and 100.0%, respectively (Shin et al., 2018). Moreover, in a study including 4000 Swiss individuals, the allele frequencies of *KEL*01, KEL*02, KEL*03,* and *KEL*06* were 0.02675, 0.95763, 0.01350, and 0.00113, respectively (Meyer et al., 2014). Another study among 39 multi-transfused patients and 22 health blood donors in Turkey reported that both groups had similar genotype frequencies for Rh, Kell, Kidd, and Duffy blood group systems. Furthermore, 98.4% were *RHD*-positive, 51% had *RHCE*C/c* genotype, 64% had *RHCE*e/e* genotype, and 95.1% had *KEL*02/02* genotype (Bakanay et al., 2013).

6.1.3.2 Duffy Blood Group System

The Duffy (Fy) blood group system consists of five antigens carried on a transmembrane glycoprotein known as Duffy antigen/receptor for chemokines (*DARC*) which is a malarial receptor. These antigens are expressed in erythroid cells, endothelial cells, and epithelial cells in many organs such as the kidneys, brain, spleen, heart, lungs…etc. Antibodies to Duffy blood group antigens have been shown to occur mainly following transfusion and less frequently because of pregnancy (Hoher, Fiegenbaum, & Almeida, 2018; Lukasik, Nowak, Czerwinski, & Wasniowska, 2019).

The two major antigens in the Fy blood group system are the antithetical antigens Fy^a and Fy^b, encoded by the two codominant alleles *FY*01* and *FY*02* differing by a single nucleotide polymorphism (SNP) at position c.125A > G (Hoher et al., 2018; International Society of Blood Transfusion "ISBT", 2021; Lukasik et al., 2019).

The Duffy-negative phenotype Fy(a-b-) is defined by the homozygous state of *FY*02N.01* allele associated with a SNP in the promotor region of the *FY* gene. This SNP suppresses the expression of the *FY* gene on red blood cells without affecting its expression in other tissues. The Fy(a-b-) phenotype is frequent in Africans, but very rare in Caucasians. Furthermore, the few cases with the Fy(a-b-) phenotype in Europeans and Asians have been found to arise from mutations in the coding region of the *FY*01* and *FY*02* alleles (Hoher et al., 2018; Lukasik et al., 2019).

In the current study, the *FY*02N.01/02N.01* genotype had the highest frequency (46%), followed by *FY*02/02* (24%), *FY*01/02* (21%), and *FY*01/01* (9%). Furthermore, the allele frequency of the *FY*01* allele was the lowest (0.195). As we mentioned, it is reported that among Caucasians, the *FY*02N.01/02N.01* genotype is very rare. Based on the results of the 1000 Genomes Project, the allele frequencies of *FY*01*, *FY*02*, and *FY*02N.01* were respectively 0.019, 0.981, and 0.964 among Africans, 0.461, 0.539, and 0.078 among Admixed Americans, 0.923, 0.077, and 0.000 among East Asians, 0.640, 0.360, and 0.000 among South Asians, and 0.398, 0.602, and 0.006 among Europeans (Hoher et al., 2018).

An old study conducted among Israeli Jews and Arabs reported that the Fy(a-b-) was observed among Arabs and Jewish immigrants from Arab countries but not among Sephardi or Ashkenazi Jews (Sandler et al., 1979). Furthermore, the study conducted among Arab blood donors in Germany reported that 2% were homozygous for the *FY*02N.01* allele, in

addition, 15.7% carried the heterozygous mutation (Flesch et al., 2020). Additionally, the Fy(a-b-) phenotype have been previously reported among 61% (Owaidah et al., 2020) and 36% ("Abstract Presentations from the AABB Annual Meeting Philadelphia, PA, October 25-28, 2014," 2014) of Saudi blood donors. However, their results were based on serological phenotyping. Similarly, another serology based study among Omani blood donors reported that 68.5% of blood donors had the Fy(a-b-) phenotype while Fy(a+b-), Fy(a+b+), and Fy(a-b+) phenotypes were observed among 9.2%, 7.4%, and 14.9% of the donors, respectively (A. Z. Al-Riyami et al., 2019). Furthermore, another Omani molecular study conducted among blood donors reported that 72% had the homozygous *FY*02N.01* genotype (A. Z. Al-Riyami et al., 2022). Among Arab Kuwaiti blood donors, 15.3% had the homozygous *FY*02N.01* genotype and 21.8% had a the *FY*02N.01* in a heterozygous state (Reem Ameen et al., 2020). Moreover, among a sample of 200 multi-transfused thalassemia major patients, the genotype frequencies of *FY*02/02*, *FY*01/02*, and *FY*01/01* were 23.5%, 47.5%, and 29%. Discrepancies between phenotypes and genotypes were found in 45 samples (Sarihi et al., 2021).

However, among the Korean population, the genotype frequencies of *FY*01/01*, *FY*01/02*, and *FY*02/02* were reported to be 88.1%, 10.7%, and 1.2%. The allele frequency of *FY*02N.01* was 0.0 (Shin et al., 2018). In comparison, among the Swiss population, the allele frequencies of *FY*01* and *FY*02* were 0.415 and 0.56288, but the *FY*02N.01* was not detected (Meyer et al., 2014). Furthermore, a study conducted in Turkey reported that among multi-transfused patients and healthy blood donors, the genotype frequencies of *FY*01/01*, *FY*01/02*, and *FY*02/02* were 28.2%, 44.73%, and 21.05%, respectively. In addition, two patients (5.26%) had *FY*01/null01* genotype and one patient (2.63%) had *FY*02/null01* genotype (Bakanay et al., 2013).

6.1.3.3 MNS Blood Group System

In terms of complexity, the MNS blood group system is second only to the Rh blood group system. The MNS blood group system consists of 48 antigens carried on glycoprotein A (GPA), glycoprotein B (GPB), or hybrids of both proteins that result from single-nucleotide substitution, uneven crossing over, or gene conversion. Two genes located on chromosome

4 encode the MNS antigens, *GYPA* and *GYPB* (Bethesda, 2005; International Society of Blood Transfusion "ISBT", 2021; M. E. Reid, 2009).

The frequencies of the M, N, S, and s antigens among Caucasians are reported to be 78%, 72%, 55%, and 89%, respectively, while among Blacks they are reported to be 74%, 75%, 31%, and 93%, respectively (Bethesda, 2005). Comparatively, in the current study, 77% were found to have the *GYPA*M* allele, 59% had *GYPA*N*, 45% had *GYPB*S*, and 90% had *GYPB*s*. Furthermore, the frequencies of M, N, S, and s antigens among Omani blood donors were reported to be 89.0%, 51.8%, 64.0%, and 82.1%, respectively (A. Z. Al-Riyami et al., 2019). A molecular study among Kuwaiti blood donors 0.3% were S-s- and the frequencies of M, N, S, and s alleles were 0.839, 0.579, 0.571, and 0.836, respectively (Reem Ameen et al., 2020). On the other hand, among Arab and Irani blood donors in Germany, the study showed that while the genotype of frequencies of *GYPB*S/S*, *GYPB*S/s*, and *GYPB*s/s* were 13.5%, 43.3%, and 43.2%, respectively among Syrians, they were 17.2%, 32.0%, and 50.8% among Arabs from the Arabian Peninsula, respectively, 4.9%, 46.3%, and 48.8% among Arabs from Northern African countries, respectively, and 13.8%, 42.8%, and 43.4% among Irani, respectively (Flesch et al., 2020). Moreover, a study conducted among Iranian thalassemia patients reported that the genotype frequencies of *GYPA*M/N*, *GYPA*M/M*, *GYPA*N/N*, *GYPB*S/s*, *GYPB*s/s*, and *GYPB*S/S* to be 52%, 38%, 10%, 45%, 41%, and 13%, respectively. In addition, discrepancies between genotype and phenotype of S/s antigens were observed in six patients, and 2 patients had discrepancies in M/N antigens (Gholamrezazade et al., 2021). On the other hand, the genotype frequencies of *GYPA*M/N*, *GYPA*M/M*, *GYPA*N/N*, *GYPB*S/s*, *GYPB*s/s*, and *GYPB*S/S* among the Korean population were reported to be 49.2%, 29.0%, 21.8%, 12.7%,87.3%, and 0.0% (Shin et al., 2018).

6.1.3.4 Kidd Blood Group System

The Kidd blood group system (JK) was first reported in 1951 (Plaut, Ikin, Mourant, Sanger, & Race, 1953). JK antigens are coded by the solute carrier family 14, member 1 (*SLC14A1*) gene on chromosome 18, which also known as the human urea transporter (Marion E Reid, 1999). This gene encodes three major alleles; *JK*A*, *JK*B*, and the silent allele *JK* (International Society of Blood Transfusion "ISBT", 2021). The location of the JK

glycoprotein in the membrane allows it to swiftly transfer urea into and out of RBCs while maintaining the osmotic stability and shape of RBCs. The Kidd glycoprotein is also produced in the kidney, where it allows the kidney to accumulate a large level of urea, which is required for the kidney to produce concentrated urine (Bethesda, 2005). People who lack the Kidd glycoprotein are unable to concentrate urine to its maximum capacity, but they are otherwise healthy, and their RBCs have a normal shape and lifespan (Bethesda, 2005). Kidd antigen-specific antibodies are a common cause of delayed hemolytic transfusion reactions and HDFN. In our study, the genotype frequencies of the JK blood group were 50%, 32%, and 18% for *JK*A/B*, *JK*A/A*, and *JK*B/B*, respectively. Furthermore, none of the patients in our sample had the rare JK null allele. The genotype frequencies of the JK alleles in this study were comparable to the phenotype frequencies reported among Caucasians which were 49% for JK(a+b+), 28% for JK(a+b-), and 23% for JK(a-b+) (Bethesda, 2005). However, they were significantly different from those reported among African Americans, which were 34%, 57%, and 9%, respectively (Bethesda, 2005). Furthermore, the genotype frequencies reported in our study were comparable with previous reports among Koreans (Shin et al., 2018) and among Arabian blood donors in Germany (Flesch et al., 2020). Moreover, the prevalence of the Kidd blood group antigens Jk^a and Jk^b were 90.64% and 69.4% among a sample of anonymous Saudi volunteer blood donors in the Jazan Province and the frequencies of the JK phenotypes were 34.69%, 12.59%, and 52.45% for Jk(a+b-), Jk(a-b+), and Jk(a+b+), respectively (Halawani et al., 2022), which were also similar to the genotype frequencies of the Kidd blood group alleles in our study. The frequencies of the Jk^a and Jk^b antigens among Omani blood donors were reported to be 82.4%, and 64.3%, respectively and the phenotype frequencies were 35.4% for Jk(a+b-), 17.3% for Jk(a-b+), 47.0% for Jk(a+b+), and 0.3% for Jk(a-b-) (A. Z. Al-Riyami et al., 2019). Furthermore, the frequencies of *JK*A* and *JK*B* among Omani blood donors were 0.822 and 0.617, respectively (Reem Ameen et al., 2020). On the other hand, among the Swiss population, the allele frequencies of *JK*A* and *JK*B* were 0.51313 and 0.48650, respectively. In addition, two Jk_{null} alleles (*JK*01N.03* and $JK*B_{null}$) were detected with frequencies of 0.00025 and 0.00024 (Meyer et al., 2014). Among alloimmunized Irani β-thalassemia major patients, the genotype frequencies were 29% for *JK*A/A*, 23.5% for *JK*B/B*, and 47.5% for *JK*A/B* (Sarihi et al., 2021), which were comparable with those reported among the patients in our study. In addition, among Turkish multi-transfused patients and blood donors, the frequencies of the Kidd blood system genotypes were 28.2%, 56.41%, and 15.38% for *JK*A/A*, *JK*A/B*, and *JK*B/B*, respectively (Bakanay et al., 2013).

6.1.3.5 Cartwright (Yt) Blood Group System

The Yt blood group system (ISBT 011), previously known as the Cartwright blood group system, consists currently of five antigens encoded by *ACHE* (International Society of Blood Transfusion "ISBT", 2021). Until 2017, this system consisted of two antithetical antigens; the high prevalence antigen Yt^a and the low prevalence antigen Yt^b. Cartwright antibodies have rarely demonstrated clinical significance (M. R. George, 2019).

In the current study, the genotype frequency of *YT*A/B* was (12%) and the frequency of *YT*A/A* was 88%. In the study conducted among Arab and Irani blood donors in Germany, the genotype frequencies of *YT*A/A, YT*A/B*, and, *YT*B/B* had been reported to be 79.9%, 18.8%, and 1.5%, respectively; however, among Arabs form northern African countries, only the *YT*A/A* genotype have been detected (Flesch et al., 2020). Furthermore, a molecular study conducted among 180 Omani blood donors reported that the genotype frequency of *YT*A/A* was 91.9% (A. Z. Al-Riyami et al., 2022). On the other hand, among Koreans, only the *YT*A/A* genotype had been reported (Shin et al., 2018).

6.1.3.6 Dombrock Blood Group System

The Dombrock blood group system (ISBT 014) consists of 10 antigens and encoded by *ART4,* located on chromosome 12. This system consists of two antithetical antigens Do^a and Do^b, and five HFAs. The identification of antibodies to the Dombrock blood group system can be challenging and they are often linked to HTRs. The frequency of Do phenotypes varies by ethnicity. Among whites, the frequencies of Do(a+b-), Do(a+b+), and Do(a-b+) are 18%, 49%, and 33%, respectively, while among Blacks they are 11%, 44%, and 45%, respectively, and among Japanese 1.5%, 22%, and 76.5%, respectively (International Society of Blood Transfusion "ISBT", 2021; Lomas-Francis & Reid, 2010).

In current study, the genotype frequencies were 47% for *DO*01/02*, 19% for *DO*01/01*, and 34% for *DO*02/02* with the allele frequencies of *DO*01* and *DO*02* being 0.425 and 0.575, respectively. The genotype frequencies are similar to the phenotype frequencies reported among Whites, but significantly different from those reported among Blacks and Japanese. Comparatively, the frequencies of the Dombrock blood group system alleles *DO*01* and

*DO*02* were 0.675 and 0.829, respectively, among Arab Kuwaiti blood donors (Reem Ameen et al., 2020). Furthermore, among Saudi blood donors, the allele frequencies were reported to be 0.432 for *DO*01* and 0.568 for *DO*02* (Bawazir, 2022) and among Arabs and Irani blood donors in Germany, the genotype frequencies were reported to be 18.6%, 50.4%, and 31.0% for *DO*01/01, DO*01/02*, and *DO*02/02*, respectively (Flesch et al., 2020). Moreover, the genotype frequencies among 251 Brazilian blood donors had been reported to be 19.9% for *DO*01/01*, 44.6% for *DO*01/02* and 35.5% for *DO*02/02* (Langer et al., 2019). However, among Koreans, the genotype frequencies of *DO*01/01, DO*01/02*, and *DO*02/02* were reported to be 1.5%, 20.2%, and 83.8%, respectively (Shin et al., 2018).

6.1.3.7 Lutheran Blood Group System

The Lutheran blood group system (ISBT 005) consists of 29 antigens including four pairs if antithetical antigens and 11 HFAs. The Lu antigens have low immunogenic antigens that can usually induce mild delayed HTRs or HDFN with few exceptions. The Lutheran blood group system antigens are encoded by *BCAM* (basal cell adhesion molecule) located on chromosome 19 (Daniels, 2009; International Society of Blood Transfusion "ISBT", 2021). Lu glycoproteins are believed to be involved in the transfer of maturing RBCs from the bone marrow to the blood stream. Although clinically Lu antigens are not very important, their genetic and functional perspectives are of interest (Daniels, 2009).

The antithetical antigens Lu^a and Lu^b are inherited in a codominant pattern. The frequency of Lu^a ranges between 6-8% among Europeans, Africans, and Northern Americans while the frequency of Lu(b-) is estimated to be <0.001 in Caucasians (Daniels, 2009).

In our study, the genotype frequencies were 2% for *LU*01/02* and 98% for *LU*02/02*, and the *LU*01/01* genotype was not detected. Comparatively, the frequencies of Lu^a and Lu^b antigens among Omani blood donors were reported to be 3.0%, and 96.7%, respectively (A. Z. Al-Riyami et al., 2019). On the other hand, among Koreans, the only reported genotype was *LU*02/02* (Shin et al., 2018). Furthermore, among Kuwaiti Arab blood donors, 0.6% had the rare Lu(a-b-) phenotype (Reem Ameen et al., 2020). Furthermore, among Arabs and Irani blood donors in Germany, the genotype frequencies were reported to be 0.2%, 3.0%, and 96.8% for *LU*01/01, LU*01/02,* and *LU*02/02*, respectively (Flesch et al., 2020).

Among Brazilian blood donors, genotype frequencies were reported to be 0.4% for *LU*01/LU*01*, 6.8% for *LU*01/LU*02* and 92.8% for *LU*02/LU*02* (Langer et al., 2019).

6.1.3.8 Colton Blood Group System

The Colton blood group system (ISBT 015), consists of four antigens carried on a glycoprotein called aquaporin 1, encoded by *AQP1*. The most significant antigens are the antithetical antigens Coa and Cob (Halverson & Peyrard, 2010; International Society of Blood Transfusion "ISBT", 2021). The phenotype frequency across most populations is reported to be 91.4% for Co(a+b-), 0.2% for Co(a-b+) and 8.4% for Co(a+b+). In addition, the frequency of Co(a-b-) is estimated to be <0.01% (Halverson & Peyrard, 2010).

Similar to or study which showed that the genotype frequencies of *CO*01/01* and *CO*01/02* were 98.0% and 2.0%, respectively, the study that was conducted among Arabs in Germany reported the genotype frequencies to be 98.6% and 1.4%, respectively (Flesch et al., 2020). Furthermore, among Arab Kuwaiti blood donors, the frequency of *CO*01* and *CO*02* were 0.998 and 0.008, respectively (Reem Ameen et al., 2020). On the other hand, among Koreans, the only reported genotype was *CO*01/01* (Shin et al., 2018).

6.1.3.9 Knops Blood Group System

The Knops blood group system (ISBT 022) consists of nine antigens carried on the Complement Receptor I (CR1) glycoprotein. This protein is found in most peripheral blood cells except platelets. The Knops blood group system antigens are not usually clinically significant (International Society of Blood Transfusion "ISBT", 2021; Moulds, 2010).

The frequency of Knops antigens Kn^a and Kn^b among Caucasians were reported to be 98% and 4% and the Kn^b antigen was not reported among Western Africans populations and was reported to be <1% among African Americans (Moulds, 2010). In our study, all patients had the genotype *KN*01/01*. However, a molecular study conducted among 180 Omani blood donors reported that the genotype frequency of *KN*01/01* was 97.7% (A. Z. Al-Riyami et al., 2022).

6.1.3.10 Diego Blood Group System

The Diego blood group system consists of 23 antigens. The antigens of the Diego blood group system are carried on erythroid band 3 protein anion exchanger 1 (AE1), which is made up of only one gene, *SLC4A1* (solute carrier family 4, anion exchanger, member 1) (International Society of Blood Transfusion "ISBT", 2021). AE1 belongs to a group of three anion exchangers or transporters found in various tissues. This protein is expression is limited to RBCs and the Kidneys. Mutations of *SLCA1* can cause RBCs with abnormal shapes resulting in hemolytic anemia and disrupt the kidney functions resulting in the accumulation of acid anions. Di^a, Di^b, and WR^a are among the most important antigens of the Diego blood group system. Apart from anti-Di^a, anti-Di^b, anti-Wr^a, anti-ELO, and and-DISK, antibodies against Diego system antigens are not clinically relevant for transfusion or HDFN (Bethesda, 2005; Figueroa, 2013).

Di(a-b+) is the most common Diego phenotype and found in >99.9% of Caucasians and blacks and around 90% of Asians (Bethesda, 2005). In our study, the genotype frequencies of *DI*B/B* and *DI*02.04/02.4* (encoding Wrb antigen) were 98% and 100%, respectively. In comparison, among Omani blood donors, molecular genotyping showed that the genotype frequency of *DI*B/B* was 99.4% (A. Z. Al-Riyami et al., 2022). However, among Koreans, the genotype frequencies of *DI*B/B, DI*A/B,* and *DI*A/A* were 92.1%, 7.5%, and 0.4%, respectively (Shin et al., 2018), while among Arab Kuwaiti blood donors, the allele frequency of *DI*A* was 0.0 (Reem Ameen et al., 2020).

6.1.3.11 Vel Blood Group System

The Vel blood group system consists of one antigen. The protein is encoded by *SMIM1* (International Society of Blood Transfusion "ISBT", 2021); however, the genetic basis of the Vel blood group system were not determined until 2013. The Vel antigen has a very high frequency (>99% in all populations). The Vel-negative phenotype is very rare and caused by a 17-bp deletion that completely blocks the protein expression. Furthermore, several polymorphisms in *SMIM1* have been reported and they were shown to affect the antigen

strength. Anti-Vel antibodies can cause severe HTRs (Arnoni et al., 2019; Cvejic et al., 2013; Storry et al., 2013; Storry & Peyrard, 2017).

In this study, 98% of the patients had the high frequency genotype (*VEL*01/01*) and two had the heterozygous genotype (*VEL*01/-01*), but no homozygous *VEL*-01* allele (*SMIM1*64_80del)* was found. The allele frequency of the VEL*-01 was 0.020. A study conducted in South Brazil reported the allele frequency of *SMIM1*64_80del* to be 0.101 (Arnoni et al., 2019). Furthermore, the study among Arab blood donors in Germany reported that the genotype frequencies of *VEL*01/01*, *VEL*01/-01*, and *VEL*-01/-01* were 98%, 1.9%, and 0.1%, respectively. Furthermore, compared to the German population, the study had a relatively higher frequency of the Vel_{null} allele, *VEL*-01* (Flesch et al., 2020). Comparatively, a study conducted among a sample of 23848 blood donors in Switzerland identified only five donors with *VEL*-01/-01* genotype and 216 donors with *VEL*01/-01* genotype (Gassner et al., 2018).

6.1.4 Alloimmunization and Autoimmunization among Frequently Transfused Thalassemia Patients

Transfusion of packed red blood cells (RBCs) is a common treatment for patients with major thalassemia for two reasons: it increases the capacity of the blood to carry oxygen and replaces non-functional RBCs with functional ones, which could improve symptoms or prevent complications. Transfusion, pregnancy, and transplantation could cause alloimmunization against blood types, but the rate of alloimmunization is higher in patients who are regularly transfused, such as thalassemia and sickle cell anemia patients (Ghasemi et al., 2016).

Alloimmunization rates in thalassemia patients have been reported to range from 5 to 30% over the world, with alloimmunization to minor blood type antigens accounting for the majority of the alloimmunization. Anti-red cell antibodies (alloantibodies and/or autoantibodies) can complicate blood transfusion. Furthermore, hemolysis is frequently associated with alloantibodies. Therefore, the development of antibodies could increase blood requirements. As a results, patients could become more susceptible to iron accumulation and its complications.

Although autoantibodies to red blood cells are less common, they can cause clinical hemolysis known as autoimmune hemolytic anemia (AIHA) and make blood cross-matching a challenging job. Patients with autoantibodies are more likely to require transfusions and immunosuppressive drugs, as well as other treatments such as intravenous immunoglobulin (IVIg) and rituximab (anti-CD20 monoclonal antibody) (Philip & Jain, 2014).

In our study, the rate of alloimmunization was 8.0% and the rate of autoimmunization was 5.0%. Among alloimmunized patients, 40% were caused by anti-cyclic citrullinated peptide antibodies (anti-CCP). Limited data are available on the frequency of RBC alloimmunization and autoimmunization in transfusion dependent Palestinian β-thalassemia patients and they are not routinely performed in blood banks. This is mainly due to shortage of the available capabilities and resources. Previous studies among thalassemia patients in the West Bank reported the rate of alloimmunization to be 12.6% (Abu Taha et al., 2019) and the prevalence of autoantibodies to be 2% (Asees et al., 2019).

In comparison to our study, a study conducted in north-east Iran reported the prevalence of alloantibodies to be 2.87% (Mohammad Hadi Sadeghian et al., 2009) while another one conducted in Tehran, Iran reported the prevalence of alloantibodies to be 10.9% and the prevalence of autoimmunization was 1.81% (Ghasemi et al., 2016). In New Delhi, India, an alloimmunization rate of 5.64% and an autoimmunization rate of 28.2% were reported (Dhawan et al., 2014). Another study conducted among patients in India, 5.6% were alloimmunized and 1% was autoimmunized (Philip & Jain, 2014). In a study conducted in Pakistan, 8.6% of patients were alloimmunized (Zaidi et al., 2015). Moreover, in Iraq alloantibodies were detected in 5.8% of the patients and autoantibodies were detected in 4% of the patients (Abdulqader et al., 2020).

Anti-CCP is an autoantibody that targets healthy tissues in the joints. Anti-CCP antibodies indicate rheumatoid arthritis, which is a progressive autoimmune disease that causes pain, swelling, and stiffness in the joints. In Lebanon, the prevalence of anti-CCP was 2.2% (Noureldine et al., 2018).

In comparison to Kuwait and Egypt, the results of this study showed a low rate of RBC alloimmunization. The observed low prevalence of alloimmunization in this study could be explained by the homogeneous ethnicity between patients and donors in our population (Abu Taha et al., 2019).

In our study, typing of the alloantibodies showed that alloimmunization was caused by antigens in the Rhesus and Kell systems, both of which are the most common cause of alloimmunization worldwide (Abdulqader et al., 2020). The most frequent alloantibodies were anti-E, anti-K and anti-D, and anti-C (65.5%, 25.0%, and 12.5%, respectively). A study conducted previously among thalassemia patients in the West Bank showed that the most frequent alloantibodies were anti-D, anti-K, and anti-E (33.3%, 25.9%, and 14.8%, respectively) (Abu Taha et al., 2019).

Antibodies against the Rh system are essential in transfusion medicine because they can cause hemolytic transfusion reactions and are the major cause of alloimmunization among transfused patients. A previous study in Iraq reported that anti-E was the most frequent alloantibody, followed by anti-C, anti-e, and anti-K (Abdulqader et al., 2020). In Pakistan, most of the alloantibodies identified were directed against the Rh system or the Kell antigen; the most frequently occurring alloantibody was anti-E (2.5%) followed by anti-K (1.8%), anti-e (1.2%), and anti-D (0.6%) (Zaidi et al., 2015). Furthermore, a study conducted in India reported Rh blood group system antigens as the main cause of alloimmunization. Anti-E, anti-D, anti-C, and anti-C^w were the most common Rh antibodies with prevalence of 17%, 13%, 13%, and 9%, respectively. In addition, 9% had antibodies against antigens of the Kell blood system and 4% had antibodies against antigens of the Kidd blood system (Dhawan et al., 2014). On the other hand, in Iran, one study reported alloantibodies against RH system (D, C, E and c) among 58.3% of alloimmunized patients and against the Kell system among 16.6% (Ghasemi et al., 2016), while another study in south Iran reported that all alloimmunization cases were caused by Rh antigens (Mohammad Hadi Sadeghian et al., 2009).

In the current study, 62.5% of the alloimmunized patients were females. Although the proportion of females was higher among immunized patients, there was no significant differences in the risk of alloimmunization between males and females. Similarly, several studies reported no significant association between gender and alloimmunization in multi-transfused thalassemia patients (Abdelrazik et al., 2016; Al-Mousawi, Al-Allawi, & Alnaqshabandi, 2015; el-Danasoury, Eissa, Abdo, & Elalfy, 2012; Guirat-Dhouib et al., 2011). On the contrary, a significantly increased risk of alloimmunization among females in South Iran had been previously reported (Karimi, Nikrooz, Kashef, Jamalian, & Davatolhagh, 2007).

Furthermore, in this study, 62.5% of alloimmunized patients were between 10-19 years old. All the patients were transfused for at least 10 years of their life and there was no significant association between age and alloimmunization. Similarly, Al-Mousawi et al. (Al-Mousawi et al., 2015) and Elhence et. al. (Elhence, Solanki, & Verma, 2014) reported that there were no significant associations between alloimmunization and age. On the other hand, a study conducted in Oman reported a significant association between age and alloimmunization (A. Z. Al-Riyami et al., 2018).

In our study, 75% of alloimmunized patients received their first transfusion before the age of 2 years. Studies have previously reported that the initiation of blood transfusion at a young age (<2 years old) can contribute to the establishment of immunological tolerance in recipients, which can protect them from alloimmunization. In their study, Bhatti et. al. (Bhatti, Salamat, Nadeem, & Shabbir, 2004) and Gader et. al. (Gader, Al Ghumlas, & Al-Momen, 2008) reported that age at first transfusion is not a strong predictor of alloimmunization.

In our study, all alloimmunized patients received blood transfusion at an interval between 2-4 weeks. Furthermore, one patient (12.5%) had anti-CCP autoantibodies. It has been previously reported that the increase in the frequency of blood transfusion might increase the risk of alloimmunization (Al-Mousawi et al., 2015; Bilwani et al., 2005; Gupta, Singh, Singh, & Rusia, 2011; Hassan, Younus, Ikram, Naseem, & Zaheer, 2004; Hussein, Ahmed Eldesoukey, Rihan, & Kamal, 2014; Pahuja, Pujani, Gupta, Chandra, & Jain, 2010). On the other hand, a lack of association between alloantibodies and autoantibodies formation and the number of transfused packed RBCs has been previously reported (Abdel-Razek et al., 2013).

Splenectomy causes a decrease in the removal of damaged red cells, thus exposing new antigens and increasing the likelihood of alloimmunization (Singer et al., 2000). However, in this study, no association between alloimmunization and splenectomy was observed and only 25% of alloimmunized patients were splenoctomized. Several studies reported no association between alloimmunization and splenectomy in multi-transfused thalassemia patients (A. Z. Al-Riyami et al., 2018; el-Danasoury et al., 2012; Guirat-Dhouib et al., 2011; Pahuja et al., 2010; Zaidi et al., 2015) while other studies reported a significant association (Al-Mousawi et al., 2015; R. Ameen, Al-Eyaadi, Al-Shemmari, Chowdhury, & Al-Bashir, 2005; Datta et al., 2015).

In our study, most immunized patients (62.5%) had O blood type. A potentially relevant finding is that alloimmunized patients had a greater prevalence of blood type O than non-immunized patients. The explanation for this is still unknown, however ABO glycosylation is increasingly recognized as interfering with cell functioning (Zaidi et al., 2015). Furthermore, all patients had history of transfusion reactions, which was previously reported (Al-Mousawi et al., 2015; Chao et al., 2013).

Arthropathy was the most common complication reported among alloimmunized patients. Arthropathy is caused by a reduction in vitamin D levels and an increase in iron overload in tissue and joints. Iron overload is caused by RBC destruction caused by alloantibodies, which causes RBC destruction and increases with blood transfusion, resulting in an increase in the rate of iron overload, which accumulates in tissue and joints, causing complications such as arthropathy, heart failure, renal impairment, and conduction defects, among others.

Examining the genotyping results of alloimmunized patients, we can see the importance of genotyping in preventing alloimmunization. In this study, the development of eight of ten alloantibodies among six patients could have been prevented by extended typing of blood group antigens of the Rh and Kell antigens prior to the initiation of transfusion. The preventable alloantibodies and genotypes of the patients were ass the following: five patients had anti-E antibodies and had *RHCE*e/e* blood type, two patients had anti-K alloantibodies and had *KEL*02/02* genotype, and one patient had anti-C antibodies and *RHCE*c/c* genotype. Furthermore, two patients had anti-D antibodies. Transfusion with RhD incompatible RBCs to RhD-negative patients due to false negative results among blood donors with weak D typing is one of the most important causes of anti-D alloimmunization. Alloimmunizaion with anti-D caused by transfusion of RBCs with weak D could be prevented through repeat testing of donor units for weak D (M. H. Sadeghian et al., 2009).

In summary, this study highlights the need to follow international treatment guidelines and protocols in order to improve the health status of thalassemia patients and minimize the complications associated with severe anemia and iron overload in body organs. In addition, the study highlights the need for RBC antigen genotyping in order to provide safe blood transfusion for patients. Furthermore, extended cross-matching for Rh antigens and Kell antigens between recipients and donors should be performed prior to transfusion. Moreover, antibody screening should be performed on a regular basis to monitor for the emergence of new alloantibodies, in order to prevent delayed hemolytic transfusion responses.

6.2 Conclusions

In conclusion, our data demonstrates that the burden of complications among thalassemia patients is relatively high. Utilizing the international guidelines can significantly improve the health of thalassemia patients.

Furthermore, although the rates of alloimmunization and autoimmunization were relatively low in our study, the most often occurring antibodies belong to the Rh and K blood groups. Therefore, extended matching of transfusion-dependent thalassemia patients and blood donors for the Rhesus antigens D, E, e, C, c, and K antigen could significantly lower the risk of alloimmunization.

6.3 Limitations

This descriptive study aims to determine the prevalence of transfusion-related complications, assess the hematological, biochemical, and hormonal profiles, determine the rate of immunization, and determine the genotypes of blood group system antigens among frequently transfused thalassemia patients using molecular genotyping methods in Palestine. Up to our knowledge, this study is the first to date on this topic in Palestine. However, the generalizability of our findings could be limited because we only included patients from the northern and central regions of the West Bank and we selected patients with the highest frequencies of blood transfusion. In addition, the sample size was considerably small compared to the number of patients diagnosed with thalassemia due to budgetary limitations.

On the other hand, being the first study to describe the distribution of blood cell antigen systems, this study provides unique insights that promote further research in transfusion medicine in Palestine. Moreover, the study provided insights on the management of thalassemia among Palestinian thalassemia patients which could be utilized to influence thalassemia care.

6.4 Recommendations

- Prevention of thalassemia should remain on the top priorities in the fight against inherited diseases due to its burden on the health system and the families of the patients. Therefore, educational and awareness campaigns aimed at informing the public about the dangers of consanguineous marriages on children and future generations are desperately needed.
- Management of β-thalassemia should follow international recommendations and protocols, especially concerning blood typing, hemoglobin levels, and iron load.
- Increased awareness, training, and resources are required in order to improve and standardize adequate blood transfusion services and iron chelation therapy.
- It is likely that the introduction of molecular genotyping for donors and recipients could enhance the safety of blood transfusion in Palestine, increase the efficacy and efficiency of red cell matching, and decrease blood inventory requirement. However, prospective outcome studies, including efficacy and cost analysis are needed before establish genotyping for accurate blood typing.
- All thalassemic patients should undergo molecular testing, which may lead to the development of a more appropriate care plan for those with the severe mutations.
- Sequencing of the *RHD* and *RHCE* genes can significantly elucidate the heterogeneity of serological reactions, especially in weak D and partial D phenotypes. Furthermore, the observed differences in allele frequencies in our population could be further understood by sequencing.
- Sequencing of samples with unclear results in order to identify identifying new genetic variants or variants that could be falsely typed in the local population.
- Understanding the frequencies of the major blood group systems other than the ABO and Rh systems among patients and donors is essential in all blood banks for routine testing and emergencies. Furthermore, extended red cell typing is crucial for facilitating the challenging task of providing antigen-negative blood, especially for patients with multiple antibodies. Therefore, wider studies among larger sample of patients and blood donors could provide more accurate and useful data that could improve the safety of blood transfusion in Palestine.

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Appendices

Appendix I: Study questionnaire in Arabic Language

المحددات الجينية على سطح خلايا الدم الحمراء لمرضى الثلاسيميا

القسم الأول: المعلومات الشخصية			
1. الاسم الكامل		2. رقم الهوية	
3. تاريخ الميلاد		4. الجنس	☐ 1. ذكر ☐ 2. أنثى
5. رقم الهاتف / الجوال		6. مكان تلقي العلاج	

القسم الثاني: المعلومات الطبية	
7. نوع الدم	
8. هل خضعت لعملية نقل النخاع؟	
9. هل خضعت لعملية إزالة الطحال؟	
10. التشخيص الطبي	☐ 1. ثلاسيميا وسطى ☐ 2. ثلاسيميا كبرى ☐ 3. أنيميا منجلية ☐ 4. أنيميا منجلية-ثلاسيميا ☐ 5. آخر _____
11. تاريخ التشخيص	اليوم / الشهر/ السنة ____/____/_____
12. العلاج بنقل الدم	☐ 1. ثلاسيميا معتمد على نقل الدم ☐ 2. ثلاسيميا غير معتمد على نقل الدم ☐ 3. آخر _____
13. العمر عند أول عملية نقل دم	
14. تكرار عملية نقل الدم	
15. هل سبق أن عانيت من تفاعلات تحسسية من نقل الدم؟	☐ 1. نعم ☐ 2. لا ☐ 3. لا أعرف

Appendix II: List of referees

Referee Number	Referee Name	Place of Work
1	Mr. Mahmoud Ruzayqat	The National Center for Cancer Diagnostics and Human Genetics – Ministry of Health
2	Mr. Khalid Al Younes	Al Quds University
3	Mr. Issa Ishtayeh	Central Public Health Laboratories - Ministry of Health
4	Dr. Loa'y Shaheen	Hematologist - Ministry of Health

Appendix III: Consent Form

Al-Quds University

Deanship of Graduate Studies

Master Program in Medical Laboratory Sciences

Title of thesis: Genotyping of Human Erythrocyte Antigens for Safe Blood Transfusion in Thalassemia Patients

Student Name: Dirgam Mufeed Yassen
Supervisor Name: Dr. Rania Abu Seir
Date:

Thank you for participating in the study, warm greetings.

Our study aim to investigate the genetic, biochemical, hormonal and hematological status of frequently-transfused Palestinian thalassemia patients. You were selected to participate in this study as you are involved in data collection for thalassemia patient. The study related to your disease.

Your participation is voluntary. There are no anticipated risks or benefits to your participation. Please sign this form if you agree to participate ……, date ……

2021-2022

تحديد الأنماط الجينية لفئات الدم لدى المرضى المعتمدين على نقل الدم لنقل دم أكثر أماناً

ملخص

إعداد: ضرغام مفيد إبراهيم ياسين

إشراف: د. رانية أبو سير

خلفية الدراسة: تعتبر إدارة مرض الثلاسيميا من نوع بيتا من التحديات الرئيسية لا سيما في البلدان محدودة الموارد. يعتبر نقل الدم العلاج الأنجع للمرضى المصابين بالثلاسيميا الكبرى، ومع ذلك، ترتبط عملية نقل الدم بالعديد من الآثار الجانبية بما في ذلك تفاعلات انحلال الدم والحساسية وتراكم الحديد والأمراض المنقولة عن طريق نقل الدم. في هذه الدراسة قمنا بتقييم الفحوصات البيوكيميائية والدموية والهرمونية ومدى انتشار مضاعفات الثلاسيميا وتكوين أجسام المناعة المتباينة ضد خلايا الدم الحمراء وأجسام المناعة الذاتية، و وتحديد الأنماط الجينية لفئات الدم لدى مرضى الثلاسيميا المعتمدين على نقل الدم من أجل المساهمة في جعل عملية نقل الدم أكثر أماناً في الضفة الغربية.

منهجية البحث: لقد تم استقطاب عينة مكونة من 100 مريض من مرضى الثلاسيميا المعتمدين على نقل الدم من خلال وحدات علاج الثلاسيميا في خمس مستشفيات حكومية بحيث تم اختيار المرضى ذوي المعدلات الأعلى لنقل الدم. تم جمع البيانات باستخدام استبيان لجمع المعلومات الأساسية للمريض واستخدام سجلات المرضى للحصول على المعلومات الطبية. بالإضافة إلى ذلك، تم جمع عينات دم من المرضى بهدف القيام بفحوصات كيمياء الدم، المؤشرات الدموية، والفحوصات الهرمونية لدى هؤلاء المرضى، بالإضافة إلى فحوصات الكشف عن وجود أجسام المناعة الذاتية وأجسام المناعة المتباينة ضد خلايا الدم الحمراء وتحديد أنواعها بالإضافة إلى القيام باستخراج المادة الوراثية من أجل التحليل الجيني ل12 فئة من فئات الدم. وقد تم إجراء التحليل الجيني من خلال طريقة تفاعل البوليمراز المتسلسل باستخدام البوادئ (برايمرات) ذات التسلسل المحدد.

النتائج: بين المرضى المئة الذين شاركوا في الدراسة، كانت نسبة الذكور 51% وبلغ معدل أعمار المرضى 21.9±10.9 سنوات. تم استقطاب غالبية المرضى (60%) من المستشفى الوطني في نابلس. أظهرت النتائج أن تركيز الخضاب لدى المرضى قبل عملية نقل الدم كان منخفضاً لدى جميع المرضى

كما كان معدل تركيز بروتين الفيريتين لدى المرضى3742.71±3670.42 نانوغرام/ديسيلتر من 2500 لدى أكثر من نصف المرضى. بالإضافة إلى ذلك، فإن اختبارات وظائف الكبد بينت أن ما نسبته 32% و42% و34% كان لديهم زيادة في مستويات بروتينات(Alanine transaminase (ALT و(Alkaline phosphatase (ALP و Aspartate transaminase (AST) على التوالي. بالإضافة إلى ذلك، بلغت نسبة المرضى الذين يعانون من خلل طفيف (نقص) في وظائف الغدة الدرقية 10% كما أن 8% من المرضى كان لديهم انخفاض في مستوى هرمون النمو و8% من المرضى كان لديهم انخفاض في مستوى الكالسيوم و 70% كان لديهم انخفاض في تركيز فيتامين د و15% كان لديهم ارتفاع في تركيز سكر الغلوكوز في الدم. بينت سجلات المرضى أن أكثر مضاعفات مرض الثلاسيميا شيوعاً بين المرض كان التهاب المفاصل وقصور الغدد التناسلية وفشل الكبد. أما نتائج التحليل الجيني لأنظمة فصائل الدم، فقد بينت نتائج التحليل أن مولد الضد D كان موجباً لدى 88% من المرضى وسالباً لدى 7% من المرضى، بينما 5% لم تكن لديهم نتائج واضحة. بالإضافة إلى ذلك فقد بلغت نسبة تواتر أليلات فصائل الدم الرايزيسية *RHCE*C*، *RHCE*c*، *RHCE*E*، و*RHCE*e* على الترتيب (0.440، 0.560، 0.165، و0.835. أما بخصوص نظام الدم من نوع Duffy، فقد كان من غير المتوقع أن ما نسبته 46% من المرضى كان لديهم الطراز الجيني (*FY*02N.01/02N.01*) بينما بلغت نسبة تواتر الأليلات *FY*01* و*FY*02* بين المرضى على الترتيب 0.195 و 0.345. بالإضافة إلى ذلك، فقد كانت نسبة تواتر الأليلات لنظام فصائل الدم MNS ما مقداره 0.585 و 0.405 على الترتيب للأليلين *GYPA*M* و *GYPA*N* ونسبة تواتر الأليلين *GYPB*S* و *GYPB*s* 0.275 و 0.725 على الترتيب. أما نسبة تواتر الأليلات *KEL*02* و*KEL*04* و *KEL*07* التابعة لنظام Kell فقد بلغت على الترتيب 0.920 و 0.985 و 0.980. أما نسبة تواتر الأليلات *YT*A* و *LU*02* و *CO*01* و *KN*01* و *DI*B* و *DI*02.04* و *VEL*01* على الترتيب 0.940 و 0.990 و 0.990 و 1.000 و 0.980 و 1.000 و 0.990. بالإضافة، فإن 2% من المرضى كانوا حاملين للطراز الجيني *Vel*01/-0.1*. بالإضافة إلى ذلك، فقد بينت نتائج الدراسة أن 8% من المرضى كان لديهم أجسام مناعة متباينة ضد كريات الدم الحمراء وكانت أجسام المناعة المتباينة لمولدات الضد E وK وD وC الأكثر شيوعاً بين المرضى، كما أن 5% كانت لديهم أجسام مناعة متباينة لمولدات الضد الذاتية.

خاتمة: بينت الدراسة أن بروتوكولات علاج ومتابعة مرضى الثلاسيميا في فلسطين لا تتبع البروتوكولات الموصى بها عالمياً. بالإضافة إلى ذلك، بينت الدراسة أهمية دراسة وتحديد أنواع الدم باختلافها سابقاً لعملية نقل الدم للتقليل من مضاعفات نقل الدم. كما أظهرت النتائج أن الأنماط الوراثية

للمرضى والمتبرعين بالدم من الممكن أن تجعل عملية نقل الدم أكثر أماناً. إضافة إلى ذلك، لقد بينت نتائج التحليل الجيني أن التوزيع النسبي لفئات الدم أمر يتطلب المزيد من الأبحاث والدراسات مستقبلياً.

الكلمات المفتاحية: المناعة المتباينة، التحليل الجيني لفصائل الدم، تراكم الحديد، بيتا ثلاسيميا، الاعتماد على نقل الدم.